

STUDIES OF HYPERSENSITIVITY TO LOW MOLECULAR WEIGHT SUBSTANCES

II. REACTIONS OF SOME ALLERGENIC SUBSTITUTED DINITROBENZENES WITH CYSTEINE OR CYSTINE OF SKIN PROTEINS*· ‡

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The concept that simple chemicals may produce allergic reactions by combining *in vivo* with tissue constituents has become progressively more widely accepted since it was proposed by Wolff-Eisner (1), and supported experimentally by Landsteiner and Jacobs (2; see also 3, 4). Despite the value of this concept for predicting and interpreting the allergenicity of many low molecular weight substances, the *in vivo* reactions that are actually involved have remained matters of speculation.

In a previous study of hypersensitivity reactions involving the 2,4-dinitrophenyl group (6), it appeared that a direct attack on this problem was offered by an application of the method developed by Sanger and others for the isolation and identification of dinitrophenyl-amino acids (for review, see reference 5). Using this method we demonstrated a combination *in vivo* of some substituted 2,4-dinitrobenzenes with lysine ϵ -NH₂ groups of skin proteins; on the basis of these results it was suggested that the formation of a covalent bond between a simple chemical and protein is required for allergenic activity on the part of low molecular weight substances (6).

In the present work we have examined the behavior of 2,4-dinitrobenzenesulfenyl chloride (DSCl) and 2,4-dinitrophenylthiocyanate (DSCN).¹ These compounds elicited allergic skin responses of the delayed type and, in con-

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‡ Paper I of this series is reference 6.

¹ The 2,4-dinitrophenyl group is hereinafter sometimes referred to as D. The substituted D compounds used in these experiments all have their respective substituents on carbon-one, and are accordingly described as follows: 2,4-dinitrofluorobenzene, DF; 2,4-dinitrobenzene sulfonate, DSO₃, etc. Dinitrophenyl-amino acids, whose terminology is discussed in footnote 3, have their amino acid component likewise as a substituent on carbon-one of the D group.

formity with our previous experience, they were found to combine chemically with skin proteins *in vivo*. Their reactions, in contrast with those described earlier, focus attention upon cystine and cysteine sulfur as additional reactive groups in skin proteins. Another sulfur-containing allergenic dinitrophenyl compound (DSO₂) was found also to react *in vivo* with these sulfur amino acids, and not with lysine ε-NH₂ groups as previously suggested (6). In order to gain further insight into the reactions with cystine and cysteine residues as they occur in living tissue, we have attempted to reproduce them *in vitro*. These attempts were successful so long as they included such highly organized materials as hair or epidermis; replacement of the latter with a gamma globulin or with glutathione led to quite different results. The *in vitro* reactions to be described emphasize, therefore, the curious complexity of the organization of cystine in keratins, a matter which is attracting much attention (7, 17).

For convenience in the following presentation, the allergenically active, substituted dinitrobenzenes which we have studied in these and in previous experiments are considered to fall into two classes: group I consists of the halogen-substituted compounds (DF, DCl, and DBr), and group II consists of the compounds with sulfur-containing substituents (DSO₂, DSCL, and DSCN).

The group II compounds examined in this study seemed to combine *in vivo* only with cysteine or cystine of skin proteins. Since these sulfur-containing amino acids are characteristically abundant in keratins and scarce in dermal proteins, the inference is drawn that epidermal proteins are predominantly involved in the molecular reactions underlying the elicitation by simple chemicals of delayed allergic reactions of the contact dermatitis type.

The behavior of several of these allergens, particularly those of group II, is presented below under the following headings: (1) elicitation of skin responses, (2) reaction with skin proteins *in vivo*, (3) reactions with gamma globulins *in vitro*, (4) reaction with epidermal proteins *in vitro*, and (5) evaluation of results with respect to free sulfhydryl groups of skin.

Materials

DSCl and DSCN were obtained from commercial sources,² and were purified by repeated recrystallizations from carbon tetrachloride and dilute ethanol, respectively. The source and means of purification for the other substituted dinitrobenzenes are given elsewhere (6).

N³-D-arginine, di-D-histidine, and N⁶-D-lysine were prepared by the methods of Porter and Sanger (8).³

² DSCl was obtained from Versatile Chemicals, Inc., Glendale, California. We are indebted to the Monsanto Chemical Co., St. Louis, for the DSCN.

³ The terminology used herein to describe D-amino acids is the same as is now in general use (5, 13), save that D is substituted for DNP to describe 2,4-dinitrophenyl. In the terms N-D-amino acid, S-D-amino acid, and O-D-amino acid, with superscripts as indicated, N, S, and O refer to the amino acid atom with which the D group is combined.

S-D-glutathione was prepared initially by the reaction of DCl with reduced glutathione (GSH) in 1 N NaOH (9); the same product was obtained by substituting for DCl the sodium or potassium salt of DSO₂. S-D-glutathione was also prepared by the reaction between equimolar amounts of DSO₂ and GSH at pH 7, under nitrogen. The compound obtained from the latter reaction was indistinguishable from the S-D-glutathione prepared according to Saunder's method (9) with respect to absorption spectrum and melting point (10). Its elemental analysis⁴ indicated that the sulfonate group is eliminated; calculated on the basis of C₁₆H₁₉O₁₀N₂S, sulfur is 6.76 per cent; found 6.59 per cent.

