STUDIES ON THE SENSITIZATION OF ANIMALS WITH SIMPLE CHEMICAL COMPOUNDS

X. Antibodies Inducing Immediate-Type Skin Reactions*

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In experiments on the sensitization of animals to certain simple chemical compounds, antibodies which induce passive anaphylaxis have been demonstrated in the serum (1). It has since been found possible to elicit reactions of the "early type", simulating the Prausnitz-Küstner reaction, in normal guinea pigs at skin sites prepared with the serum of sensitized animals.[1] The effect has been obtained with acyl chlorides, acid anhydrides, picryl chloride, and 2:4 dinitrochlorobenzene. Experiments of this type are described in the present paper together with related observations on reactions occurring in the skin of normal guinea pigs as a result of interaction between antibodies and common antigens.

EXPERIMENTAL

Sensitization of Animals and Production of Antisera.—For obtaining sera of satisfactory potency, it was necessary with most animals to extend the sensitizing injections over 5 to 12 weeks or more, with intervening rest periods. The simple chemicals dissolved in olive oil or in corn oil were injected intradermally into male albino guinea pigs,[2] each injection consisting of 0.05 cc. of the vehicle containing 1/20 mg. of the incitant in most cases (*o*-chlorobenzoyl chloride, citraconic anhydride, phthalyl chloride, 2:4 dinitrochlorobenzene), but 1/80 mg. of picryl chloride, and 1/5 mg. of phthalic anhydride. With the last substance, a concentrated solution in dioxane was added to olive oil (*cf.* Jacobs (6)). Once sensitivity had become established, the reactions developing upon subsequent injections were examined early (between 30 minutes and 2 hours) as well as the next morning, since there is evidence for two maxima corresponding respectively to the early and the delayed-type reactions (7, 9). Although the presence of effective concentrations of transfer antibodies could be determined only by trial bleedings and by testing the serum as described below, the occurrence of intense early reactions (with at times subsidiary reactions of the early type around old injection sites (*cf.* 6–8)) seemed to give the best indication as to probability of a high serum antibody level. It may be men-

* The work here presented was largely done in association with the late Dr. Karl Landsteiner, and was cited as item No. 344 in the latter's bibliography (2).

[1] These have been reported briefly in reference 3; similar effects with antiprotein sera have also been found (4).

[2] Of the several stocks of albino guinea pigs employed, progeny from the colony established genetically as highly susceptible to experimental sensitization with 2:4 dinitrochlorobenzene (5), and subsequently pen-inbred, may have been somewhat superior in regularity of production of transfer sera.

tioned that the choice of olive oil or corn oil as vehicle for the chemical substances either in sensitizing or in eliciting reactions was shown to be immaterial for the results.

To describe a typical experience with ortho-chlorobenzoyl chloride:—

One lot of 13 male albino guinea pigs was injected intradermally with 1/20 mg. of the chemical dissolved in 0.05 cc. corn oil on 10 occasions over a period of 3 months, arranged in 4 courses with intervening rest periods of 16 or 17 days—on days 1, 4, 8, 12; 29, 36, 43; 59, 65, 73; 89. Twenty-eight trial bleedings made during this time revealed the transfer antibody in 11 of the 13 guinea pigs; the best 4 animals were exsanguinated for serum—1 on the 46th day, another on the 81st day, 2 on the 97th day. The various bleedings were tested in the skin of normal animals as described later; the test dose of *o*-chlorobenzoyl chloride was 1 to 2 mg. given by the subcutaneous route. Reactions developing in 45 to 60 minutes with this substance were considered satisfactory. In a similar but more prolonged experiment with citraconic anhydride, a total of 11 injections was made over 19 weeks, and every guinea pig in the lot of 10 eventually developed a good content of transfer antibody. In all, inclusive of series treated over shorter periods of time, 44 satisfactory transfer sera were secured from a total of 80 animals sensitized to citraconic anhydride. In the case of this substance, sites prepared on normal recipients with such sera commonly reacted in 3 to 10 minutes.

The sera reacting with picryl chloride, developed by repeated intradermal injections of olive oil solutions, were nearly always less potent than those prepared in the same way to acid anhydrides and acyl chlorides (*e.g.*, transfer sites in normal recipients reacted in 40 to 60 minutes after subcutaneous injection of picryl guinea pig serum) and were encountered not too frequently. For instance, in the course of sensitizing injections on 13 guinea pigs, spaced approximately as described above, a total of 10 trial bleedings between the 37th and 72nd days demonstrated the antibody in 9 of the animals. Similar sera but of much higher activity were encountered almost regularly in animals which, between 4 and 7 weeks previously, had received intramuscularly an emulsion of picryl guinea pig serum, or picryl guinea pig stromata, along with paraffin oil and killed tubercle bacilli.[3]

With 2:4 dinitrochlorobenzene, transfer antibodies were obtained following intracutaneous injections (10 injections of 1/20 or 1/40 mg. of the chemical in 0.05 cc. olive oil over a period of 9 weeks), although the concentration of antibody in the sera was often low. Even in effecting sensitization by means of painting the skin ten times with a 2 per cent solution in alcohol, these antibodies were clearly, although feebly, detectable now and then. Another method which has yielded more active sera consisted of several intracutaneous injections of 1/50 mg. of the incitant dissolved in paraffin oil, to which killed tubercle bacilli had been added. After injections on five occasions over an 18 day period, 7 positive sera were found among 18 guinea pigs bled between the 28th and 40th days.

Because transfer reactions with drug hypersensitivity sera were so readily elicited by the corresponding protein conjugates, antiprotein sera were prepared in guinea pigs (4). (The analogous work of Ramsdell (13), not then known to us, will be described later.) Horse serum (0.1 cc. of a 1:5 dilution) was injected intradermally, approximately in the sequence

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[3] These emulsions were made with the aid of commercial preparations derived from lanolin, namely Aquaphor (Duke Laboratories) or Falba (Pfaltz and Bauer) and contained 10 mg. conjugate and 0.5 mg. dried tubercle bacilli in 1 cc., the amount injected into each animal, distributed between 4 or 5 sites. Such emulsions were used in preference to injecting the aqueous and oily phases separately as we did originally in sensitizing guinea pigs to picryl chloride by means of a protein conjugate and a suspension of killed tubercle bacilli in paraffin oil (10). The preparation of Aquaphor emulsions incorporating dead tubercle bacilli in paraffin oil was devised by Freund as a method which confers substantial adjuvant effect on several antigenic materials (11, 12; *cf.* 4).

shown above for the first two courses with *o*-chlorobenzoyl chloride; on the 48th day, 1 week after the last injection, the sera of all 8 guinea pigs were found to give a transfer effect. A second method, usually giving more active sera, was the procedure developed by Freund (11) namely a single injection into the muscles of the neck of an emulsion of horse serum, Aquaphor, and killed tubercle bacilli in paraffin oil; the animals were bled 4 or preferably 6 weeks or longer afterwards.

In view of the results obtained with horse serum, the preparation of anti-ragweed sera was undertaken. This proved possible (4) by intracutaneous injections of alumina flocs of pollen extract or, apparently less regularly, by subcutaneous injection of defatted pollen incorporated in an Aquaphor emulsion with paraffin oil and dead tubercle bacilli. The alum precipitates were made from a "7 per cent" extract of defatted low ragweed (*Ambrosia elatior*) pollen in Coca's alkaline fluid prepared without phenol, by adding 1/9 volume of 10 per cent potassium alum solution and sufficient N/10 NaOH to neutralize the mixture; 0.1 cc. portions of such freshly prepared suspensions were injected into the skin twice a week for 3 weeks; then further injections were given after a month's rest, and the sera were taken between the 9th and 12th weeks. Transfer sera against ragweed, of apparently higher titer, have since been obtained in guinea pigs by Kulka and Hirsch (14).

Trial bleedings were made by heart puncture 1 to 3 days, rarely later, following a routine sensitizing injection and were tested in normal guinea pigs as described later. The donors chosen were exsanguinated between the 2nd and 10th days after the preceding sensitizing injection, commonly around the 5th day. The sera were stored in the refrigerator at 4–7°C. without addition of preservative.

Sensitizing Chemicals.—Ortho-chlorobenzoyl chloride and citraconic anhydride, Eastman preparations, were redistilled *in vacuo*. Phthalyl chloride, a Kahlbaum preparation, was purified by fractional freezing. Injection of these three substances was made with solutions in oil prepared immediately beforehand. Phthalic anhydride was a commercial preparation (Eastman). Picryl chloride was twice recrystallized from a benzene-alcohol mixture, while 2:4 dinitrochlorobenzene was recrystallized from alcohol.

