ELICITATION OF DELAYED ALLERGIC SKIN REACTIONS WITH HAPTENS: THE DEPENDENCE OF ELICITATION ON HAPTEN COMBINATION WITH PROTEIN*

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Allergic skin reactions of the delayed type may be conveniently considered to involve two distinct processes: first, induction of the sensitized state, and second, elicitation of the allergic reaction in the sensitized individual. With regard to the induction of sensitization, it is generally believed that low molecular weight substances (haptens) can function effectively in this respect if they combine *in vivo* with tissue constituents, presumably proteins. This belief, which had long been reasonably maintained on the basis of analogy with the conditions required for the production of antisera by haptens, achieved a high degree of plausibility as a result of the systematic studies of Landsteiner and Jacobs (1, 2).

The question as to whether hapten combination with protein is involved in the second process, namely, elicitation of the reaction, has received little attention. In many studies of the elicitation process, haptens which are homologous in structure to the hapten used for inducing sensitization often have produced widely divergent reactions: some homologues have elicited skin lesions, while other have not (3–5). This diversity of responses has been interpreted quite consistently in terms of specificity arising from the configurational conformity of the eliciting compound to the sensitizing hapten, little attention being paid to other factors that might modify the responses. However, from a consideration of these earlier studies, it appeared possible that, in addition to configurational features, at least one other factor—namely, combination of the eliciting hapten with protein—might be involved in accounting for the diverse responses sometimes obtained in eliciting reactions with haptens which are structurally similar to the substance used for inducing sensitization. Accordingly, we have sought to determine with a group of 2,4-dinitrophenyl compounds whether combination with protein is important in determining the effectiveness of haptens in producing delayed allergic skin lesions in sensitized guinea pigs and men.

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A previous attempt to evaluate the protein-combining characteristics of some haptens was made by Brownlie and Cumming who examined the *in vitro* combination of egg albumin and some amino acids with a group of trinitrophenyl compounds whose capabilities in eliciting lesions in sensitized guinea pigs had previously been thoroughly studied by Gell (4, 6).

EXPERIMENTAL AND RESULTS

In order to determine the capacity of homologous 2,4-dinitrophenyl compounds[1] for eliciting allergic responses in sensitized individuals, each compound was tested, by percutaneous application, on guinea pigs and men that had been previously sensitized by means of exposure to 2,4-dinitrofluorobenzene (DF).

Haptens.—These compounds were so chosen that they differed from each other only in the substituent attached to the 1-carbon. DF was prepared by the method of Cook and Saunders (7), and was purified by 3 successive vacuum distillations.[2] The other D compounds were obtained from commercial sources, and were purified by repeated recrystallization.

Guinea Pigs.—The guinea pigs used were male albinos, weighing from 300 to 500 gm. They were maintained on Rockland guinea pig pellets, were given water *ad libitum* and fresh vegetables once a week. The animals were sensitized by 5 or 6 intracutaneous injections of DF given on consecutive or alternate days. Each injection was about 0.14 ml. in volume, and corresponded to about 4.2 μg. DF. The solution used for these injections was prepared by 100-fold dilution with saline of a stock solution of DF in ethanol.

Seven to 14 days after the last injection, the animals were subjected, after clipping the hair, to multiple skin tests, each test consisting of an application to the skin of one drop of a solution of a different D compound. As many as 12 tests were carried out at the same time on one animal. This procedure required the use of a large skin area over the dorsal surface of the animal. To determine whether inconsistencies might arise from variations in reactivity of one skin area as compared with another, multiple tests (each consisting of one drop of 0.01 M DCl in corn oil) were carried out at the same time over longitudinal and transverse axes on the dorsal skin surface of sensitized animals. It was found that in guinea pigs weighing 400 gm. and over, the reactions were quite uniform within a roughly rectangular area 8.0 × 7.0 cm., symmetrically placed with respect to the midline, and with its long axis parallel to the animal's vertebral column.

Two days after the solutions were applied, the test areas were depilated[3] and about 3 hours afterwards the skin tests were read. The readings are recorded as follows: 0, no reaction; ±, an equivocal reaction; 1+, slight but definite reaction; 2+, moderately strong reaction; 3+, strong reaction.

Non-sensitized guinea pigs served as controls. Three to five such animals were tested with each D compound in identical manner, and at the same time, as were the sensitized animals. These controls exhibited no reactions, except for occasional, very faint erythematous reactions to 0.2 M DOH and DNH_2 and to 0.01 M DF, DCl, and DBr. When the sensitized animals gave entirely negative responses or a faintly erythematous reaction indistinguishable from that

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[1] The 2,4-dinitrophenyl group is hereafter referred to as D. Hence, DH, DCH_3, etc., refer respectively to *m*-dinitrobenzene, 2,4,-dinitrotoluene, etc.