RESULTS

Elicitation of Skin Responses.—

Ten male albino guinea pigs weighing 300 to 600 gm., and four human subjects were sensitized with DF and were subsequently challenged with skin applications of dilute solutions of DSCl and DSCN, using as a solvent equal volumes of toluene and mineral oil. The procedures and time intervals involved in sensitization and skin testing were those previously described in detail (6). Controls consisted of similar skin tests performed with the same solutions on non-sensitized guinea pigs and humans. All reactions were read 48 hours after application of test solutions and were graded from 0 to 3+, using criteria previously described in detail (6), and mentioned below (Table I).

DSCN, 0.01 M, produced strongly positive reactions, varying between 2+ and 3+ in the sensitized guinea pigs and the sensitized humans. The same concentration of DSCl produced feeble reactions in guinea pigs and negative responses in man; 0.02 M DSCl, however, produced allergic reactions of moderate intensity in 8 guinea pigs and weakly positive (1+) reactions in 4 humans. The latter reactions in humans displayed very little erythema but had distinct vesicles.

The intensities of the reactions produced by group II compounds were compared with the intensities of those elicited by group I compounds in a series of patch tests in 5 guinea pigs and 1 human, all individuals having been previously sensitized with DF. It appeared (Table I) that reactions elicited by DSCN equalled in intensity those elicited by group I. DSCl, however, was somewhat less effective in the guinea pig and conspicuously weaker in man. Since DSCl is rapidly hydrolyzed by water (11), it is probable that its feeble allergenic activity on human skin is due, at least partly, to decomposition by sweat. DSO₂, the remaining member of group II, has previously been found to be active in man and in the guinea pig, requiring for activity in the latter species a detergent in the solvent (6).

Reaction with Skin Protein in Vivo.—

The procedures used in treating the depilated skin of normal guinea pigs with dinitrophenyl compounds, in removing the epidermis and hydrolyzing it, and in examining the hydrolysates chromatographically, have all been described previously (6). DSCl was sprayed on the skin in a saturated solution (5 per cent or less); the solvent consisted of 1 volume of mineral oil, and 9 volumes of either ethylene chloride, toluene, or "diethyl carbitol." DSCN was dissolved to make a 5 per cent solution in 1 volume of corn oil and 9 volumes of acetone. About 24 hours

⁴ The analysis was done by F. Schwarzkopf Microanalytical Laboratory, Middle Village, New York.

after spraying, the animals were sacrificed, the skins removed and, after the hides had been flattened on a hot plate at 50°C. for 2 minutes, the epidermis was gently teased from the dermis (12). Teasing did not accomplish such a separation without preliminary warming. In this respect, the DSCI- and DSCN-sprayed skins resembled normal skin and DSO₂-sprayed skin; by contrast, with skins sprayed with 10 per cent solutions of DF, DCl, and DBr, warming the hides was unnecessary, teasing alone being sufficient to separate the two layers of skin (6). This difference is commented on below.

Usually, about 500 mg. samples of epidermis (wet weight) were hydrolyzed with 6 N HCl for 24 hours at 110°C. The hydrolysates were then diluted with water and extracted with ether. As in previous experiments, the aqueous fraction in each case was examined. This fraction may be assumed to contain the bulk of dinitrophenyl-amino acids formed when dinitrophenyl compounds react with polar side chains of non-terminal amino acids in an intact

TABLE I
Elicitation of Skin Reactions with Dinitrophenyl Compounds in Individuals Sensitized with 2,4-Dinitrofluorobenzene (DF)

Group	Compound	Concentration*	Results†					
			Guinea pig No.					Human
			1	2	3	4	5	
		<i>moles/liter</i>						
I	DF	0.01	3+	3+	3+	3+	3+	3+
	DCl	0.01	3+	3+	3+	3+	3+	3+
	DBr	0.01	3+	3+	3+	3+	3+	3+
II	DSCN	0.01	3+	3+	2+	2+	2+	3+
	DSCI	0.01	1+	2+	±	0	1+	0
	"	0.02	3+	2+	1+	1+	1+	±

* Solvent in all cases was a mixture of toluene and mineral oil in equal volumes.

† Skin responses were graded as follows: 0, no reaction; ±, equivocal reaction; 1+, slight erythema; 2+, moderate erythema and edema; 3+, marked erythema and edema. Non-sensitized guinea pigs and humans gave no reactions to any of the above test solutions.

protein. In rejecting the ether-soluble fraction, it is possible that we have discarded traces of other dinitrophenyl-amino acids, corresponding at most to the substitution of dinitrophenyl in α -NH₂ groups of terminal amino acids.

The aqueous fractions of the hydrolysates of both DSCI- and DSCN-sprayed skins were observed to yield a single retarded yellow band with an average retardation factor (R_F) of 0.4 on buffered silica gel columns, using 2 M KH_2PO_4 as the stationary phase and M66⁵ as the developing solvent. In repeated examinations of such preparations the R_F of this substance has varied from 0.6 to 0.3. When these bands were eluted and hydrolyzed for an addi-

⁵ The solvents used in silica gel chromatography are described with the symbols now in wide usage (5). Thus, M66 is 2 volumes methyl ethyl ketone, 1 volume ether, saturated with water; CB30 is 7 volumes chloroform, 3 volumes *n*-butanol, saturated with water.

tional 20 hours in 6 N HCl at 110°C., and the aqueous fraction of the ether-extracted hydrolysate examined again, the R_F was noted to have decreased to 0.25. A band corresponding to N⁶-D-lysine, *i.e.* having an R_F on these columns of 0.08 (range 0.07 to 0.10), was not observed.