Protein Conjugates.—For the preparation of *o*-chlorobenzoyl and phthalyl conjugates, 10 cc. of guinea pig serum was treated with 0.7 cc. of N Na_2CO_3 and shaken for several minutes with 0.5 cc. of chloroform containing 0.05 cc. of *o*-chlorobenzoyl chloride or phthalyl chloride; a small additional amount of chloroform was later added and the shaking continued for a time. The aqueous solution was separated from the chloroform layer and the conjugated protein brought to flocculation by careful dropwise addition of N HCl. Where the amount of precipitate was rather heavy, several volumes of saline were added. After centrifuging and discarding of the supernatant solution, the precipitate was dissolved in a small amount of saline with addition of N NaOH to neutrality, any insoluble residue being removed. The dry weight was determined upon a portion precipitated with alcohol.

"Citraconyl guinea pig serum" was made by shaking 0.05 cc. of citraconic anhydride with 10 cc. serum for several minutes, any precipitate forming meanwhile being dissolved by partial neutralization with N NaOH. The mixture was precipitated by addition of N HCl, the conjugated protein separated by centrifugation and washed with saline; it was then dissolved in saline by adding N NaOH to pH 7.0.

The preparation of "picryl guinea pig serum" followed the method already described (1). Picryl casein was made similarly, with a 7 per cent solution of Hammarsten's casein dissolved with sodium carbonate instead of serum, but precipitation with alcohol was omitted. In analogous manner, starting with dinitrofluorobenzene, "dinitrophenyl guinea pig serum" was prepared (*cf.* 1), here likewise with omission of the alcohol precipitation.

Preparation of Skin Sites; Recipient (Acceptor) Animals.—Male albino guinea pigs weighing between 350 and 450 gm. served as recipients. Injections of guinea pig sera (0.15 cc. or less)

were made intradermally on the back between the loose skin over the pad of fat in the nuchal region and the skin over the pelvic girdle, and distant by 22 to 30 mm. from the middorsal line. In the routine testing of sera, as many as 8 sites were prepared and tested simultaneously on one animal: this must be done with discretion when it is to be expected that 5 or 6 sites may react, not only because strong reactions may spread and become confluent but also because a portion of the antibody introduced at each site escapes into the circulation and contributes to a systemic reactivity. When multiple like sites react, the extent of the individual reactions is apt to be less than when the sera are tested singly; a similar situation was encountered by Fischel and Kabat (15) in studying the transferred Arthus phenomenon in the rabbit.

With a given serum, the development and extent of the transfer reactions vary with the individual recipient and with age (weight). In addition, there may be differences in strains, since of the 2 stocks of guinea pigs chiefly employed animals of the one described in footnote 2 have given more intense reactions. Male albino guinea pigs weighing between 350 and 450 gm. have been used almost exclusively in the present experiments, but animals of 200 to 275 gm. weight of either sex give larger and more striking reactions under the same conditions of test; conversely, guinea pigs weighing 500 gm. and over have rarely proved satisfactory, exhibiting erythema and edema only meagerly. As mentioned, in any weight group and sex, individual variation occurs, and although only a few have failed to give indubitable responses, some animals have proved to be poor "acceptors," giving responses of feeble intensity and small diameter. When in a small experiment histamine dihydrochloride (0.05 cc. of 1:1200 dilution calculated as the free base) was injected into the skin of two selected classes of animals, namely individuals which had given large and prominent transfer reactions and those which had not, it turned out that members of the former group were all characterized in 10 to 15 minutes by distinguishably larger, colorless swellings; but the method did not reveal sufficient gradations to suggest its use for practicable preselection of recipients. To recognize individual differences among acceptors, it has been our practice in routine work to include a skin site prepared with a standard serum of the same sort as or, preferably, one of different specificity from that under test; in the latter case, an evaluation of the individual recipient may be made at some subsequent time without influencing the reaction under study.

Testing of Antisera.—Testing was usually done 1 or 2 days after the preparatory injections, as soon as the sites were essentially normal in appearance. (With sterile guinea pig sera, toxic reactions were seldom encountered.) The specific reactions, developing in the prepared sites and spreading peripherally, with erythema and edema, could be elicited either by local application of suitable test materials or by injection at a remote point. Where the simple chemical incitant is readily reactive, for instance with proteins, injection of amounts of about 1 mg. (citraconic anhydride, phthalyl chloride) or 2 mg. (phthalic anhydride, *o*-chlorobenzoyl chloride) in a bland oil beneath the skin of the abdomen commonly produced a clear reaction within 3 to 45 minutes. In the same way, picryl chloride (1 mg.), which, chemically, reacts more slowly, led to observable reactions after 45 to 200 minutes; subcutaneous administration of the still less reactive 2:4 dinitrochlorobenzene was not effective (see footnote 5). Upon local injections of oil solutions into the prepared sites (in which case low concentrations are necessary to avoid toxic effects and the volume must be small), reactions ensued but these tended to be small and blurred, probably because of slow release of the chemical substance. With some substances, however, scratch tests made through dioxane solutions across the prepared sites[4] could be used to advantage (citraconic anhydride, phthalic anhydride). With citraconic anhydride, scratches were made through a drop of 10 to 25 per cent solution in dioxane placed on the skin; similarly adequate concentrations of most other substances had some primary toxicity

[4] Or scratches may be made elsewhere on the normal skin, with the consequence that the latent period is somewhat longer before reaction at the serum site starts.

for skin, so that the application was best confined to the scratch line. For this purpose, scratches were made with a No. 26 hypodermic needle (attached to a loaded syringe) having a very small pendent droplet of the solution on the needle tip. With phthalic anhydride, a scratch test with a 25 per cent solution in dioxane was used, and this served well for testing either phthalic anhydride (*cf.* 16) or phthalyl chloride sera. In some other cases, scratch tests across prepared specific sites also evoked reactions (40 per cent picryl chloride in dioxane, initiating a reaction in 50 to 120 minutes, and 15 per cent phthalyl chloride in olive oil, producing a reaction of small size that started within 30 minutes). The reaction is readily induced when soluble protein conjugates are substituted for the free chemical: local injections of rather small quantities may be made directly into the prepared area, or the conjugate in larger amount (1 to 50 mg.) may be injected subcutaneously at a remote site or intraperitoneally or intravenously. Central wheals are most evident following the local injections of conjugates. When sites have been prepared with sera of low antibody content, the tests have seemed to be best made by the subcutaneous injection of fairly large amounts of protein conjugate (say 50 to 75 mg.). With sera to 2:4 dinitrochlorobenzene, the antibody has so far been satisfactorily demonstrable only with the corresponding dinitrophenyl protein conjugate.

Characteristics of the Reaction.—After the preparatory injection, an interval of 7 to 10 hours is necessary for the skin site to become typically reactive (see also 17). Tested prior to this time, the serum site may show at best some transient slight degree of increased volume, the same effect as may be noted when a conjugate (or protein antigen) and the corresponding antiserum are injected as a mixture; in both of these cases, the potential reactivity of the site is "discharged" and no reaction will ensue at a later retest (*cf.* 18). (In the rabbit, the situation appears to be different: Ramsdell (13) found the rabbit ear to respond as soon as antiserum was deposited in the tissue, and Fischel and Kabat in a recent study (15) injected antibody and antigen into the same area of rabbit skin at an interval of 30 minutes in order to develop the delayed response characteristic of the Arthus phenomenon.)

After the skin site has become capable of reaction there is a latent period—typically 3 to 30 minutes—between administration of the substance and the first appearance of a perceptible reaction. Of the various factors which influence the length of this latent period, apart from the question of the nature of the chemical substance, the foremost are the concentration of antibody in the antiserum, and the adequacy of the method of testing as to route and amount of substance; of lesser bearing is individual variability among recipient animals. Variations in the latent period arising from the different reactivities of the various chemicals may be nullified by testing with preformed conjugates of the chemical substances with proteins, and the sera can then be compared under equivalent conditions. That the latent period may reflect the strength of the antiserum was observed in consecutive trial bleedings from animals undergoing active sensitization and in several of the experiments in which various dilutions of one antiserum were used to prepare skin sites (see p. 504).