[2] Additional DF was subsequently procured from Jasonols Chemical Corp., Brooklyn.

[3] Depilation was done with any one of several commercial depilators.

of the control animals, the reaction is recorded as 0. Despite the subjectivity inherent in this procedure, the distinction between a weakly positive and a negative reaction can be usually made.

Man.—Of the four human subjects, two (H.N.E. and L.O.) were sensitized by accidental exposure in the laboratory to DF and the two others (K. and H.) were deliberately sensitized by applying to their skin two drops of a 10 per cent acetone solution of DF.[4] The latter two were tested 2 weeks after their initial sensitizing exposure. Skin tests were performed on the human subjects by means of conventional patch tests: a piece of filter paper (Whatman No. 1) about 0.5 × 0.5 cm. was saturated with the solution of D compound, placed on the volar aspect of the forearm and covered with an adhesive plaster.[5] The adhesive covers and filter paper were removed 24 hours afterwards. The readings were made at 24 and 48 hours after

TABLE I

Elicitation of Skin Reactions with Various D Compounds in DF-Sensitized Guinea Pig and Man

Compound (D = 2,4-dinitrophenyl)	Concentration	Solvent*	Results‡	
			Guinea pig	Man
	mols/liter			
DH	0.2	A	0,0,0,0,0	0,0
DOH	0.2	A	0,0,0,0,0	0,0
DCH_3	0.2	A	0,0,0,0,0	0,0
DNH_2	0.2	A	0,0,0,0,0	0,0
DF	0.01	A	2+,2+,2+,2+,1+,1+,0	3+,3+,3+,2+
DCl	0.01	A	2+,2+,2+,2+,1+	3+,3+,3+,2+
DBr	0.01	A	2+,2+,2+,1+,1+	3+,3+,2+,2+
DSO_3Na	0.025	B	0,0,±,±	2+,2+,2+,3+
DSO_3Na	0.05	Bd	2+,2+,1+,1+,±	

* Solvents: A, equal volumes acetone and corn oil; B, ethylene glycol monomethyl ether (methyl cellosolve); Bd, 10 per cent Tween 80 in methyl cellosolve.

‡ Each recorded value represents the result of a single skin test.

application of the patch tests. Only the latter are reported since no systematic differences between the 24 and 48 hour readings were noted. The readings were graded in the same way as were the guinea pig tests.

Non-sensitized human controls were similarly tested to determine whether the D compounds produce irritant skin lesions in the concentrations used. In the concentrations reported in Table I, the D compounds produced no reactions in the human controls, with the exception of DH, as noted below.[6]

All elicitation tests on sensitized individuals, human and guinea pig, were initially carried out with 0.01 M solutions. Those compounds which failed to produce lesions at this concentra-

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[4] We wish to thank Dr. W. S. Tillett for permission to carry out some of the studies on human beings on the Third (New York University) Medical Division of Bellevue Hospital.

[5] Elastopatch, Duke Laboratories, Inc., Stamford, Conn.

[6] Of 11 human controls, none reacted to 0.01 M DF or DCl, but 5 reacted with a faint erythema to these compounds at 0.025 M. Of 5 other control subjects, none reacted to 0.01 M or 0.2 M of DNH_2, DCH_3 or DOH; however, 2 of 3 controls reacted to 0.2 M DH with a very faint erythema. No reactions were produced in 3 controls by 0.01 M or 0.025 M DSO_3.

tion were subsequently reexamined at 0.2 M, except in the case of DSO_3 which is described in detail below.

The results of the skin tests, with the highest concentrations used, are given in Table I. It is clear that 4 of the D compounds (D-H, -OH, $-CH_3$, $-NH_2$) failed to produce lesions,[7] while the 4 other compounds (D-F, -Cl, -Br, $-SO_3$) succeeded in producing skin lesions.[8]

In Fig. 1 are represented two dimensional projections of the van der Waals contours of the D compounds, drawn to scale using accepted bond angles, covalent radii, and van der Waals radii (8, 9). The small structural differences among these compounds indicate that their configurational features, *per se*, do not account for the great differences they display in eliciting lesions in the skin of sensitized guinea pig and man.

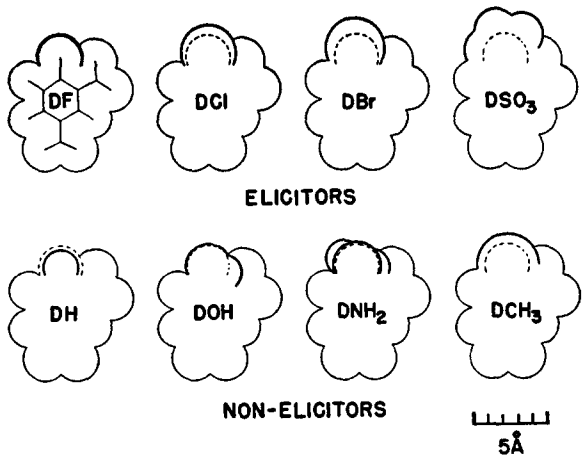

FIG. 1. Scale drawings of van der Waals contours of D compounds. The varying substituents on the 1-carbon are indicated by the bold lines. The F atom of DF, the sensitizing hapten, is indicated by the dotted line in each of the other drawings, to facilitate comparison.