²⁰In view of these results with DSCl and DSCN, we reexamined DSO₃, an allergenic dinitrophenyl compound which was previously described as reacting *in vivo* with skin protein to give on silica gel columns a very faint yellow band with an R_F like that of N⁶-D-lysine (6); this previously described band was too faint and too irregularly observed to permit critical examination in the form of a mixed chromatogram with N⁶-D-lysine. In the earlier protocols slow yellow bands with the same behavior as those now obtained from DSCl- and DSCN-treated skins were recorded, but their significance was not previously appreciated. The experiments with DSO₃ have recently been repeated. Three guinea pigs were sprayed with this compound and epidermal hydrolysates were chromatogrammed. There was observed in each instance the same distinct yellow band (average R_F 0.4) as is obtained with DSCl and DSCN. When M66 solutions of the material which gave initially only a single yellow band of R_F 0.4 were permitted to stand at room temperature unshielded from light for several days and then chromatogrammed again, there occasionally appeared a very faint yellow band of R_F 0.16–0.10. The latter, which we previously confused with N⁶-D-lysine, has not been identified. It probably represents a degradation product of the faster moving material, since it has been observed amongst the products of numerous attempts to prepare S-D-cysteine by the reaction of DSO₃ with cysteine (10). However, as in the case of DSCN and DSCl, N⁶-D-lysine is not recovered from skin treated *in vivo* with DSO₃.

The identity of the yellow band of average R_F 0.4 was established by systematic comparison with those yellow dinitrophenyl-amino acids which might be expected to be present in the acid-soluble fraction. These are: N¹-D-lysine, N⁶-D-lysine, N¹-D-arginine, di D-histidine, and S-D-cysteine (5). The results of the comparison are summarized in Table II. Although the variability of the R_F of the unknown material is considerable, it clearly differed from the acid-soluble dinitrophenyl-amino acids listed above with the exception of S-D-cysteine.

The possibility that the unknown band corresponds to S-D-cysteine was further examined by studying acid hydrolysates of S-D-glutathione. This indirect approach was necessary since we, as well as others (13), have been unable to obtain purified preparations of S-D-cysteine; S-D-glutathione, on the other hand, is a readily synthesized, stable, crystalline compound (9, 10; see also above) which presumably yields S-D-cysteine on acid hydrolysis (13). The glutathione derivative was hydrolyzed in 6 N HCl at 110°C.; shorter periods of hydrolysis were required for this compound than are necessary for

proteins (13). When hydrolyzed for 6 hours, the aqueous fraction of the hydrolysate yielded with the M66-2 M KH_2PO_4 solvent system a single yellow band of R_F 0.4. With more prolonged hydrolysis (24 hours), there was obtained a single yellow band of R_F 0.25. Furthermore, with the CB30-2 M KH_2PO_4 solvent system the hydrolysate was stationary. Thus, the S-D-glutathione hydrolysate reproduced the behavior of the unknown in two solvent systems and duplicated the alteration in R_F that results from prolonged hydrolysis (Table II). The foregoing results suggest that the group II compounds react

TABLE II
Comparison of Dinitrophenyl-Amino Acid Formed *in Vivo* by DSCI, DSCN, and DSO_3 with Other Acid-Soluble Dinitrophenylamino Acids

Materials examined	Solvent system	
	M66-2 M KH_2PO_4	CB30-2 M KH_2PO_4
	R_F	R_F
Epidermis treated with group II		
24 hrs.' hydrolysis	0.4	Stationary
44 " "	0.25	
N ¹ -D-lysine	0.05*	0.1
N ⁶ -D-lysine	0.08	
N ¹ -D-arginine	0.2	
di D-histidine	Fast	
S-D-glutathione		
6 hrs.' hydrolysis	0.4	Stationary
24 " "	0.25	

* Estimated from reference 13.

in the living skin to yield a substance corresponding to S-D-cysteine.⁶ Other evidence, to be presented below, adds considerable support to this argument.

The above observations on skins that had reacted *in vivo* were made with hydrolysates of epidermis alone. In additional experiments separate samples of both epidermis and dermis were obtained from animals that had been sprayed 24 hours before sacrifice with solutions of DSO_3 , DSCI, and DSCN. The epidermal samples had wet weights of 225, 255, and 290 mg.; the corresponding dermal samples weighed 510, 500, and 480 mg. No yellow dinitrophenyl-amino acids were obtained from the dermal hydrolysates. Each corresponding epidermal hydrolysate yielded only the yellow dinitrophenyl-amino acid identified with S-D-cysteine.

⁶ We are uncertain as to whether the sulfur of DSCI and DSCN is retained in the dinitrophenyl derivative of cysteine. With DSO_3 , however, it is probable that the S-D-cysteine formed lacks the sulfur of the dinitrophenyl reagent. This view is based on sulfur analysis of the S-D-glutathione prepared from DSO_3 (see above).

