ELICITATION OF ALLERGIC CONTACT DERMATITIS IN THE GUINEA PIG

The Distribution of Bound Dinitrobenzene Groups within the Skin and Quantitative Determination of the Extent of Combination of 2,4-Dinitrochlorobenzene with Epidermal Protein in Vivo*, ‡

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Plates 49 and 50

(Received for publication, July 24, 1958)

The biological characteristics of hypersensitivity reactions of delayed type are, in general, much the same as are those of many tissue responses known to be mediated by antibodies. For example, the requirement for structural specificity on the part of agents which evoke delayed allergic reactions seems to be just as exacting as is the case for antigens in classical immunological systems (1). There is, however, increasing evidence that serum antibodies are not required for delayed type reactions (2–4).

Delayed type hypersensitivity may be induced and elicited with certain simple chemicals as well as with such complex materials as tuberculin, diphtheria toxoid, and egg albumin (5–7). With the latter substances, allergic skin responses are commonly elicited in the sensitized host by means of intradermal injections. This procedure is limited by the fact that many inflammatory allergic skin responses which are clearly dependent on serum antibody may persist long enough after injection of sensitizer to overlap the time at which typical delayed-type reactions ordinarily appear. On the other hand, delayed responses elicited by application of sensitizers on the skin surface (allergic contact dermatitis) are free of this limitation because in none of the reactions known to be dependent on serum antibody does application of

* This work was supported, in part, by research grants from the National Institute for Allergy and Infectious Diseases, United States Public Health Service, and from the Standard Oil Company (New Jersey), New York City; it was also supported, in part, by the Research and Development Division, Office of The Surgeon General, Department of the Army (contract No. DA-49-007-MD-695), sponsored by the Commission on Cutaneous Diseases, Armed Forces Epidemiological Board.

‡ Portions of this study were presented before the American Society for Clinical Investigation (*J. Clin. Invest.*, 1953, **32**, 564), and before the American Association of Immunologists (*Fed. Proc.*, 1954, **13**, 491).

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sensitizer on skin surface lead to persistent inflammation (2). Among the more complex sensitizers only tuberculin is capable of eliciting a response following its application on the skin—and, then, only in sensitized man. As is well known, however, a multitude of simple chemical sensitizers produce delayed allergic reactions following surface contact in sensitized guinea pigs and man. Contact dermatitis elicited with simple chemical sensitizers furnishes, therefore, an especially unambiguous system for experimental study of mechanisms concerned with delayed type hypersensitivity: it permits the use of chemically defined sensitizers under conditions which minimize the possibility that antibodies, in or from serum, participate in a critical manner in the reaction.

The inflammatory lesion of allergic contact dermatitis develops just in that portion of the skin whose surface has been in contact with sensitizer. The piece of skin which underlies the area of surface contact is, therefore, a readily accessible tissue in which occur many of the significant reactions which constitute the molecular basis for this phenomenon.

As part of a study of these underlying reactions we have, in the present work, sought answers to the following questions: (*a*) how much of the sensitizer applied to the surface combines chemically with skin substance? and how much of the chemically combined material is accounted for as being conjugated with skin protein? (*b*) what structures within the skin are occupied by the determinant group? (*c*) how does the amount and distribution of chemically combined sensitizer in skin change in respect to time? For the most part, each of the foregoing questions has been examined both in normal, non-sensitized, guinea pigs and in guinea pigs with contact skin sensitivity specific for the low molecular weight sensitizers employed.

In the present study the sensitizers used were 2,4-dinitrofluorobenzene and 2,4-dinitrochlorobenzene. Analysis of skin for localization and measurement of dinitrophenyl groups was carried out after removal of organic solvent-extractable materials. This approach was justified by earlier work which demonstrated a strict correlation, amongst a group of 2,4-dinitrobenzenes, between the capacity of a substance to elicit the lesion of allergic contact dermatitis and its ability to combine chemically *in vivo* with skin proteins (8, 9).

The distribution of dinitrobenzene groups in skin was studied in paraffin sections by means of radioautography and a specific histochemical staining procedure. For radioautography and for quantitative analyses of skin, 1-C^{14}-2,4-dinitrochlorobenzene was used.