In Vitro Combination with Protein.—A systematic attempt to determine the capacity of haptens to combine with tissue protein was made by Landsteiner and Jacobs (2) who compared a large number of chloro- and nitro-substituted benzenes in regard to (*a*) decomposition velocity in sodium alcoholates and (*b*) formation of substitution products with aniline. Although their results were highly significant in establishing as plausible that those haptens which could induce sensitization could probably combine with tissue protein, it was

[7] These 4 compounds are hereafter referred to as non-elicitors.

[8] These 4 compounds are hereafter referred to as elicitors.

felt that study of actual combination with protein, as is now feasible by a variety of means, is a more relevant procedure than is derivatization of aniline.[9]

It must be emphasized that study of combination with protein through the formation of covalent (irreversible) bonds rather than reversible binding to protein was the object of the present procedure. The former type of linkage was anticipated to be of greater relevancy than the latter type because at least one of the non-elicitors (DOH) is bound reversibly by serum albumin (10), and probably the other non-elicitors are as well. In order, therefore, to minimize the effects of reversible binding, the protein chosen for these *in vitro* studies was a gamma globulin.

Briefly stated, the procedure followed consisted of shaking a protein solution with an ethanolic solution of D compound for about 16 hours, precipitating all the protein through the addition of more ethanol, extracting all unreacted D compound from the precipitated protein, and, finally, determining the extent of derivatization on a solution of the thoroughly washed protein by measurement of the nitrogen concentration[10] and absorption spectrum. All procedures were carried out at room temperature. Preliminary experiments indicated that in some instances combination with protein was accompanied by a number of competitive side reactions—*e.g.*, the formation of DOH. In order, therefore, to maintain constant the concentration of unreacted D compound in solution during the entire experiment, a large excess was added—sufficient to insure its persistence as solid phase. Preliminary survey of the effect of pH on combination with protein also indicated that high pH was favorable in promoting protein combination. Thus, at pH 8.0 (borate buffer) DF combined readily, whereas DCl and DSO_3 failed to combine with protein (room temperature, 16 hours).

A typical experiment consisted of mixing 1.1 ml. of a saline solution containing 20 mg. bovine gamma globulin[11] and 27 mg. K_2CO_3 with 1.0 ml. of a saturated ethanol solution of D compound. Excess D (solid phase) was added (usually a total of 20 mg.) to assure continuous saturation of the system. In the case of DSO_3, this hapten was dissolved in water, no alcohol being necessary in the system. The above mixtures were prepared in duplicate. The tubes were shaken vigorously for a few minutes and then were rocked overnight at room temperature. The following day about 8 volumes of ethanol were added, precipitating any protein which still remained soluble. The precipitated protein was washed extensively with ethanol, acetone, and ethylene glycol monomethyl ether (hereafter referred to as methyl cellosolve) until the washes were colorless and clear. The precipitates were then dissolved in 0.1 N NaOH, insoluble material was removed by brief centrifugation, and the protein reprecipitated by the addition of trichloroacetic acid. Re-solution and re-precipitation were carried out twice to insure the complete removal of any adsorbed, unreacted D compounds. Finally, the precipitates were dissolved in 0.1 N NaOH. An aliquot of each solution was then diluted in 0.1

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[9] In relation to the effectiveness of certain simple chemical compounds in inducing precipitin formation in rabbits, when injected without prior combination with protein, Gell and his coworkers have previously measured the reactivity of some haptens with alanine (28).

[10] Kjeldahl analyses of DOH and DNH_2 indicated that, with the procedure used for determining nitrogen, the nitro groups of any D substituents in the protein would contribute little, if any, nitrogen. Even if these groupings had contributed all their nitrogen, the results would not be significantly different from those herein reported.

[11] Armour Laboratories, fraction II from bovine plasma.

N NaOH and an absorption spectrum obtained in the Beckman spectrophotometer, model DU (11). On other aliquots, in duplicate, nitrogen was determined by micro-Kjeldahl analysis (12). Samples of bovine gamma globulin were treated in an identical manner, save for omission of D compound, thereby providing control material. All absorption spectra were obtained in from 30 to 90 minutes after the solutions were prepared. This schedule was carefully adhered to because of slight spectral instability of protein in 0.1 N NaOH: in the time interval used, this instability introduces an error not greater than two per cent (13). It was anticipated that a considerably greater error would arise from instability of D amino acids (especially N^5-D-lysine) in this solvent (14); however, it was found that N^5-D-lysine was stable in 0.1 N NaOH during this time interval, and, in fact, for several hours longer.