Reactions with Bovine Gamma Globulins in Vitro.—It was previously found that the halogen-substituted 2,4-dinitrobenzenes (DF, DCl, DBr), and DSO_2 , reacted at pH 10–11 with lysine $\epsilon\text{-NH}_2$ groups of bovine gamma globulins (6, 14). These experiments have now been extended by examining the reactions of DSCl and DSCN with the same protein under similar conditions. For these and the previous experiments bovine gamma globulins have been chosen to represent the behavior of globular proteins.

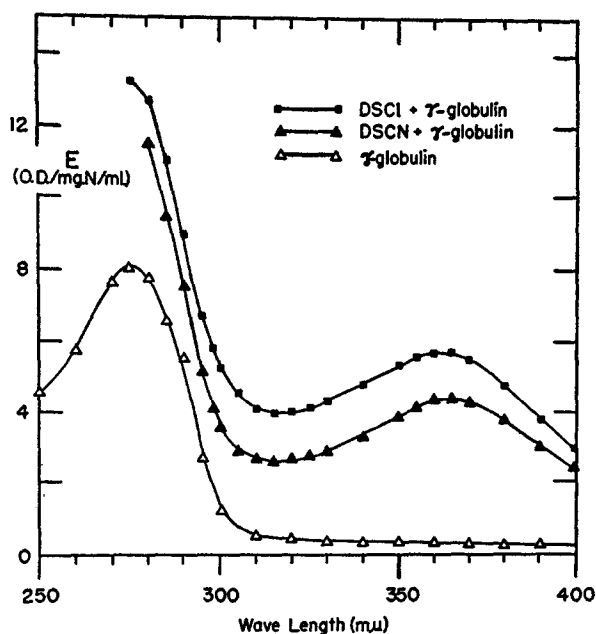


FIG. 1. Ultraviolet absorption spectra of bovine gamma globulins and of dinitrophenyl-bovine gamma globulins prepared with DSCl and DSCN. Solvent was 10 N HCl. Extinctions (E) are recorded as optical densities (O.D.) per milligram of N per milliliter.

100 mg. of bovine gamma globulins⁷ in 5.0 ml. saline, 0.2 M with respect to K_2CO_3 , was mixed with 100 mg. of DSCl or DSCN in 5.0 ml. of ether. The mixture was shaken rapidly for about 20 hours at room temperature. Ethanol was then added, precipitating any protein which had remained soluble, and the precipitate was washed thoroughly with ethanol, acetone, and methyl cellosolve. The effective removal of unreacted dinitrophenyl compounds was indicated by serial examination of the wash fluids with an acetone "spot test" (10). Finally, the washed protein was dissolved in 10 N HCl. Nitrogen concentrations were determined on aliquots by micro Kjeldahl analysis; on other aliquots, after accurate dilution, ultraviolet absorption spectra were obtained in a Beckman spectrophotometer. An absorption spectrum was also obtained on a control sample of bovine gamma globulins which was similarly treated except for the omission of dinitrophenyl compounds.

The absorption spectra (Fig. 1) demonstrate that combination with protein had occurred. In order to gain some insight into the nature of the protein

⁷ Armour Laboratories, fraction II from bovine plasma.

groups combining with the dinitrophenyl compounds, the optical density of the untreated protein was subtracted from that of an equivalent concentration of the treated protein, and the resulting data were normalized by expressing the optical density at all wave lengths as a fraction of the optical density at λ max. ($365 \text{ m}\mu$). The data thus treated yield average absorption spectra of the dinitrophenyl-amino acids present in the treated proteins.

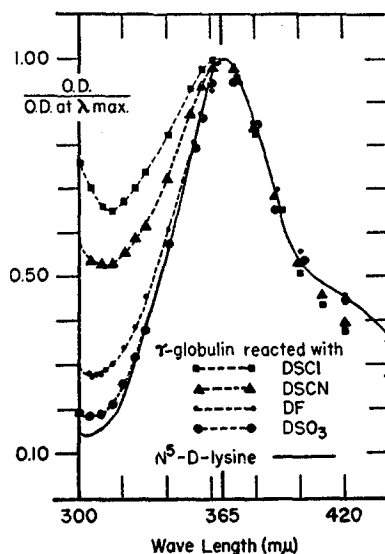


FIG. 2. Comparison of ultraviolet absorption spectra of N^5 -D-lysine and of dinitrophenyl-amino acids of dinitrophenyl-bovine gamma globulins prepared with DSCI, DSCN, DF, and DSO_3 . The latter were obtained by subtracting from the optical densities of the treated protein the optical densities of an equivalent concentration of untreated protein, at corresponding wave lengths. The data on the dinitrophenyl-globulins prepared with DSO_3 are taken from reference 14. Solvent was 10 N HCl . Optical densities (O.D.) are expressed as a fraction of the optical densities at λ max. ($365 \text{ m}\mu$).