Almost independently of the manner of testing, which influences chiefly the amount of reactive material available and the rapidity of absorption, and thereby the latent period before the reaction starts, there usually appear first discrete dots of color, rash-like, over an area of 1 or 2 cm., then additional pinkish patches, and confluence, all within 5 minutes (*cf.* 4); the onset of edema is apparent even then. In the most striking cases, the effect starts as an even, bluish (cyanotic) discoloration with transient but prominent erythematous streaks across the blue area; hyperemia and edema start almost at once at the periphery, and the pinkish color extends slowly into the bluish center which, incapable of developing edema for some minutes, probably because of intense vasoconstriction, forms an umbilicate center in the reaction area. In either case, the erythema spreads from the central area peripherally and pseudopodially, rapidly at first, followed more slowly by progressive edema which gradually replaces the erythema. The erythema may be maximal 10 to 20 minutes after onset of the reaction, while the

greatest diameter and swelling of the local area are attained still later. The area involved in a reaction varies with the strength of the serum and with the particular animal; as mentioned above, when one animal has several like sites reacting at the same time, the areas are usually smaller. Reactions as large as 97 × 50 mm., involving practically the entire flank, have been encountered, but even with large reactions it is rare that the middorsal line is crossed. Except for occasional faint recrudescence of erythema a few hours later, which may be confined to the central part of the area previously reacting, there is typically no further change, the edema receding in its turn and the skin appearing normal on the next day; following intense reactions, brownish pigmentation may be visible for a day or so. (With antisera against horse serum, and sometimes with antisera against *o*-chlorobenzoyl chloride, there may be definite effects still present on the following day.)

When a typical, homologous reaction has once been induced, the site will not respond to a second testing, reflecting depletion of antibody. In the case of intense reactions, there may be a second factor, to be referred to later: the skin area does not fully recover its capacity to react, even to a different antigen-antibody system, for perhaps 6 to 8 days. When reactions are partial because of the use of insufficient antigen or allergenic chemical, a prompt retesting with adequate material may call forth a second response, but the sites tend to become discharged (*cf.* Table VIII, test and retest of recipient 3). A few instances have been encountered in which sites have been discharged without exhibiting visible reaction, the specific substance corresponding to the antibody having been given subcutaneously in small amount on the day prior to the adequate test; on the other hand, the situation shown in test and retest of recipient 4, Table VIII, is met with more often. These effects within a localized area, it will be recognized, have counterparts in the desensitization of animals (or organs) in the passively induced anaphylactic state.

If no reaction is induced, the area remains responsive for 5 or 6 days, sometimes rather well so for 3 weeks or more. While the length of fixation varies with the particular serum, probably reflecting its antibody content, the capacity of the local site to react is found to lessen, gradually, after the first few days.

Exhaustion of Cutaneous Reactivity by Local Reactions.—It has been stated that an intense reaction may result in a temporary exhaustion of the capacity of a skin area to respond to restimulation. This exhaustion is perhaps best shown by inducing and charting the area of rather large transfer reactions on several animals. At various times thereafter on different animals, starting 2 days later, small amounts of an antiserum of another specificity may be injected along the margin and in the center of one of the areas that reacted previously. When this antibody is brought to reaction 1 day after its injection, sites at the periphery of the old area usually show a distinct crescent shape, with the concave side determined by the border of the initial area of reaction; thin, pincer-like projections of erythema may then spread into the area to complete a circular outline, and the extended crescent may slowly fill in, thereby producing a disc-like reaction, the newer segment not infrequently having lesser color and elevation. Sites made in the very center of the old area show substantially greater inhibition, and on the 3rd or 4th day after the primary reaction may show no more than fine dots of erythema over an area of perhaps 20 × 20 mm. This undoubtedly reflects the greater intensity of reaction in the center of an area (at the site of antibody deposition), just as does the central vasoconstriction seen at times in the primary reaction, or central whealing in human Prausnitz-Küstner skin tests. By the 7th day, the old reaction area is nearly normal, and at the 9th day is indistinguishable from other areas of skin.

A more widespread, but less complete, exhaustion of the skin's capacity to react has been noted as well. When several successive reactions in different skin areas are to be developed at short intervals, as in the type of experiment shown in Table VI, but not illustrated therein, the last reaction is apt to be feebler in color and less sharply demarcated than its predecessors.

TABLE I*

Passive Local Sensitization of Normal Skin by Sera from Sensitized Guinea Pigs

Sera injected intracutaneously on the back in amounts of 0.15 cc. Test substances, dissolved in 0.25 cc. corn oil, injected 2 days later under the skin of the abdomen.

Recipient	Test injections		Sera used in preparing skin sites			
	Time	Substance	Anti-*o*-chlorobenzoyl chloride	Anti-citraconic anhydride	Normal guinea pig serum 1	Normal guinea pig serum 2
No.	*hrs.*					
1	0	*o*-Chlorobenzoyl chloride 2 mg.	i = 10′ 26 × 24 ++, sl. el., 19′ 53 × 40 +, m. swol., soft, 67′	Neg.	Neg.	Neg.
	4½	Citraconic anhydride 1 mg.	No change	i = 7′ 60 × 54 +, el., very soft, 20′	Neg.	Neg.
2	0	Citraconic anhydride 1 mg.	Neg.	i = 4′ 28 × 28 +++, well el., 13′ 29 × 28 +, m. el., 22′ Color receded, 70′	Neg.	Neg.
	4½	*o*-Chlorobenzoyl chloride 2 mg.	i = 14′ 25 × 25 +, sl. el., 30′ Same, well el., 55′ Color receding, 105′	No change	Neg.	Neg.
3	0	Citraconic anhydride 1 mg.	Neg.	i = 4′ 30 × 25 ++, el., 14′ 36 × 29 +, el., 24′ Color receded, 70′	Neg.	Neg.
	4½	*o*-Chlorobenzoyl chloride 2 mg.	i = 17′ 24 × 22 +, el., 30′ 29 × 23 +, v. m. el., 55′ Color receded, 105′	No change	Neg.	Neg.

* In this and other tables, the interval (in minutes) between administration of the chemical and appearance of a reaction is designated by "i." The other entries show first the reaction area (cross diameters given in millimeters), then the degree of erythema (± to +++), finally the extent of edema. The key to abbreviated words is: sl. el., slightly elevated; v. m. el., very markedly elevated; swol., swollen; al. cls., almost colorless; ps., pseudopodial; tr., trace. Estimation of the edema seemed adequate, for it proved best to examine the area by very gentle palpation and to avoid folding of the skin.

Transfer Effects with Compounds of High Reactivity

Sera having a suitably high concentration of antibodies, obtained by injection of acyl chlorides and acid anhydrides, were injected in amounts of 0.1 to 0.15 cc. intracutaneously into the skin of the flank or back of normal guinea pigs. The skin sites so prepared reacted specifically in the manner described, when, on the following day, the corresponding incitant was injected locally into

the area or elsewhere, injections under the abdominal skin being used preferentially. The reactions could be elicited not only by the simple substances but also by protein conjugates of the respective chemical substances; *e.g.*, "phthalyl guinea pig serum," and the like.

A representative experiment is shown in Table I. Four skin sites were prepared on the backs of normal guinea pigs by injection of sera, two being from untreated guinea pigs and the others from animals sensitized respectively to *o*-chlorobenzoyl chloride and citraconic anhydride. After the lapse of 2 days the two substances, dissolved in corn oil, were injected subcutaneously in turn at an interval of several hours. Reactions developed after latent periods of 4 to 17 minutes. It is seen that regardless of which substance was injected first only the corresponding serum site reacted. The second round of testing demonstrated that sites failing to respond to the prior test material had remained capable of reacting.

Transfer Effects with Allergens of Lesser Chemical Reactivity

Transfer antibodies were also produced by allergens of lesser chemical reactivity, even though with these substances sera of satisfactory potency were encountered less frequently. The reactions of sera obtained after courses of injections with picryl chloride are shown in Table II, in comparison with a serum obtained to *o*-chlorobenzoyl chloride. Here likewise, regardless of the order of injecting the test substances, the corresponding serum site alone reacted. It will be noted that the reactions induced by picryl chloride developed slowly and only after fairly long latent periods (70 to 170 minutes), in contrast to the earlier response to the more highly reactive substance *o*-chlorobenzoyl chloride. When, however, the testing was done with preformed conjugates (recipients 3 and 4), the site prepared with serum to picryl chloride reacted promptly and much like the site prepared with serum against *o*-chlorobenzoyl chloride. The difference in reaction times seen with recipients 1 and 2 was therefore not a reflection of significantly different concentrations of antibody in the two sera but rather of the chemical individuality of the simple allergenic substances under test. Indeed, with antisera produced by treatment with 2:4 dinitrochlorobenzene, which is chemically less reactive than picryl chloride (*cf.* 1), the simple substance itself gave no more than a much delayed, transient graying of a prepared site (10 hours after testing).[5] In contrast, the antibodies were readily and typically demonstrable by the use of conjugates, as in Table III.