TABLE II

Combination of D Compounds with Protein

Compound	*In vitro* combination with bovine gamma globulin*			*In vivo* combination with skin protein—D amino acid recovered
	Final protein color	E‡ at 360 mμ	$\frac{E \text{ at } 360 \text{ m}\mu}{E \text{ at } 290 \text{ m}\mu}$	
		× 10^{-3}		
None§	Colorless	0.3	0.03	—
DH	"	0.3	0.03	None
DOH	"	0.2	0.02	"
DCH_3	"	0.5	0.05	"
DNH_2	"	0.3	0.03	"
DF	Yellow	40.7	1.65	N^5-D-lysine
DCl	"	38.0	1.62	"
DBr	"	48.8	1.88	"
DSO_3Na	"	25.4	1.77	"

* The recorded values are averages of replicate (2 to 4) analyses.

‡ E is expressed as O.D./μg N/ml.

§ Control (bovine gamma globulin without D compound).

The absorption spectrum of the bovine gamma globulin control is compared with the absorption spectrum of derivatized gamma globulin, prepared with DF, in Fig. 2. The extinction (expressed as O.D. per μg. N per ml.) at 360 mμ is used to indicate the extent of substitution of dinitrophenyl groups in protein. The ratio of optical densities, or of extinctions, at 360 mμ/290 mμ is another means of determining the extent of derivatization. These values, and the gross color of the final protein precipitate, are given in Table II for all the D compounds. The data which appear in Table II show that the elicitor D compounds formed derivatives with gamma globulin, while the non-elicitor compounds failed to do so.

Data which are consistent with the foregoing have been reported with a different hapten, namely "tetryl" (2,4,6-trinitrophenylmethylnitramine). Gell (4) found that in guinea pigs sensitized with tetryl, 8 of 22 di- and trinitrobenzenes produced delayed allergic skin reac-

tions, and 14 did not. Subsequently, Brownlie and Cumming (6) studied 5 of the elicitors and 2 of the non-elicitors with respect to their ability to combine *in vitro* with egg albumin and amino acids, as determined spectrally. Of the 5 elicitors, 4 combined and 1 behaved equivocally; of the 2 non-elicitors neither combined.

In Vivo Combination with Protein.—The foregoing results provide grounds for supposing that the elicitor compounds combine with tissue protein while non-elicitors do not. However, because these results were obtainable only at

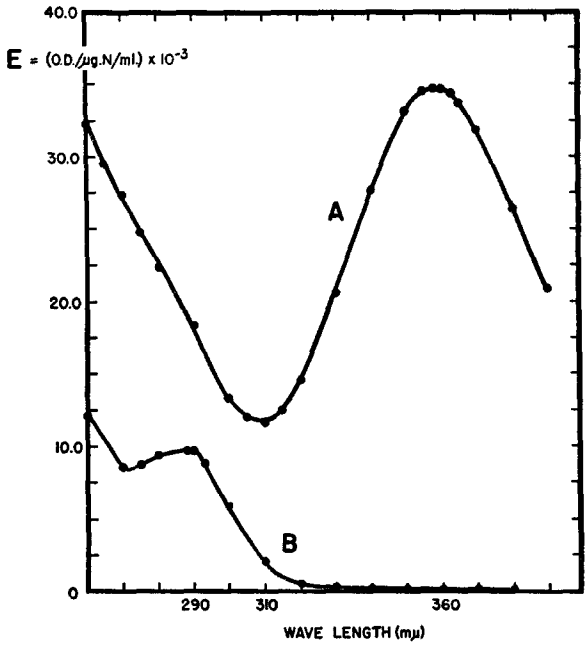

FIG. 2. Ultraviolet absorption spectra of bovine gamma globulin control (*B*) and bovine gamma globulin combined with DF (*A*). The latter is not included in the data of Table II since it was prepared in a manner slightly different from that used as routine: Continuous rocking overnight was neglected, the tubes remaining unagitated during this time interval. The proteins were dissolved in 0.1 N NaOH and examined in from 30 to 90 minutes afterwards.

such high pH (around 10), they cannot be considered as indicating anything more than what *might* take place in tissue. The effort was made, therefore, to obtain direct evidence for combination with tissue protein by isolating derivatized amino acids from skins that had been treated *in vivo* with D compounds. For this attempt, recourse was had to the technique developed by Sanger (15) for the characterization of protein end groups.