In Fig. 2, the results of such a calculation are contrasted with comparable data obtained from the reactions of DF and DSO_3 with bovine gamma globulins. The absorption spectrum of N^5 -D-lysine, similarly plotted, may be considered as representative of those dinitrophenyl-amino acids whose dinitrophenyl group is substituted in a free NH_2 group (15). In all the dinitrophenyl-proteins prepared at pH 10-11 substitution of NH_2 groups occurred, as indicated by an absorption maximum at $365 \text{ m}\mu$.⁸

⁸ The deviation of the normalized spectra of the dinitrophenyl-proteins from that of N^5 -D-lysine in the region 300 to $340 \text{ m}\mu$ is pronounced in the case of DSCI, less with DSCN, slight with DF, and probably insignificant with DSO_3 (Fig. 2). In view of the general difference in spectral properties between O-D-compounds and N-D-compounds (15), it seems likely that

In support of this interpretation, the hydrolysate of a dinitrophenyl-protein prepared with DSCl yielded a band of R_F 0.08, identical with that of N^6 -D-lysine, on buffered columns with M66, and a mixed chromatogram with N^6 -D-lysine gave one band.⁹

The reactivities of some of the dinitrophenyl compounds with some globular proteins have been examined also at more physiologic pH values. DSO_3 , which may be taken to represent group II, has been found not to react with gamma globulins at pH 7 (14). In contrast, group I compounds are reactive at about this pH value. Thus, DF is known to react with NH_2 groups at pH 8 (13), and even DBr (the least reactive member of group I) reacts slightly with serum albumins at pH 7.4 (16).

In the present work the reaction of DCl with bovine gamma globulins was studied. After reacting 24 hours at room temperature and pH 7.1 in phosphate buffer, and after thorough extraction of unreacted reagent, the protein (dissolved in 0.1 N NaOH) gave an ultraviolet absorption spectrum with an absorption maximum at 365 $m\mu$. From the optical densities at 365 $m\mu$ and 290 $m\mu$ it was calculated that about 3 dinitrophenyl groups were substituted in each protein molecule of 160,000 molecular weight. The calculation was based on the assumptions that the optical density at 365 $m\mu$ was due entirely to N^6 -D-lysine and that the optical density at 290 $m\mu$ was due to both protein and N^6 -D-lysine. When the dinitrophenyl-globulin thus prepared was hydrolyzed and chromatogrammed only one yellow band was obtained, and this was indistinguishable from that of N^6 -D-lysine.

Reactions with Epidermal Proteins (Keratins) in Vitro.—The reactions of group I compounds with skin proteins *in vivo* can be duplicated qualitatively *in vitro* by reacting these compounds with bovine gamma globulins at either high or physiological pH values (6, 16; see above for DCl reaction at pH 7). As shown in the preceding sections, however, this is not the case with group II. In an effort to account for this difference, we have examined in some detail the reactions *in vitro* of group II compounds with epidermal proteins at pH values of 7 and 10. DCl, chosen to represent group I, was examined in the same manner for purposes of comparison. The epidermal proteins present in hair and strips of depilated epidermis are assumed to be chiefly keratins.

Epidermal strips were teased from the dermis of warmed hides (50°C., 2 minutes) which had been obtained from non-sensitized depilated guinea pigs. Hair from albino guinea pigs was washed with water and defatted by extraction with alcohol and ether in a Soxhlet apparatus. When epidermis was used, 200 mg. of tissue (wet weight) was suspended in 1.0 ml. of

these deviations may reflect varying degrees of substitution of D in hydroxyl groups. Thus, DSCl is known to be reactive with aliphatic alcohols (11 *a*, *b*); it might also react directly with the tyrosine benzene ring under these conditions (11 *b*). DF, on the other hand, reacts with tyrosine hydroxyl but not with serine hydroxyl (13).

⁹ We have no information as to whether the ϵ - NH_2 derivative of lysine obtained with DSCl, and presumptively with DSCN, retains the sulfur of the latter reactants. With respect to DSO_3 , it has been shown that sulfur is eliminated when free NH_2 groups are substituted at pH 10 (14).

saline, and mixed with 3.5 millimoles of dinitrophenyl compound in 10 ml. of organic solvent.¹⁰ When hair was employed, 100 mg. was mixed with about 0.3 to 1 millimole of dinitrophenyl compound in an appropriate solvent.¹⁰ pH values of 10 and 7 were attained with either K_2CO_3 , phosphate buffer, or an amberlite resin (IRC-50).¹¹ No difference was noted between reactions carried out under nitrogen or in the presence of oxygen. After being shaken rapidly at room temperature for 24 hours, the epidermal strips or hairs were washed with alcohol, acetone, and dioxane and finally were hydrolyzed and chromatogrammed with the M66-2 M KH_2PO_4 solvent system. The results are recorded in Table III, in which bands of R_F 0.07 to 0.10 are listed as N⁵-D-lysine and bands of R_F 0.25 to 0.6 (average 0.4) are recorded as S-D-cysteine. In the former cases the results were confirmed by mixed chromatograms.

It is apparent (Table III) that at high pH values all the elicitors reacted with lysine $\epsilon-NH_2$ groups but not with sulfur groups. At pH about 7, how-

TABLE III
Reactions in Vitro of Dinitrophenyl Compounds with Proteins

Compound	D-amino acids recovered chromatographically from reactions with					
	Epidermis		Hair		Bovine gamma globulins	
	pH 7	pH 10	pH 7	pH 10	pH 7	pH 10
DSCI	S-D-cysteine	N ⁵ -D-lysine	S-D-cysteine	N ⁵ -D-lysine	—	N ⁵ -D-lysine
DSCN	" *	"	"	"	—	" †
DSO ₃	" *	"	"	"	None‡	" §
DCI	N ⁵ -D-lysine	"	N ⁵ -D-lysine	"	N ⁵ -D-lysine	"

* In occasional runs a very faint band of R_F about 0.15 was seen (see text).