[5] For this effect, the skin was painted with 6 drops of a 10 per cent solution in olive oil at some distance from the prepared site, since this concentration has a high primary toxicity for normal skin. The highly reactive substance 2:4 dinitrofluorobenzene injected directly into prepared sites may have neutralized the antibody in the very center of the prepared area, but not the peripheral zone.

TABLE II

Passive Local Sensitization of Normal Skin by Sera from Sensitized Guinea Pigs

Experiment as in Table I, except that picryl chloride was injected in 0.5 cc. corn oil and the soluble protein conjugates were given in 1 cc. saline.

Recipient	Test injections		Sera used in preparing skin sites		
	Time	Substance	Anti-picryl chloride, No. 1	Anti-*o*-chlorobenzoyl chloride	Normal guinea pig serum
No.	*hrs.*				
1	0	Picryl chloride 2 mg.	i = 86′ el. papules over 17 × 12, 100′ 22 × 19 +±, knobby, v. m. el., 120′ 26 × 26 ++, enor. swol., umbilicate, 145′ 31 × 29 ++, enor. swol., 192′	Neg. “ “	Neg. “ “
	5	*o*-Chlorobenzoyl chloride 2 mg.	No change “ “	i = 22′ 29 × 24 ++±, m. el., 40′ 35 × 29 ++±, “ “ 100′	Neg. “
2	0	*o*-Chlorobenzoyl chloride 2 mg.	Neg. “	i = 18′ 25 × 19 ++±, sl. el., 38′ 27 × 20 +±, “ “ 133′	Neg. “
	3	Picryl chloride 2 mg.	i = 110′ small scattered dots, 110′ 22 × 22 large spots +++, 124′ 33 × 25 +, not el., 184′ 53 × 40 ±, “ “ 290′	No change “ “ “ “	Neg. “ “
3	0	Picryl casein 45 mg.	i = 10′ 45 × 35 + (spotty), cls. center, 17 × 15 10′ 48 × 48 +±, cls. center 12, 13′ Much faded, 50′	Neg. “ “	Neg. “ “
4	0	*o*-Chlorobenzoyl guinea pig serum 50 mg.	Neg. “	i = 10′ 25 × 20 +, sl. el., 5′ 25 × 22 +++, v.m.el., 40′	Neg. “
			Anti-picryl chloride, No. 1	Anti-picryl chloride, No. 2	Normal guinea pig serum
5	0	Picryl chloride 1 mg.	i = 70′ 37 × 32 +, prac. confluent, el., 99′ 70 × 48 ±, v. well el., 200′	i = 170′ 36 × 35 ±, sl. el., 200′ 50 × 40 cls., el., 290′	Neg. “ “

In this experiment, along with sites receiving the dinitrochlorobenzene serum, there have been included sites prepared with two guinea pig sera taken after a course of injections with phthalyl chloride. Upon testing with "2:4 dinitrophenyl guinea pig serum," the homologous site, it will be seen, reacted promptly and typically, while one of the phthalyl sera gave a delicate cross-reaction, coming to expression more promptly and definitely when the amount

TABLE III

Experiment as in Table II

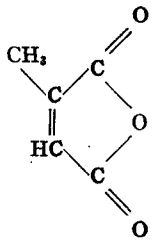
Recipient	Test injections		Sera used in preparing skin sites				Time
			Anti-2:4 dinitrochlorobenzene	Anti-phthalyl chloride No. 1	Anti-phthalyl chloride No. 2	Normal guinea pig serum	
No.	*hrs.*						
1	0	Dinitrophenyl guinea pig serum 10 mg.	i = 12′				
			22 × 20 +, sl. el.			Neg.	19′
			28 × 24 +, el.			"	40′
			28 × 24 ±, "			"	120′
					i = 168′		
				Neg.	Delicate patchy erythema over 18 × 13	"	208′
				"	19 × 15 +, (mottled), not el.	"	268′
	24	Phthalyl guinea pig serum 10 mg.		i = 9′	i = 9′		
			Neg.	23 × 21 ±, flat	25 × 25 ±, sl. el.	Neg.	12′
				26 × 26 +, sl. el.	33 × 30 +±, v. m. el.	"	27′
2	0	Dinitrophenyl guinea pig serum 50 mg.	i = 7′				
			31 × 29 +, m. swol.	Neg.		Neg.	16′
					i = 30′		
			33 × 31 +, enor. swol.	"	24 × 24 ±, delicate erythema, ps. margin, sl. el.	"	35′
				"	26 × 26 +, sl. el.	"	40′
	24	Phthalyl guinea pig serum 50 mg.		i = 6′	i = 6′		
			Neg.	30 × 30 +++, m. el.	40 × 40 +++, m. el.	Neg.	10′
			"	50 × 35 ++, v. m. el.	48 × 45 ++, v. m. el.	"	20′
			"	55 × 42 ±, swol.	55 × 50 ±, swol.	"	30′

of conjugate was increased fivefold (recipient 2), and the other phthalyl serum did not respond to either test dose. Injection of "phthalyl guinea pig serum" on the following day caused both of the homologous test sites to respond within 10 minutes, and without evident diminution in the case of the serum which had exhibited the cross-reaction. Despite the partial crossing, which will be discussed later, the reactions exhibited, therefore, the attributes of serological specificity, in that the homologous antigen-antibody reaction was the greater.

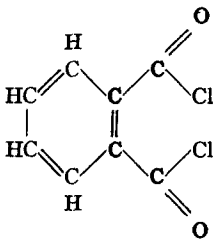
Specificity of the Transfer Reactions

Reactions of absolute specificity were presented in Tables I and II. In several cases cross-reactions of varying magnitude were seen, paralleling those

encountered in actively sensitized animals. Observed instances and some expectancies are recorded in Table IV. The cross-reactions can be ascribed largely to a substantial degree of configurational identity (see formulae), particularly among the products formed upon conjugation of the respective substances (*e.g.*, phthalic anhydride, phthalyl chloride, citraconic anhydride) with proteins. These cross-reactions were not inconsiderable but were weaker than the homologous ones and on the second injection the reactions were specific, an effect comparable to a serum made specific by absorption with a heterologous antigen. Such a case is illustrated in Table V, which shows an outstanding instance of cross-reactivity, almost surely reflecting similar chemical configurations, namely that between citraconic anhydride and phthalyl chloride (or similarly phthalic anhydride).



Citraconic anhydride



Phthalyl chloride

With respect to cross-reactions seen in actively sensitized guinea pigs, it is evident that treatment of guinea pig skin with various simple substances, for sensitizing and for testing, will lead to the formation of corresponding conjugates all having in common a moiety, probably protein, with cavy specificity. Experience with artificial conjugated antigens (19), however, has shown the wisdom of selecting different protein components for the antigens to be used for testing and those used for immunizing in order to avoid reactions that depend upon structures other than the attached chemical radical. Such for instance would be areas of the protein surface having altered properties because of immediately contiguous sites of coupling, or spatial structures at the points of attachment, including the basal portion, but not necessarily the entirety, of the attached radical, together with the neighboring portions of the protein molecule. Cross-reactions attributed to both these types of configuration rather than to the whole structure of the attached radical, have been described (19: see pp. 158, 159).

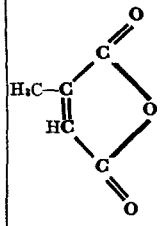
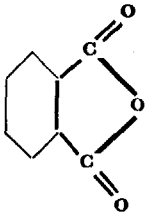
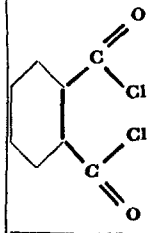
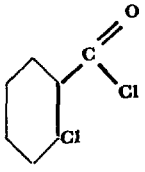
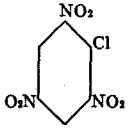
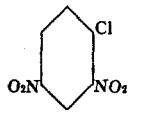
Among our animals, phthalyl chloride in particular led to broad sensitivity, although the homologous reaction was strongest by far. It would seem that some of the cross-reactions may have occurred by reason of configurational structures smaller than the entirety of the simple substance; *e.g.*, reactions in those animals sensitized to phthalyl chloride given by picryl chloride or by picryl casein, the latter at times even leading to anaphylactic shock upon intravenous injection. Citraconic anhydride and *o*-chlorobenzoyl chloride, and their respective conjugates with proteins, have been far enough apart in structure not to give cross-reactions.