Sanger has shown that DF combines with free NH_2 groups of proteins and that after acid hydrolysis of the derivatized protein, dinitrophenyl amino acids[12] may be effectively separated from one another and identified chromatographically on silica gel columns. Accordingly, the procedure adopted was as follows: The dorsal surface of a non-sensitized guinea pig was clipped and then depilated over an area about 8 × 8 cm. About 3 hours after depilation the bare skin was sprayed (in a well ventilated hood) with about 0.5 ml. of a 10 per cent solution of D compound (*W/V*), the solvent consisting of 9 volumes acetone and 1 volume corn oil; the only exception was DSO_3Na which was sprayed as a 5 per cent solution in methyl cellosolve. By the following day irregular areas of superficial necrosis were sometimes noted on the animals sprayed with DF, DCl, and DBr. In the case of DH, 3 out of 6 animals died overnight, an occurrence which is not surprising in view of the well known toxicity of this compound. About 16 hours after spraying, the animals were sacrificed, the treated skin was promptly excised, and the epidermis separated from the dermis. With DF-, DCl- and DBr-sprayed skins the separation of epidermis was readily accomplished by gently peeling it from the dermis. With all other compounds the removal of epidermis was easily carried out after placing the whole skin on a hot plate at 50°C. for 2 minutes (16). Weighed samples of epidermis were then hydrolyzed with 6 N HCl in a sealed tube at 110°C. for 24 hours. With DF- DCl-, DBr-treated samples, 100 to 200 mg. wet weight epidermis were hydrolyzed; with all others, 700–1200 mg. wet weight epidermis were hydrolyzed. The latter large samples were obtained by combining the epidermal scrapings of two or three animals. After hydrolysis, the samples were diluted with water, extracted with ether, and the fractions were taken to dryness *in vacuo*. The dried acid-soluble and ether-soluble fractions were then taken up in appropriate solvents and run on silica gel in Zechmeister chromatography tubes of 1.0 cm. diameter. The silica gel was prepared by the method of Gordon, Martin, and Synge (17), following the recommendations of Tristram (18).[13]

In view of the purpose of the present experiment, the demonstration of any one D amino acid was deemed sufficient to establish that derivatization of epidermal protein had taken place. Since N^5-D-lysine was anticipated as being the D amino acid most likely to be found in high yield, this derivative was sought for in all cases. N^5-D-lysine, prepared according to the procedure of Porter and Sanger (19), was always run as a standard in a control column alongside the columns carrying the epidermal hydrolysates.

The acid-soluble fractions were examined on columns with 2 M KH_2PO_4 as the stationary phase, as recommended by Blackburn (20), with ether—66 per cent (*V/V*) methylethylketone as the moving phase.[14] On such columns the standard N^5-D-lysine has a retardation factor (R.F.) of 0.08 to 0.10.[15] Bright yellow bands with the same R.F. as N^5-D-lysine were obtained with the acid-soluble fractions of epidermal hydrolysates from animals sprayed with DF, DCl, and DBr. A very faint, but unequivocal, yellow band with this same R.F. was obtained with the corresponding material from animals sprayed with DSO_3. With the latter, 1.2 gm. of pooled epidermis (wet weight) from two animals sprayed with a total of 150 mg. DSO_3 yielded a barely perceptible band of R.F. 0.1. However, with DF, DCl, and DBr, epidermal samples of 100 to

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[12] Dinitrophenyl amino acids are referred to by Sanger and others as DNP amino acids. For the purpose of consistency, however, these substances are hereinafter referred to as D amino acids.

[13] We wish to thank Dr. E. A. Kabat of Columbia University for many helpful suggestions in regard to the chromatographic technique.

[14] The solvents are described with the terminology adopted by Tristram (18).

[15] The retardation factor (R.F.) is used as in the conventional sense: the ratio of the movement of the middle of a band to the simultaneous movement of the surface of the developing solvent in the tube above the packed silica gel.

200 mg. (wet weight) regularly gave intensely yellow bands with the R.F. of N^5-D-lysine. Mixed chromatograms prepared from standard N^5-D-lysine and acid-soluble fractions obtained from animals sprayed with DF, DCl, and DBr gave only a single band of R.F. 0.08 to 0.1.

When the foregoing solvent system was used to develop the acid-soluble fractions of epidermal hydrolysates from animals sprayed with non-elicitor compounds, no slow yellow bands were observed. Similarly, the corresponding ether-soluble fractions of these hydrolysates failed to yield any evidence of D amino acids when developed with chloroform and with chloroform-1 per cent (*V/V*) *n*-butanol. These negative results were obtained even with very large samples (0.7 to 1.0 gm. wet weight) formed by combining the epidermal scrapings from 2 or 3 animals. Negative results in the case of the non-elicitors were obtained even when such large samples were subjected to milder hydrolytic conditions (21) in order to minimize the destruction of the more labile D amino acids, had they been present. In most instances a faint band of R.F. greater than 1.0 was observed, but these, as pointed out previously (15, 21), are artifacts which do not represent D amino acids.