† Identified by absorption spectrum.

§ From reference 14.

ever, the results duplicated the reactions *in vivo* in that DSCI, DSCN, and DSO₃ reacted with cystine sulfur but not with lysine $\epsilon-NH_2$ groups, and DCI combined with lysine $\epsilon-NH_2$ groups but not with cystine sulfur.¹²

¹⁰ The solvents used in these reactions were as follows: DSO₂-water, or methyl cellosolve or methyl cellosolve containing a detergent, tween 80, to the extent of 10 per cent; DCI ethanol; DSCI and DSCN-ether or "diethyl carbitol."

¹¹ We wish to thank Dr. Edward Palmes of this department for suggesting the use of a resin in these reactions.

¹² Considering the over-all procedure, the minimal amounts of N⁵-D-lysine and S-D-cysteine that may be hydrolyzed and then visualized on columns of 1.0 cm. diameter are about 0.1 micromole and about 5 micromoles, respectively. The latter value for S-D-cysteine was arrived at by determining the smallest amount of a hydrolysate of a known amount of S-D-glutathione that could be visualized as a yellow band of R_F 0.4 (0.4-0.25), using the M66-2M KH_2PO_4 solvent system.

This large difference in sensitivity introduced some doubt as to the validity of the observation that DCI did not combine with cystine in hair: thus, since DCI reacts readily with free NH_2 groups, it seemed possible that the relatively large amount of N⁵-D-lysine formed could prevent visualization of a band corresponding to S-D-cysteine. This possibility was excluded

In view of the results obtained with epidermal proteins at pH 7, DSO₃ and DCl, selected to represent group II and group I, were reacted at pH 7 for 24 hours at 25°C. with glutathione in both reduced and oxidized forms (referred to below as GSH and G₂S₂). From absorption spectra of the individual reactants and the final reaction mixtures, it appeared that neither DSO₃ nor DCl reacted with G₂S₂. Both compounds, however, reacted with GSH forming a yellow compound having the same absorption spectrum as S-D-glutathione. These reactions with glutathione are reported in detail elsewhere (10).

It is of considerable interest that the —S—S— group of oxidized glutathione did not react with either DCl or DSO₃, whereas the —S—S— groups of keratins (hair and non-viable epidermis) reacted readily with DSO₃ but not at all with DCl. Thus, those disulfide groups of keratins which react with group II clearly differ in reactivity from the disulfide of oxidized glutathione. It might be assumed that traces of sulfhydryl, in equilibrium with disulfide, account for the unusual reactivity of the disulfide groups of keratin with group II. This does not seem to be the case, however, since we have found that DCl has a fourfold greater reaction rate with the sulfhydryl of reduced glutathione than does DSO₃ (10).

On the basis of the foregoing, it was expected that if DCl were reacted at pH 7 with epidermal strips in the presence of an agent capable of reducing —S—S— groups to SH, S-D-cysteine should be recovered in addition to N⁶-D-lysine. Such a reaction was accordingly carried out under nitrogen with conditions as described above, but with Na₂SO₃ added to the reaction mixture. When the epidermis hydrolysates were chromatogrammed with the M66-2 M KH₂PO₄ solvent system there were observed, in addition to the N⁶-D-lysine band (R_F 0.08), yellow bands of average R_F 0.4 (range 0.25-0.7), corresponding to the bands of S-D-cysteine (Table IV).

The reaction of DSO₃ with hair keratin provided an additional opportunity to test the validity of the contention that the yellow bands of average R_F 0.4 (with the M66-2 M KH₂PO₄ solvent system) correspond to S-D cysteine. Peracetic acid treatment is known to oxidize selectively cystine —S—S—linkages of proteins to the corresponding sulfonic acid, or sulfonamide, residues (17). Consequently, the reaction between DSO₃ and hair that had been previously treated with peracetic acid would fail to yield the colored derivative in question, if the reaction depends upon the presence of cystine disulfide.

by the following experiment: 100 mg. defatted hair was acetylated by the method of Alexander and Whewell, a procedure which blocks about 70 per cent of the free NH₂ groups of wool (24). The acetylated hair was then treated with one millimole of DCl at pH 7 as described above. The reacted hair was hydrolyzed and the total yield was placed on a buffered silica gel column. There was observed only a barely visible yellow band with the R_F of N⁶-D-lysine. A band corresponding to S-D-cysteine was not obtained (Table IV).

Defatted hair was stirred vigorously for 24 hours at room temperature with peracetic acid (17); the hair was then thoroughly washed with water and, after drying, was reacted at pH 7.0 with DSO₃, using the same quantities of materials and solvents as above. At the end of 24 hours the reacted hair was colorless (in contrast to the yellow hair produced when this reaction is carried out with unoxidized hair), and when hydrolyzed and chromatogrammed no yellow bands were observed.

The foregoing experiments with modified keratins are summarized in Table IV. They provide additional evidence that the unknown derivative yielded by compounds of group II corresponds to S-D-cysteine.

Evaluation of Results with Respect to Free SH Groups of Skin.—DCl is even more reactive with SH than with NH₂ groups (9, 10; see also above reactions

TABLE IV
Reactions in Vitro of Modified Keratins

Keratins modified by*	Modified keratins reacted with†	Dinitrophenyl-amino acids observed chromatographically
Reduction with Na ₂ SO ₃	DCl	N ⁵ -D-lysine + S-D-cysteine
Peracetic acid oxidation	DSO ₃	None
Acetylation‡	DCl	Faint trace N ⁵ -D-lysine; no S-D-cysteine

* Reduction with Na₂SO₃ was performed on strips of depilated epidermis. Other procedures were performed on hair.