The cross-reactions found upon transfer with serum (Table IV) have practically duplicated in scope, and in relative intensity, those observed in the actively sensitized animals. Thus, the sera from phthalyl chloride–treated animals exhibited the broadest range of reactivity, giving cross-reactions even with picryl and dinitrophenyl groupings (*cf.* Table III), likewise when the picryl groupings were attached to casein. The available transfer sera to *o*-chlorobenzoyl chloride exhibited less crossing than did tests on guinea pigs actively sensitized with it,

TABLE IV

Cross-Reactions with Transfer Sera (Combined Table)

The testing was done by the subcutaneous route, 1 or 2 days after local preparation of the skin. Conjugates were used in amounts of 10 to 100 mg., while the simple substances were employed in 2 mg. test doses except for 1 mg. in the case of citraconic anhydride. The relative strengths of the reactions are shown by the symbols ± to +++.

Test substance	Transfer sera prepared against					
	Citraconic anhydride	Phthalic anhydride	Phthalyl chloride	*o*-Chlorobenzoyl chloride	Picryl chloride	2:4 Dinitrochloro-benzene
						
Citraconic anhydride	+++	+	± to +±	0	x	
Phthalic anhydride	±	+++	+++	0[a]	0	0
Phthalyl chloride	0 to +±	+++	+++		x	
Phthalyl guinea pig serum	0 to ++	+++	+++	0[a]	0	0?
o-Chlorobenzoyl chloride	0		0[a]	+++	0	
o-Chlorobenzoyl guinea pig serum	x	0	+ (often slow)	+++	0	
Picryl chloride	x		0 to tr.	0	+±	
Picryl casein	0	0	+±	0[a?]	+++	+
Dinitrophenyl guinea pig serum		0[a]	0 to +	0	± to +	++±

[a] The cross-reactions of actively sensitized guinea pigs suggest that with some sera reactions may yet be found.

[x] From cross-tests on actively sensitized guinea pigs, reaction would not be expected.

despite its partial configurational relationship with substances such as phthalyl chloride and phthalic anhydride. Closer interpretation of such cross-reactions as shown in Table IV require, of course, studies with sera of precisely known antibody content. Skin testing with preformed conjugates would offer a choice of protein moieties and accordingly the opportunity of testing whether reactions are directed only toward the attached chemical radical.

In studying cross-reactivity of sera by passive local sensitization of normal skin, it is to be pointed out that the capacity of a skin area to react may become temporarily exhausted, for

TABLE V

Passive Local Sensitization of Normal Skin by Sera from Guinea Pigs Sensitized to Cross-Reacting Substances

Sera injected intracutaneously in amounts of 0.15 cc. Test substances (1 mg.) given subcutaneously in 0.25 cc. corn oil on the 2nd and 3rd days respectively.

Recipient	Test injections		Sera used in preparing skin sites			
	Time	Substance	Anti-citraconic anhydride	Anti-phthalyl chloride	Normal guinea pig serum 3	Normal guinea pig serum 4
No.	*hrs.*					
1	0	Citraconic anhydride	i = 8′ 75 × 62 ++,el.,soft, 23′	i = 13′ 22 × 22 ±, flat, 43′	Neg.	Neg.
	24*	Phthalyl chloride	Neg.	i = 40′ 27 × 27 +++, el., 75′	Neg.	Neg.
2	0	Citraconic anhydride	i = 7′ 75 × 48 ++, el., 27′	i = 12′ 23 × 23 +, sl. el., 27′	Neg.	Neg.
	24*	Phthalyl chloride	Neg.	i = 45′ 25 × 25 +, sl. el., 90′	Neg.	Neg.
3	0	Phthalyl chloride	i = 21′ 29 × 27 +++,m.el., 43′	i = 8′ 42 × 35 el., +++, enorm. 43′	Neg.	Neg.
	24*	Citraconic anhydride	i = 3′ 79 × 40 ++, sl. el. 15′	Neg.	Neg.	Neg.

* All sites appeared normal at 24 hours.

as long as 6 to 8 days, following a reaction; this circumstance may lead one at times to erroneous conclusions when a subsequent stimulation fails to develop another reaction in the area.

Transfer Effects with Common Antigens and the Corresponding Antisera

The fact that the reactions can be elicited by injection of protein conjugates and not only by the simple compounds themselves naturally suggested a comparison with sera produced with other types of antigenic substances. Actually, reactions of the same type were seen when guinea pig sera prepared against horse serum and against low ragweed were compared directly with a serum obtained after a course of intracutaneous injections of citraconic anhydride (Table VI). No matter what the sequence of injecting the corresponding

test substances was, at each test the homologous prepared site was the only one to respond; further, the responses occurring in the different local areas

TABLE VI

Passive Local Sensitization of Normal Skin by Various Guinea Pig Sera

Sera injected intracutaneously 2 days before, in amounts of 0.05 cc. (No. 1) or 0.15 cc. (No. 2). Test substances injected subcutaneously.

Recipient	Test injections		Sera used in preparing skin sites			
	Time	Substance	Anti-citraconic anhydride	Anti-ragweed	Anti-horse serum	Normal guinea pig serum
No.	*hrs.*					
1	0	Horse serum 1 cc. 1:2	Neg.	Neg.	i = 15′ 31 × 25 ++, m. el., 29′ 44 × 30 +, m. swol., 51′ 44 × 30 ±, v. sl. el., 120′	Neg.
	2	Ragweed extract 1 cc. "2.5%"	Neg.	i = 8′ 22 × 18 +, 13′ 25 × 20 ±, el. 29′	No change	Neg.
	4	Citraconic anhydride 1 mg. in 0.25 cc. oil	i = 4′ 70 × 34 ++, el., gray umbilicate center 17 × 16, 14′ 75 × 46 cls., m. el., 35′			Neg.
2	0	Citraconyl conjugate with guinea pig serum 15 mg. in 1 cc. saline	i = 12′ 25 × 25 ++, el., 25′ 23 × 23 al. cls., sl. el., 180′	Neg.	Neg.	Neg.
	3	Ragweed extract 1 cc. "7%"	No change	i = 15′ 25 × 25 +, 42′ 27 × 27 ±, 80′ tr., 180′	Neg.	Neg.
	6	Horse serum 1 cc. 1:2		No change	i = 23′ 30 × 25 +, el., 48′	Neg.

followed closely a common pattern. One will note that "citraconyl guinea pig serum," a preformed conjugate, elicits the reaction just as does the highly reactive chemical substance itself.

Other types of common antisera produced in guinea pigs and rabbits were investigated in the same manner (4) and upon intracutaneous injection were found to prepare cavy skin for

local reactions. In the case of immune sera from rabbits, as contrasted with sera from guinea pigs, zonal phenomena have been encountered with respect to the relative quantities of antibody and antigen to elicit optimal responses in guinea pig skin. Apart from problems posed by the toxicity of rabbit serum—less than half of the guinea pigs injected with undiluted rabbit antiserum have proved useful in that they have shown only transient, mild erythema with suitable recovery within 2 or 3 days—we have usually found it advisable to prepare the skin with rabbit immune sera diluted 1:5 to 1:30 (*cf.* 4). Both guinea pigs and rabbits, after injections of dead tubercle bacilli in a hydrocarbon vehicle, have produced sera, largely of anticarbohydrate specificity, which give immediate local reactions of the type described above following subcutaneous injection of old tuberculin; this study is still in progress.

Passive Sensitization of the Entire Skin

For preparation of the skin, the local injection of antibody may be replaced, less satisfactorily, by subcutaneous, intraperitoneal, or intravenous injection of larger quantities of the same immune serum, the skin being then generally reactive (and responsive to successive tests over a period of several days). Here also, the reactions are erythematous, edematous, and transient, but they have been less sharp and discrete than in passive local sensitization of the skin. For instance, quantities of the order of 25 cc. given intraperitoneally 1 or 2 days before testing of the skin can be used in place of local injections; *e.g.*, with citraconic anhydride immune serum. In this case, the skin responses can be developed either by scratch testing with citraconic anhydride or by the intracutaneous injection of preformed conjugate, as "citraconyl guinea pig serum."