Since hydrolysis was carried out in 6 N HCl it was considered unlikely that derivatization of amino acids could take place during the hydrolysis by unreacted D compound. Nevertheless, in order to completely exclude this possibility, a sample of epidermis (200 mg. wet weight) from the untreated skin of a normal guinea pig was hydrolyzed in the presence of a large excess of DF (about 75 mg.). After hydrolysis under standard conditions (24 hours, 110°C.), dilution and ether extraction, the material was chromatographed, as described above. No trace of any D amino acid was detected. Consequently, any D amino acid recovered from the hydrolysates of skins that had been treated with D compound could only have been formed *in vivo*.

The results of the *in vivo* experiments are summarized in Table II. Since the elicitors formed N^5-D-lysine it is evident that these compounds combined with epidermal protein *in vivo*. DSO_3, however, performed relatively feebly in this regard as compared with the more effective elicitors—DF, DCl, and DBr. The non-elicitors, contrariwise, yielded no detectable N^5-D-lysine or other D amino acid, even when large amounts of tissue were subjected to mild acid hydrolyses.

The choice of epidermal samples, rather than whole skin, for isolation of D amino acids was based upon the following preliminary experiment. The skin of a non-sensitized guinea pig, after having been sprayed 16 hours before sacrifice with about 100 mg. DBr, was gently scraped to give epidermal and dermal fractions. Microscopic examination of portions of these fractions (after fixation in Zenker's solution and staining with hematoxylin and eosin) revealed that the epidermal part was histologically intact and uncontaminated by dermis; however, very small amounts of epidermis were present in the dermal fraction in the form of deep projections of hair follicles and as a few small, widely scattered, islands of epidermis. The dermal fraction was next crudely fractionated by shaving fine slices from its superficial surface with a razor. Histologic examination indicated that this procedure had removed, approximately, the most superficial one-third of the dermis. Four crude fractions were thus obtained: epidermis, whole dermis, superficial dermis, and deep dermis. Samples of each of the foregoing fractions, weighing 456, 490, 425, and 548 mg. wet weight, respectively, were then hydrolyzed and the acid-soluble fractions of the hydrolysates examined chromatographically. The epidermal fraction yielded considerable N^5-D-lysine, roughly estimated by visual comparison with standard N^5-D-lysine to be of the order of 100 to 1000 μg. The dermal fractions, however, failed to yield any N^5-D-lysine. It is estimated from the sensitivity of the procedure,

therefore, that the latter fractions had less than 20 to 30 μg. of N^5-D-lysine in about 400 to 500 mg. of tissue.

Further Considerations of DSO_3.—Elicitation of skin lesions with D compounds produced similar results in both guinea pig and man (Table I), except for the case of DSO_3. This compound, at a concentration of 0.01 M, had failed to produce lesions in man, but at 0.025 M lesions were produced in man and not in the

TABLE III

*Elicitation of Skin Reactions with DSO_3**

Concentration	Form in which applied	Solvent	Results‡	
			Guinea pig§	Man§
mols/liter				
0.01	DSO_3Na	Methyl cellosolve	—	0,0,0
0.025	DSO_3H‖	" "	0,0,0,0,0	3+,2+
"	DSO_3Na	" "	0,0,0,0,0	3+,2+,2+,2+
0.05	"	" "	±,±,0,0,0	
"	"	" " + D¶	2+,2+,1+,1+,±	
0.15	"	" "	1+,±,±,0,0	
"	"	" " + D¶	2+,2+,1+,1+,1+	
0.05	"	Water	±,0,0,0,0	
"	"	" + D¶	2+,1+,1+,1+,±	
0.15	"	"	0,0,0,0,0	
"	"	" + D¶	2+,1+,+,±,0	
0.10	"	Methyl cellosolve	(0,0,0,0,0)**	
"	DSO_3H‖	" "	(0,0,0,0,0)**	

* All test solutions used produced no reactions in non-sensitized control guinea pigs and human beings.

‡ Each recorded value represents the result of a single skin test.

§ In all individuals, sensitization induced by DF, except as noted in footnote**.

‖ Used as supplied commercially, not recrystallized.

¶ Detergent—Tween 80—present in solvent to extent of 10 per cent.

** In these animals sensitization had been induced by 10 intradermal injections of 5μg. DSO_3 each (over a 3 week period); the animals reacted to 0.025 M DCl in methyl cellosolve as follows: 3+,3+,2+,2+,1+. The same DCl solution produced no reactions in 3 non-sensitized control animals.

guinea pig. The results obtained by examining DSO_3 in several solvents at a variety of concentrations are summarized in Table III. Water and methyl cellosolve were the major solvents used since DSO_3 was not sufficiently soluble in less polar substances.