† All reactions at pH 7.

‡ See footnote 12.

with glutathione). Since SH groups are abundant in the deeper layers of epidermis (stratum mucosum) (18), the failure to obtain evidence of their reaction *in vivo* with DCl, and other group I elicitors, requires explanation. In evaluating this problem three alternative explanations have been considered.

(a) DCl applied to the skin surface may penetrate superficially, combining only with epidermal protein of the cornified layers in which SH groups are present, if at all, to only a slight extent (18, 19). The restriction of dinitrophenyl-protein conjugates to this region is not an appealing possibility since it implies that subsequent specific interactions with antibodies take place in this essentially non-viable tissue. Furthermore, this localization would seem not to be applicable to those other allergenic chemicals which are generally supposed to undergo metabolic alterations prior to combining with tissue protein.

(b) S-D-cysteine may actually be formed, but its recognition could be

obscured by the relatively large amount of N^ε-D-lysine which is also formed. This possibility cannot be excluded in view of the disproportionate sensitivity of the chromatographic procedure for these derivatives.¹²

(c) A further possibility was suggested by the observation previously alluded to; *viz.*, that in contradistinction to normal skins and skins sprayed with group II, the epidermis of skins treated with group I compounds at high concentration is teased from the dermis easily, without prior warming. This vesicant-like effect indicated that at the 0.5 M concentration previously used (6) group I compounds produced considerable tissue damage, possibly associated with the disappearance of SH groups from epidermis. In order to evaluate this possibility, treated skins were examined histochemically for their sulfhydryl content.

Normal and sensitized guinea pigs were clipped, depilated, and 5 hours later were painted with several dinitrophenyl compounds at various concentrations, as indicated in Table V. Twenty-four hours later the animals were anesthetized with nembutal and small pieces of treated skin were excised and, without prior fixation, promptly cut on a freezing microtome into sections of 50 μ thickness. The sections, on glass slides, were dipped for 20 seconds into a 5 per cent aqueous solution of zinc acetate (20), then drained, and two drops of 2 per cent sodium nitroprusside were placed on them. With this procedure SH groups react within 2 or 3 minutes to give a bright red color. After rinsing in water, clearing with xylol, and covering with permount and a glass coverslip, the sections retained the characteristic color unchanged for several months. In order that uniformly good results be obtained, the time elapsing between resecting the skin and staining the section had to be not more than 20 minutes; generally this time interval was 5 to 6 minutes. Skin removed from a normal animal not painted with a dinitrophenyl compound was examined as a control.

In Table V, the color reactions of the stained skin sections are summarized together with the results of chromatographic analyses of hydrolysates of epidermis removed from other areas of treated skin from some of the same animals. In skins sprayed with high concentrations of group I elicitors, the SH groups of epidermis were absent. The basis for this effect is obscure; it is probably not due simply to free acid released when these compounds react with NH₂ groups, since 0.5 M HCl, applied to the skin in a comparable manner, has neither a vesicant-like action, nor does it visibly alter SH reactivity in the epidermis. The SH groups may even have disappeared *because* they combined with these elicitors; the resulting S-D-cysteine may then not have been observed because of the limitations of silica gel chromatography referred to above.¹² The latter limitations applied also when a lower concentration of DCl was used (0.01 M), even though in this instance SH group reactivity was not noticeably reduced. From the foregoing, it is apparent that the chromatographic procedure used does not permit exclusion of the possibility that S-D-cysteine is formed in epidermis which has been treated *in vivo* with DCl, DF, and DBr.

Parenthetically, it was of interest that, in a sensitized animal, biopsies from areas having maximally intense allergic responses displayed epidermal SH reactivity to a normal degree (Table V).

TABLE V
Free SH Groups in Epidermis after Treatment with Dinitrophenyl Compounds in Vivo

Group	Compound applied to skin	Concentration <i>moles/liter</i>	Solvent*	Nitroprusside reaction†	Amount of epidermis hydrolyzed <i>mg.</i>	D-amino acids identified chromatographically‡
I	DCl	0.5	A	0	570	N ⁶ -D-lysine
	"	0.05	"	1+	430	" very faint
	"	0.01	"	4+	—	
	"	0.01	"	4+	—	
	DF	0.05	"	0	506	N ⁶ -D-lysine, faint
	"	0.01	"	4+	—	
II	"	0.01	"	4+	—	
	DSO ₃	0.18	B	4+	780	S-D-cysteine
	"	0.18	Bd	4+	402	"
	DSCl	0.20	C	4+	—	(")
	DSCN	0.20	C	4+	—	(")
	HCl	0.5	D	4+		

* Solvents: A, 9 volumes acetone,, 1 volume corn oil; B, ethylene glycol monomethyl ether (methyl cellosolve); Bd, 9 volumes methyl cellosolve, 1 volume tween 80; C, 9 volumes toluene, 1 volume mineral oil; D, water saturated with tween 80 (5 to 10 per cent).

† Results graded from 0 (no color developed) to 4+ (intense red color in stratum mucosum and hair follicles, the same as seen in normal skin).