Transfer experiments of this nature, with antiprotein sera, were conducted previously by Dienes, who injected large amounts of high titered homologous antiserum into normal guinea pigs by the intraperitoneal route and studied the development of the "evanescent type" of skin reaction in response to intracutaneous injection of the corresponding antigen (20; *cf.* 21). Ramsdell (13) likewise on occasion gave antibody intraperitoneally, or subcutaneously, and made a test injection of antigen in the ear or intracutaneously on the abdomen; at the same time, trypan blue was injected into the blood stream in order to facilitate reading of the reactions; her chief experiments are described below. More recently, Fell, Rodney, and Marshall (22) have employed trypan blue similarly in transfer experiments in the rabbit.

Transfer Reactions and the Use of Vital Dye

Ramsdell (13, 17) carried out transfer experiments in guinea pigs and rabbits with immune serum against horse serum prepared in both species, and cleverly employed an intravenous injection of trypan blue immediately prior to the test injection of antigen, so that the area participating in the immediate skin reaction became delineated by local accumulation of dye in consequence of alteration in capillary permeability owing to the antigen-antibody reaction (*cf.* 23). In her experiments, the ears were found to offer the most sensitive test areas, but a few tests were made on the belly skin on the guinea pig. The ear of both the rabbit and the guinea pig could be passively sensitized by local

injection of antibody, and the reaction elicited by the injection of antigen into the same or a contiguous site (13, 17, 24, 25). Other findings are described later; here it may be remarked only that the method was sufficiently sensitive to demonstrate reversed passive reactions, that is, the occurrence of reactions immediately upon injection of antibody into the skin, the antigen having been injected intravenously at a prior time.

Using in place of trypan blue the less diffusible dye pontamine sky blue 6B, freed from salts as described by Parsons and McMaster (26), we made several attempts to employ an intravenous injection of the vital dye in the hope of identifying in the sera of our sensitized guinea pigs antibodies present in lesser concentrations than were detectable visually. It eventuated, however, that sub-erythema reactions were not demonstrable by use of the dye. (More promising were some trials at building up the antibody concentration in a limited area of the skin, by depositing in it on each of 3 successive days three closely adjacent blebs of undiluted antiserum, and testing on the 4th day.)

In these experiments, various dilutions of a few types of guinea pig antisera in saline or in normal guinea pig serum were used for preparing skin sites, and some recipient animals were tested with the specific allergenic material alone, others with this and dye in addition.[6] With preparatory injection of antibody in the skin of the back, it eventuated that blueness developed in the various sites with intensities (and with speeds) varying with the preparatory concentration of immune serum, and the manner of development of the reactions was portrayed delicately, but the dye would accumulate only in areas of antibody concentrations capable of showing visible reactions without the dyestuff. A preliminary, direct comparison of the ear and the skin of the back with use of vital dye yielded essentially equivalent effects, and while our presumptive hope might have been realized by more extended tests on the ear skin (*cf.* 17), these were not pursued, for the latter site was not suitable for the bulk of the work, such as comparative testing of several sera at one time, and the dye method itself was obviously inapplicable when successive tests were to be made on one recipient.

Antibody Concentrations; Quantitative Relations in Eliciting the Reaction

As for the concentration of antibody in guinea pig sera which effect local cutaneous transfer, reactions in the case of the better sera were obtained in sites prepared with 1:8 to 1:30 dilutions in saline or normal serum. Antibodies were at times demonstrable in ring tests with soluble protein conjugates (as also in artificial agglutination tests using as agglutinogen soluble protein conjugates adsorbed to collodion particles[7]), but in other cases have not been surely

[6] The technique, as adapted from unpublished data which Dr. Philip McMaster kindly made available, is as follows: 1.5 mg. pontamine sky blue 6B in 1 cc. saline was injected intrajugularly into a prepared recipient guinea pig of about 300 gm. weight (5 mg. per kilo) at a time when the reaction could be expected to commence within the succeeding 20 to 30 minutes; accordingly, in some cases the subcutaneous test injection preceded rather than followed the dye injection. Injection of the dye should, however, be made prior to the development of a visible erythematous reaction, for local edema hinders accumulation of the dye.

[7] The collodion particles were kindly supplied by Dr. Jules Freund (27).

detectable except by actual trial in skin transfer experiments, which therefore has been the method of choice (*cf.* 28, 25).

Anaphylactic antibodies were present in far higher concentration than in the sera studied previously (1), where 1 cc. amounts of the best antisera to picryl chloride, and usually 3 cc. of the common antisera, were necessary to give, upon injection into a normal female guinea pig, demonstrable sensitization of uterine horns in the Schultz-Dale test; and transfer was not then feasible with sera from animals treated with 2:4 dinitrochlorobenzene. The higher concentrations of antibody now developed to both of these substances have been, we may conclude, the result of successive restimulations with the allergens over a longer period and of a method allowing readily a more careful selection of sera. In particular, it was now found that upon transfer of antiserum developed to 2:4 dinitrochlorobenzene (4 cc.) the normal recipient animal developed definite anaphylactic symptoms when injected intravenously on the next day with 20 mg. dinitrophenyl guinea pig serum. With the other substances studied in the

TABLE VII

Passive Anaphylactic Transfer with an Antiserum Obtained after Injections of Citraconic Anhydride

Serum injected by intraperitoneal route, diluted to a total volume of 3 cc., 24 hours before the test shown; the recipients weighed 340 gm.

Recipient	Volume of serum injected	Intravenous injection of 5 mg. citraconyl guinea pig serum
No.	*cc.*	
1	2.0	Typical anaphylaxis. Death 3′
2	1.5	" " " 4′
3	0.5	" " " 4′
4	0.5	Coughs, jerks, severe convulsion; survived
5	0.2	Negative

present paper, the antibody level attained will reflect their greater chemical reactivity, as well as the length of the sensitization period. As seen in Table VII, fatal anaphylactic shock was demonstrable on the day following transfer of 0.5 cc. of one serum developed to citraconic anhydride. The antibody levels may be compared with the findings in the quantitative experiments of Kabat and Boldt (29) on homologous passive transfer with sera from guinea pigs immunized with ovalbumin: with their sera of high antibody content secured by means of the Freund adjuvant technique and supplementary courses of injections as well, it may be calculated that 0.1 to 0.15 cc. was necessary to lead to fatal anaphylaxis. With an anti-horse serum guinea pig serum prepared by us with the same adjuvant method, but less intensively, only 0.2 cc. was necessary to induce a sensitivity that resulted in fatal shock within 5 minutes when 0.1 cc. horse serum was injected intrajugularly 24 hours later.

The effect of varying the amounts of an antiserum and of its corresponding test substance (citraconic anhydride) is shown in Table VIII. As less of the test substance was employed, both the size of the reactions and the degree of edema decreased, and this effect was the more marked the smaller the amount of antibody used for preparing the site. When so little chemical allergen was used that reactivity was not elicited (recipient 4), the site remained as it were poised, and responded to a later adequate test. When, however, the amount of allergen was such as to induce some partial reaction (recipient 3), this amount sufficed to exhaust the reactivity. The same situation held when the experiment was repeated with an anti-horse

serum guinea pig serum and varying amounts of horse serum were used to elicit the reaction. In both cases, the smaller amount of antibody required a greater amount of allergen or protein in order to cause a reaction, a relationship which appears to differ from that obtaining in the transferred Arthus phenomenon in the rabbit (15). Had the concentration of antibody in the serum been considerably higher, as in rabbit immune serum, the highest test dose might well have given only a transient graying of the skin, and complete discharge ("flash" reaction).

TABLE VIII

Effect of Varying the Amounts of Antiserum and Allergen on the Transfer Reaction

Skin sites prepared with 0.15 cc. of dilutions in saline of an antiserum developed by courses of injections with citraconic anhydride. One day later, the various amounts of citraconic anhydride were deposited subcutaneously in 0.25 cc. of corn oil. On the day after the recorded reaction, the skin sites again appeared normal. Only one measurement is given when both diameters were alike.