It is of interest to contrast the elicitation of skin reactions by DSO_3 with the elicitation by DF, DCl, and DBr. The latter group of effective elicitors regularly produced positive reactions at concentrations of about 0.005 to 0.001 M in the guinea pig, and about 0.005 to 0.00005 M in man. By contrast, the mini-

mal concentration required to elicit reactions with DSO_3 (with detergent omitted from the solvent) was about from 50- to 500-fold greater than with the other elicitors.

In order to determine whether the different solvents used were responsible for the foregoing differences in eliciting ability, a comparison was carried out between 0.01 M DF in a mixture of equal parts acetone and corn oil (solvent A, Table I), and in methyl cellosolve, using five sensitized guinea pigs and one sensitized human. Positive reactions were obtained in both solvents in all individuals. The intensities of the reactions were possibly slightly less with methyl cellosolve than with the mixed, less polar, solvent; nevertheless, it was apparent that the solvent, *per se*, did not account for the relatively feeble eliciting capacity of DSO_3.

The relative ineffectualness of DSO_3 as an elicitor may possibly be attributable either to (*a*) weak capacity for derivatizing protein, or, (*b*) poor penetration of epidermis. With respect to the former possibility, it does indeed appear from the *in vitro* studies (Table II) that DSO_3 introduces somewhat fewer D groups into bovine gamma globulin than do the better elicitors (DF, DCl, DBr)—about 40 and 60 D substituents per protein molecule, respectively.[16] This difference, however, is rather small, and seems insufficient to account for the great differences in the capacity to elicit skin reactions. It seems more likely that poor epidermal penetration is an important factor in accounting for the weak performance of DSO_3 as an elicitor. This latter possibility is particularly supported by the pronounced effect exerted by the detergent "Tween 80" (Table III). Thus, with water as the solvent, with Tween 80 present 0.05 M is an effective concentration for eliciting a positive reaction, but with the detergent absent, a concentration of 0.15 M is ineffectual in producing skin lesions.[17]

In regard to the foregoing remarks on epidermal penetration, it is of interest to note that when DSO_3 was injected intradermally into normal guinea pigs, in a manner comparable to that used for inducing sensitization with DF, all the animals, on subsequent examination, reacted positively to percutaneous testing with DCl (0.025 M in corn oil), but gave no reaction to DSO_3Na or to DSO_3H, the latter, also applied percutaneously, as 0.025 M in methyl cellosolve (see Table III, footnote**). Thus, when *injected intradermally*, DSO_3 was capable of inducing sensitization in guinea pigs.

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[16] Calculated on the basis of the molecular weight of bovine gamma globulin being 160,000, and the molar extinction coefficient of N^5-D-lysine at 365 mμ being 16,800 in 0.1 N NaOH (22).

[17] In view of these considerations of epidermal penetration, the following question may well be raised: Is poor epidermal penetration also a possible factor in accounting for the behavior of those D compounds which have been found not to elicit in concentrations as high as 0.2 M? This does not seem to be the case because the solubilities of the latter compounds are, unlike the highly ionic DSO_3, very much the same as the solubilities of the most effective elicitors. Furthermore, animals sprayed with DOH or with DNH_2 showed diffuse yellow staining of the cut surface of the dermis to a depth of about 1 mm. from the epidermal surface. In the case of DH, the death of 3 out of 6 animals within 16 hours of spraying (in a well ventilated hood) is considered to be assurance that considerable epidermal penetration had taken place.

DISCUSSION

The foregoing results demonstrate that the differences in ability to produce allergic skin responses displayed by a group of homologous haptens, having virtual identity of configuration, arise from corresponding capacities to derivatize skin protein. It is worthy of emphasis that, with the group of compounds studied, derivatization of protein involves the formation of covalent bonds. Although decisive data are not yet at hand, it would seem that reversible protein binding is probably not concerned with the production of delayed allergic skin lesions by simple chemicals. This would seem to be the case since DOH, a non-elicitor, undergoes reversible binding with serum albumin (10); and it is highly probable that the other non-elicitors are also bound reversibly by this protein, though perhaps not so well as DOH. Whether the unimportance of reversible protein binding is due to the absence from skin of a suitable protein (such as serum albumin), or whether it is due to the ineffectualness of the relatively weak bonds involved in reversible protein binding cannot be determined from the information at hand. In any event, it appears that for haptens to elicit allergic skin reactions of the delayed type one of several necessary conditions to be fulfilled is that the eliciting compound combine with skin protein through the formation of irreversible bonds of the covalent type.