‡ Results in parentheses obtained from other animals.

|| These results were obtained in strongly positive allergic skin reactions (3+) in guinea pigs sensitized with DF; all other results obtained in non-sensitized animals.

DISCUSSION

In the foregoing experiments it was shown that DSO₃, DSCl, and DSCN combined *in vivo* with skin protein through reaction with sulfur of cystine or cysteine. These compounds did not react *in vivo* with free NH₂ groups of protein, probably because they require for this reaction a higher pH value than is likely to occur in viable skin. Since cystine and cysteine are characteristically abundant in epidermal proteins and are absent or very scanty in the major proteins of the dermis (21), these findings imply that the combination of the foregoing compounds with skin protein is predominantly localized in the epidermis.¹³ This suggestion is supported by the failure to detect dinitro-

¹³ Since O-D-amino acids are colorless they would not have been detected if formed. This circumstance does not vitiate the argument for epidermal localization since DSO₃ is not re-

phenyl-amino acids in hydrolysates of dermis from skins treated *in vivo* with group II allergens. In view of the reactivity of these compounds with both free SH and with —S—S— groups of hair and epidermis, it is not possible to infer a particular intraepidermal localization.

Because lysine is abundant in collagen, as well as in keratin (21), the combination *in vivo* of group I compounds with free NH₂ groups of protein offers, *per se*, no insight in regard to the localization of their conjugation with skin protein. However, we previously found that in DBr-sprayed skin (*in vivo*) dinitrophenyl-lysine could be recovered from epidermis but not from dermis (6), indicating that with group I elicitors also the combination with tissue protein is predominantly, and perhaps exclusively, localized in the epidermis.

The allergic skin lesion produced by the dinitrophenyl elicitors may be assumed to depend upon specific interaction between a dinitrophenyl-protein conjugate and an appropriate antibody. On the basis of the reactions *in vitro* described above the protein involved in conjugation is most likely a keratin, at least in the case of group II compounds, and, accordingly, the conjugates would seem to require for activity little or no solubility or mobility. The ability of apparently immobile epidermally situated dinitrophenyl-protein conjugates to elicit allergic skin responses permits the speculation that either the epidermis of sensitized individuals synthesizes the required antibodies, or that the antibodies, formed elsewhere, can penetrate into epidermis. In regard to these possibilities it is of interest to call attention to the claim that lymphocytes are constituents of the normal epidermis (22).

Since we have not been able to obtain definitive information with regard to the formation of an S-D-cysteine derivative by DF *in vivo*, we are unable to evaluate the possibility that production of lesions in DF-sensitized individuals by group II elicitors corresponds immunologically to a cross-reaction. In this connection, it is of interest to consider that in the case of the analogous quantitative precipitin reaction involving the dinitrophenyl group combined with protein we have found that considerable specificity of anti-dinitrophenyl antibodies is directed against protein structures in the immediate region of attachment of the dinitrophenyl group (23).

The results of the present work have an obvious bearing on efforts to assess the allergenicity of simple chemicals on the basis of reactions *in vitro*. From the point of view of such attempts, the compounds of group I present few difficulties since they react *in vivo* much as they do *in vitro* with most proteins over a wide range of pH values. The group II compounds, however, required *in vitro* the use of keratins and restricted pH values in order to duplicate their

active with protein hydroxyls (14), and DSCl and DSCN seem to require high pH values (about 10) for reaction with alcohols in aqueous systems (unpublished observations). Furthermore, tyrosine, serine, and threonine are much more abundant in keratins than in collagens and elastins (21).

behavior in the living skin. The latter observations indicate some of the significant variables in procedures concerned with evaluating allergenic activity of low molecular weight substances.

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SUMMARY

2,4-dinitrophenylsulfenyl chloride (DSCI) and 2,4-dinitrophenylthiocyanate (DSCN) elicited allergic reactions of the delayed type when applied to the skin of guinea pigs and of human beings who had been sensitized by prior exposure to 2,4-dinitrofluorobenzene (DF). DSCI and DSCN, together with 2,4-dinitrobenzene sulfonate (DSO₃), constitute a clearly defined group of allergenic dinitrophenyl compounds in that they all combined with skin protein *in vivo* through reaction with cysteine or cystine. *In vitro*, these compounds combine with free SH groups, and with —S—S— groups of hair and epidermis, but not with —S—S— groups of oxidized glutathione or of bovine gamma globulins. DSO₃, DSCI, and DSCN did not react with amino groups *in vivo*, but did react with protein amino groups *in vitro* at pH values of about 10.

Another group of dinitrophenyl compounds (DF, DCl, and DBr) previously had been shown to combine with lysine ϵ -NH₂ groups of epidermal proteins. In the present work it was found that these compounds do not react with the disulfide groups of these proteins, either *in vivo* or *in vitro*. Moreover, they did not seem to react with SH groups of viable skin, although they are highly reactive with sulfhydryl *in vitro*. This apparent discrepancy between reactivity with SH groups *in vitro* and *in vivo* may be due to the fact that the chromatographic technique employed was relatively insensitive for the sulfhydryl derivative.

When a compound of either group was applied to the skin surface, dinitrophenyl-amino acids were recovered from the epidermis but not from the dermis.

The results are discussed from the viewpoint of the epidermal localization of dinitrophenyl-protein conjugates.

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