Recipient	Test dose	Skin sites prepared with serum				Time
		0.1 cc.	0.05 cc.	0.025 cc.	0.0125 cc.	
No.						
1	2 mg.	i = 8′	i = 8′	i = 8′	i = 8′	
		24 +±, m. el.	21 +, sl. el.	21 +, sl. el.	17 +, sl. el.	20′
		27 × 24 ±, " "	22 ±, el.	22 ±, el.	18 ±, v. sl. el.	55′
2	0.5 mg.	i = 18′	i = 18′	i = 18′	i = 18′?	
		17 +±, not el.	14 +±, not el.	14 +, not el.	12 ±, not el.	30′
		20 ±, " "	18 ±, " "	18 ±, " "	12 al. cls., " "	70′
3	0.1 mg.	i = 26′	i = 26′	i = 26′	i = ?	
		18 ±, (incomplete), not el.	11 ±, not el.	7 ±, not el.	0?	33′
		24 al. cls., el.	20 ±, " "	13 tr., " "	V. sl. reac.	70′
4	0.02 mg.	Pos.?	Pos.?	tr.	tr.	55′, 180′
3 (retested)	1 mg. 1 day later	Sl. diffuse color over 28 × 16, Sl. color	0 Sl. color	0 V. sl. color	0 Prac. 0	15′ 55′
4 (retested)	1 mg. 1 day later	25 +++,m.el. 29 ±, " "	20 +++,sl.el. 25 ±, m. el.	18 +++,el. 22 al. cls., el. (soft)	13 faintly brownish, not el. 18 cls., el. (soft)	20′ 55′

Observations on the Nature of the Antibody

There is, as described, a relatively high concentration of anaphylactic antibody in the transfer sera. The question whether various antisera of one specificity exhibit a constant relationship between the quantities conferring passive anaphylactic transfer and local cutaneous preparation of the skin was not answered definitely, because of technical obstacles, but no sharp differences were observed (*cf.* 17). This relationship has been questioned in several reports, as (14), and in studies of some human antibodies (24, 25, 30).

In a few experiments with heating of the transfer serum, there was no clear-cut heat lability such as is seen in the case of the human antibodies termed "reagins" with reference to their property of sensitizing normal human skin (*cf.* 31) and as is reported to occur in the sera of cows actively sensitized with extract of ragweed pollen (32). Rather, we observed that with progressive heating the activity of the sera declined gradually. With an antiserum developed by injections of citraconic anhydride, heating at 60°C. for 30 minutes evidently destroyed half or more of its activity, but the serum was still weakly active when the heating was prolonged at 60° to 1 hour or was done at 63° for 30 minutes. With a guinea pig antiserum to horse serum, the activity was reduced by two-thirds upon being kept at 65° for 2 hours, but there was almost no alteration upon heating at 56° for 4 hours, a treatment usually found adequate to "destroy" the reaginic antibodies in human sera. The effectiveness of heat in altering human reaginic sera likewise in part may depend upon concentration of antibody: thus in the experience of Schmidt and Lippard (31) heating at 56°C. had to be prolonged to 7 hours with one human serum out of thirteen, and it was found that this particular serum had the highest concentration of antibody. As far as the guinea pig antisera are concerned, it would seen unwise at present to view the portion first rendered ineffective by heat as belonging in a special "labile" category.

DISCUSSION

In the foregoing it is shown that guinea pigs sensitized to some simple chemical compounds have circulating antibodies which are capable, upon transfer, of inducing skin reactivity of the immediate type.[8] After the manner of testing used with human reagins, the reaction can be elicited in the prepared sites either by local application of reactive materials as in the Prausnitz-Küstner test or by injection at a distant point as in the "*Fernauslösung*" reaction of Jadassohn and others (*cf.* 33, 34, 35). The reaction in guinea pig skin consists of erythema, often spreading pseudopodially, and marked edema, all developing in the course of some minutes under proper conditions of test and receding within an hour or so. The reaction undoubtedly is the same as the "evanescent" type studied by Dienes (20, 21) in guinea pigs sensitized to egg white, and it reproduces the "wheal-and-erythema" type of reaction observed by Jacobs *et al.* (6, 8, 9) on animals actively sensitized to anhydrides.

Early reactivity upon contact with the incitant is not commonly encountered in hypersensitiveness to simple chemicals, although such effects have been observed. Very definite reactions occurring after a short time were found in

[8] The experiments here presented deal with the transfer of reactions of the immediate type; studies on the transfer of reactions of the delayed (contact dermatitis) type have been communicated preliminarily (3), in which case the mechanism of transfer involves the transfer of white cells from the sensitized animals rather than of their serum.

guinea pigs by Jacobs (9), who used as sensitizers anhydrides such as citraconic anhydride or, with less effect, an acyl chloride. In human beings immediate reactions have been described with salvarsan (36, 37) and, in a case of exquisite hypersensitivity to formaldehyde, Horsfall experienced reactions within 10 minutes after contact with 1:30,000 formaldehyde solution.[9] Also, in a patient sensitive to phthalic anhydride Kern (16) saw wheal reactions within 20 to 30 minutes after making a scratch test, and in a more extensive study Feinberg and Watrous (38) observed immediate reactions to chloramine-T (sodium *p*-toluenesulfonechloramide) and to halazone (*p*-sulfonedichloramidobenzoic acid) in factory workers who had become sensitized to these compounds. Also, patients giving immediate reactions to sulfathiazole (39) and to sulfadiazine (40, 41) have been studied experimentally. Kern (16) with phthalic anhydride, Feinberg and Watrous (38) with chloramine-T and halazone, Shaffer, Lentz, and McGuire (39) with sulfathiazole, Whittemore and de Gara (40) and Sherman and Cooke (41) with sodium sulfadiazine, and Ensbruner (37) with salvarsan—see also Biberstein (42)—have all reported passive transfer of reactions of the immediate type in skin sites prepared with serum from highly selected allergic individuals. Such positive reports are, however, relatively rare in the extensive studies on human allergy, and most observers have failed in their attempts. Consequently for long only minor consideration was given in the literature to affirmative statements.

The contrast between the ease with which transfer can be effected in experimental animals, once the method has been established, and the generally negative transfer experiments in human beings is probably to be explained by the necessity of securing sera with a sufficiently high concentration of antibodies and by working with substances of high chemical reactivity. With regard to the first point, the animals that produced active serum had been subjected to fairly prolonged treatment, like the factory workers mentioned by Kern, and by Feinberg and Watrous, and the potency of the sera varied according to the time at which they were drawn. Secondly, as to the nature of the substances used, potent transfer sera (and sera allowing passive transfer of anaphylaxis (7, 1)) were most readily obtained, and reactions in prepared skin sites took place most quickly with substances of high reactivity, such as acyl chlorides and anhydrides, and in this category would be included both phthalic anhydride (16) and chloramine-T (38), although interpretation of the latter is at the moment puzzling.

But even with the less reactive compounds the presence of transfer antibodies was demonstrable: although the reactions following injection of the simple compounds might be delayed for some hours or even fail to develop, typical effects were seen when the releasing injection was made not with the

[9] Horsfall, F. J., Jr., personal communication.

simple compound but with a protein conjugate thereof. In this manner transfer reactions were effected not only with the sera of guinea pigs treated with picryl chloride but also with guinea pig antisera against the typical human allergen 2:4 dinitrochlorobenzene. Animals being sensitized with the less reactive allergens, therefore, may possess such circulating antibody but may fail to reveal it either by early responses to intracutaneous test or through transfer attempts with their serum, unless there are available readily reactive conjugates or analogues of the substances in question. It may be anticipated, therefore, that the detection of early-type reactivity in human cases likewise will depend on the nature of the chemical allergen or on the use of preformed conjugates for testing.

Since the corresponding protein conjugates served so well to elicit the reactions, antisera to horse serum and to ragweed extract were prepared in guinea pigs for comparison. Such sera gave the same type of reaction, but as in the case of drug hypersensitivity sera it was necessary to continue the immunization of the guinea pigs for 7 to 9 weeks; with the adjuvant technique of Freund (11), however, adequate concentrations of antibody to horse serum were obtained regularly. Rabbit antibody of several specificities (4) was found to sensitize guinea pig skin in the same way; for preparing skin sites it proved best to dilute powerful precipitating sera five- to thirtyfold. The effect of this, according to the values for antibody content given by Kabat and Boldt (29), would be to reduce the antibody concentration down towards the level that is met with in guinea pig immune sera.