The general validity of the foregoing interpretation may seem questionable in view of the many clinical instances of production of delayed allergic skin lesions by simple substances which appear to be incapable of derivatizing protein; *e.g.*, penicillin, etc. With respect to these situations it seems reasonable to postulate that metabolic degradation products, in contrast to the original substance, may be able to combine with protein. This suggestion has been made previously by Landsteiner and di Somma (23) in relation to the induction of sensitization by picric acid and by Mayer (24) on the basis of extensive cross-reactions exhibited, on skin testing, by a number of structurally diversified aromatic amines. It is this possibility, among others, which would limit the usefulness of the *in vitro* determination of protein-combining capacity as a guide to the sensitizing potential of simple chemical compounds.

The reasons underlying the apparent obligatory nature of protein combining are obscure. A number of possible explanations have, however, been tentatively explored. One possibility is that combination with protein is required to reconstitute the "complete" haptenic configuration involved in antibody formation. If this were the case, then N^5-D-lysine, which is the derivatized amino acid formed in greatest abundance, might well be a highly effective elicitor. However, N^5-D-lysine, as 0.01 M and 0.2 M ethanol solutions, proved incapable of producing lesions in sensitized guinea pigs, and in sensitized human beings has produced only occasional equivocal reactions, at the higher concentration only.[18]

[18] Gell, for similar reasons, tested "tetryl"-sensitized guinea pigs with picryl glycine. No lesion was produced (4).

A second possible explanation arises from consideration of the "framework" theory (26, 25) which has proven so useful in the interpretation of the precipitin reaction (27). If the assumption is made that the formation *in vivo* of large aggregations of mutally "multivalent" antigen and antibody is the basis for the production of delayed allergic skin reactions, then hapten combination with skin protein may serve to furnish the polyhaptenic "antigen" particle which is demanded by the theory. Examination of this possibility was undertaken by skin testing sensitized individuals with the following compounds, all of them having two or three haptenic groups per molecule: (*a*) 2,2′,4,4′-tetranitrodiphenyl; (*b*) 2,2′,4,4′-tetranitrodiphenyl disulfide; (*c*) 2,4,6-tris(2,4-dinitrophenylazo)resorcinol; and (*d*) 2,7-bis(2,4-dinitrophenylazo) chromotropic acid.[19] With the terminology employed herein these compounds may be designated, respectively, as follows: D-D, D-S_2-D, (D-azo)$_3$ resorcinol, and (D-azo)$_2$ chromotropic acid. These compounds have so far been examined at concentrations less than 0.05 M; they have, however, failed to produce skin lesions, except for D-S_2-D which gave occasionally an equivocal reaction in sensitized man.

The foregoing possibilities have not yet been examined in sufficient detail to determine whether or not they are valid. Nevertheless, a third possibility which must be considered is that combination with protein, particularly with the scleroproteins which are so abundant in skin, retards the diffusion of hapten from the skin. Such a kinetic effect may be of particular importance because of the relatively long time required for the evolution of these lesions. If this view is correct, then non-elicitors are incapable of producing lesions because, being uncombined with fibrous protein, they diffuse relatively rapidly from the skin. To a very limited extent, this hypothesis is consistent with the results of some preliminary attempts to specifically inhibit, with the non-elicitors, the production of skin reactions. These attempts were based upon the anticipation that non-elicitor D compounds might inhibit, by competitive interaction with antibody, the production of lesions by elicitor D compounds. A number of attempts to demonstrate such inhibition, using in the test solutions molecular ratios of non-elicitor to elicitor as high as 1000 to 1, have so far failed. This possibility—namely, that rates of disappearance from skin determine the essential difference between elicitors and non-elicitors—is being studied further with the aid of isotope-labelled D compounds. These studies will be reported in a future communication.

Although the foregoing three speculations have been advanced as though each were an independent possibility, it must be noted that they are not mutually exclusive.

We wish to thank Dr. Norton Nelson for his helpful interest in the course of this work.

[19] Gell tested tetryl-sensitized guinea pigs with dipicrylamine (4). He, likewise, obtained a negative result.

SUMMARY

Eight 2,4-dinitrophenyl compounds with a high degree of configurational uniformity were examined in regard to their ability to elicit delayed allergic skin reactions in guinea pigs and men who had been sensitized by previous exposure to 2,4-dinitrofluorobenzene. Four of the eight compounds produced reactions (elicitors) and four others did not (non-elicitors). It was found that the elicitors combined with protein *in vitro* and *in vivo*. The non-elicitors, however, failed to combine in either situation. It is concluded that a necessary condition for the elicitation of delayed allergic skin reactions by haptens is the combination of the latter with skin protein through the formation of bonds of the covalent type. No choice is yet possible amongst the several possible explanations which are advanced to account for the obligatory character of protein combination.

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