Transfer of antiprotein sera from the rabbit and guinea pig into guinea pigs for the study of cutaneous reactivity had been made by Dienes (20, 21) and by Ramsdell (13, 17), as described above. The method selected by Dienes—intraperitoneal introduction of large amounts of antiserum—allowed careful observation of the "evanescent" type of reaction and served well for his special studies but in our hands has not yielded nearly so sharp reactions as has the local preparation of the skin. Ramsdell, working with sera which probably contained lesser concentrations of antibody than we have selected for use, but employing an intravenous injection of trypan blue to delineate local cutaneous reactivity and selecting a sensitive site (the ear skin), likewise injected antibody intraperitoneally, or subcutaneously, and tested the skin; she could demonstrate even reversed passive reactions. Later, the ear skin of normal guinea pigs was sensitized locally by injection of antibody, and the reactions were elicited a day later by injecting antigen into the prepared site and trypan blue intravenously or giving intravenously a mixture of antigen and trypan blue (17). By the latter procedure, Tuft and Ramsdell (24, 25) could detect antibodies in human sera from a case of horse-serum sickness,[10] and at times

[10] Karelitz and Glorig (30) obtained reactions with such sera in the Prausnitz-Küstner test in human skin.

antibody in the serum of asthmatic patients (43). Ramsdell, then, was the first to reproduce in guinea pig skin reactions "giving a counterpart in the experimental animal of the Prausnitz-Küstner reaction in man." The technique of the present paper (4) has since been used by Kulka and Hirsch (14) for testing rabbit antibody prepared against ragweed extract, and most recently Parventjev, Goodline, and Virion (44) developed reactions in guinea pig skin sites prepared with rabbit antiserum to a bacterial nucleoprotein (from *Hemophilus pertussis*), by testing the skin sites 1 day later with local injections of the antigen.

It would not seem improbable that the antibodies concerned in the cutaneous transfer in the guinea pig are the same as or are closely related to anaphylactic antibodies. This question has not been worked out on a sufficiently large scale. Several of the sera here used were tested for passive anaphylaxis, quantities as small as 0.25 to 0.5 cc. sufficing to induce the anaphylactic state, demonstrable by fatal shock upon intravenous injection of protein conjugates. When sera were heated, the skin-sensitizing activity was seen to fall off gradually as heating was prolonged or the temperature was increased, without evidence of the clear-cut heat lability said to be characteristic of human reagins. After being maintained at 60° C. for 30 minutes, a procedure deleterious to reagins, a serum showed the loss of half or more of its skin-sensitizing ability, but was not entirely inactivated in the same period of time at 63° C. Whatever the nature of the antibody, it has proved possible with guinea pig antiserum against phthalic anhydride to reproduce in the guinea pig the transfer effects observed by Kern (16) with human serum from a patient sensitive to phthalic anhydride.

The relative persistence of the transfer antibodies in guinea pig skin—6 days to 3 weeks or more—also deserves mention, for it approaches the behavior of human reagins deposited in the skin of normal human subjects, and stands in apparent contrast to observations on the shorter period of retention of rabbit agglutinins and rabbit precipitins after injection of these into normal rabbit skin (45). There are some other points of difference, whether or not referable chiefly to different antibody levels attainable in the two species. For instance, in the rabbit there appears to be essentially no incubation period before the prepared site is capable of reaction (13), whereas there is an incubation period in the guinea pig of 7 to 8 hours. Again, the rabbit shows a transferred Arthus reaction, absolutely or in degree unlike the guinea pig. Species differences obviously come into play, and so they may in other instances, such as in comparisons of human and guinea pig antibodies.

It is evident that the production of transfer antibodies, although secured by use of a sensitizing procedure (repeated injections into the skin), is in part at least independent of the concomitant development of delayed-type skin reactivity, for these antibodies were found also in guinea pigs that had been injected intraperitoneally with picrylated stromata of guinea pig erythrocytes

but did not exhibit any contact-type skin sensitivity. In the same way, the immune sera recently reported by Gell, Harington, and Rivers (46) as having been developed in rabbits by courses of injections with interesting new sensitizing substances may be expected to behave like our guinea pig antisera in transfer experiments.

The sera which are useful are those with a sufficiently high level of antibody;[11] the output of antibody fluctuates and may well fall or remain below detectable levels. Hence a low or undetectable concentration of circulating antibody need not reveal a correspondingly low state of sensitivity of the tissues of the actively sensitized animals (for instance, with respect to the anaphylactic state or skin reactivity), a correlation which has often been sought or inferred to exist (*e.g.*, 14, 47).

Turning to the subject of specificity it may be said that the essentially specific nature of the reactions was clearly demonstrated in several cases, for instance in the reactions of citraconic anhydride and *o*-chlorobenzoyl chloride (Table I) and of the latter substance and picryl chloride (Table II), where no overlapping was observed. In other instances in which cross-reactions were encountered, *e.g.*, phthalyl chloride–citraconic anhydride (Table V, *cf.* Table IV), specificity was clearly demonstrable on a second injection made at a later time, since a prior testing with the heterologous substance left a substantial residual reactivity for the homologous compound, which is evidently akin to partial absorption *in vitro*. The strongest cross-reactivity met with, namely between citraconic anhydride and phthalic anhydride or phthalyl chloride, is plausibly referable to chemical similarity of the substances, all being derivatives of dibasic acids with adjacent carboxyl groups. There were, besides, cross reactions between compounds without obvious relationship in constitution, which occurred as well in the actively sensitized guinea pigs; *i.e.*, a response to picryl chloride or to picryl casein being seen in animals sensitized to phthalyl chloride and in some measure in those sensitized to *o*-chlorobenzoyl chloride, and to *o*-chlorobenzoyl chloride in animals sensitized with phthalyl chloride. Here the explanation may be that the reactions were due more to proteins altered in a similar manner than to the radicals attached to these.

In our hands, reverse transfer was not fruitful, namely securing a reaction by injecting allergen or conjugate some hours to a day prior to the local or intravenous injection of antiserum. Although positive effects had been obtained by Ramsdell (13) in guinea pigs following intravenous injection of the antigen, with trypan blue to outline the reactions, we did not find the unmodified procedure at all promising, despite the fact that there is much to recommend it for some human (Voss (48, 49), Karelitz and Glorig (30)) and animal (50) experiments. In one experiment patterned after Voss, the best effect observed

[11] This is well expressed in the individual differences observed among sera from cases of chloramine-T sensitivity by Feinberg and Watrous (38: Table III).

was a colorless swelling over 28 mm., 3 hours after 1.5 cc. anti-horse serum guinea pig serum had been given intravenously, in a site injected the day before with 0.1 cc. undiluted horse serum; another site injected with 0.02 cc. horse serum remained essentially unaffected.

Following a transfer reaction, a temporary local exhaustion of the capacity of the skin to react was noted, lasting perhaps 6 to 8 days. It would seem that this exhaustion of a skin area may be a secondary phenomenon, not reflecting failure of the antigen-antibody system to react. It may well be a vivid example of the mechanism underlying so called "non-specific antianaphylaxis," *e.g.*, the occasional failure of tissues, as segments of small intestine, from guinea pigs sensitized to a multiplicity of antigens, to respond in the Schultz-Dale test to more than the first antigen tested (51–54). Its counterpart was probably encountered by Ramsdell (13) in the ear of a rabbit actively sensitized to horse serum, in which after a primary reaction to a 1:10,000 dilution of antigen the ear remained insensitive to reinjection for 2 days. It calls to mind, with regard to the "wandering" of urticarial reactions on a patient, the frequently encountered "skipping" of skin areas that have recently reacted (*cf.* 55). Other instances, in man, of temporary local refractoriness following whealing in normal skin are known and have been studied, both after specific Prausnitz-Küstner tests (56) and after transcutaneous introduction, by electrophoresis, of certain drugs, pilocarpine and eserine in particular (55); such effects and those to be seen in guinea pig skin following specific reaction would appear to be closely alike. Pertinent theoretical conclusions have been presented by Alexander, Elliott, and Kirchner (55).

SUMMARY

Evidence is presented to show that guinea pigs actively sensitized to simple chemical compounds form serum antibodies capable of sensitizing the skin of normal guinea pigs. Skin sites prepared as for the Prausnitz-Küstner test develop immediate-type ("evanescent") reactions with erythema and edema, upon subsequent injection of the corresponding simple compounds or protein conjugates thereof, and give effects resembling transferred reaginic reactions as seen in human beings. The antibodies were obtainable after sensitization by acyl chlorides, acid anhydrides, and also substances of lesser reactivity, picryl chloride and 2:4 dinitrochlorobenzene, which are human allergens. Observations are reported on the specificity of the antibodies and on various details of the reaction.

Like effects result when antiprotein immune sera and their corresponding antigens are employed for the test, making it highly probable that the antibodies secured after sensitization to drugs result from immunization by conjugates formed *in vivo*.

The sera obtained after sensitization with simple chemical compounds readily

confer passive anaphylaxis, and their capacity for sensitizing the skin declines gradually with progressive heating.

It was observed that following a reaction of substantial degree in guinea pig skin the area involved does not fully recover for some days its capacity to react, the effect being a manifestation, it would seem, of what has been termed "non-specific antianaphylaxis."

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