Experimental

Materials.—The 2,4-dinitrobenzenes[1] were obtained from commercial sources. DNCB was recrystallized from ether. ε-*N*-DNP lysine was prepared according to Porter and Sanger (10).

[1] Abbreviations used are as follows: DNCB, 2,4-dinitrochlorobenzene; DNFB, 2,4-dinitrofluorobenzene; DNCB*, 1-C^{14}-2,4-dinitrochlorobenzene; DNP, 2,4-dinitrophenyl group combined with protein or amino acid.

1-C^{14}-2,4-dinitrochlorobenzene[1] was prepared at Isotopes Specialities, Glendale, California, by nitration of 1-C^{14}-chlorobenzene,[2] and was crystallized twice from ether. The melting point determined in this laboratory was 50°–51°C. (Fisher-Johns melting block), and a paper chromatogram developed with *n*-butanol saturated with water revealed a single C^{14} peak which migrated close to the solvent front (Rf — 0.9), as did an authentic sample of 2,4-dinitrochlorobenzene. The peak contained 98 per cent of the C^{14} applied to the paper; a second peak was not detected. About 85 per cent of the product formed by DNCB* in the skin *in vivo* was characterized as a 2,4-dinitrophenyl derivative. Accordingly, no effort was made to identify impurities which might have been present to the extent of 2 per cent.

Radioactivity Counting.—Determinations of radioactivity were made in a flow gas counter on dried samples which were prepared on polyethylene planchets of 2.54 cm. diameter.[3] Counts were corrected for self-absorption, using 4 mg. as a reference weight for normalizing data. DNCB* could not be counted directly because of small but significant and variable evaporative losses. Accordingly, for purposes of counting, samples of this material were quantitatively converted into the sodium salt of 2,4-dinitrophenol by heating with dilute NaOH for 3 to 4 hours at 85°C. By this method of counting, the specific activity was 2.35×10^8 counts per minute (C.P.M.) per millimole. The same specific activity was found with C^{14}-ε-*N*-DNP-lysine prepared from DNCB* and lysine.[4] With the conditions employed, counting efficiency was estimated to be 28 per cent.

Animal Procedures.—The guinea pigs used were male albinos of 400 to 800 grams body weight. In some animals, sensitization of the contact dermatitis type was induced by intradermal injections of 2,4-dinitrochlorobenzene or 2,4-dinitrofluorobenzene (8). In a few experiments, described below, other methods for inducing sensitivity were employed. Unless otherwise stated, skin applications were made by dropping about 70 μl. of 0.01 M 2,4-dinitrochlorobenzene in 1:1 (V/V) acetone-corn oil onto freshly clipped skin areas on the dorsum.[5] Immediately afterwards the treated areas were gently stroked with a thin glass stirring rod for about 10 seconds so that the solutions finally covered a skin area of about 5 $cm.^2$. The glass rod generally removed about 0.2 per cent of the amount applied, as determined by C^{14} counting. Treated guinea pigs were maintained one to a cage in order to minimize losses of applied solution by their rubbing against one another.

Animals were sacrificed by intraperitoneal injections of sodium pentobarbital, and skin sites were excised about 5 minutes after injection, at which time animals were comatose.

Preparation of Skin for Histologic Study.—Excised skin was cut into strips about 3×20 $mm.^2$. Each of the strips was shaken at room temperature for 24 hours with 80 ml. of 9:1 ethanol-10 per cent formalin (V/V). The latter solvent was changed ½ hour, 2 hours, and 16 hours after initial immersion in order to extract free, *i.e.* unreacted, sensitizer as the tissue was being

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[2] Obtained from Tracerlab, Boston.

[3] Obtained from Formold Plastics Co., Chicago.

[4] DNCB* was diluted tenfold with DNCB and reacted with lysine by the method of Porter and Sanger (10). The C^{14}-ε-*N*-DNP-lysine was twice recrystallized from 6 N HCl.

[5] In non-sensitized guinea pigs applications made in this manner produce, 24 hours later, either no visible response or (in about 20 per cent of animals) an exceedingly faint discoloration. Since entry of sensitizer into skin and its subsequent reaction with protein is dependent, among other things, on its affinity for its solvent and penetrability of the solvent, the definition of a non-irritant solution of sensitizer must take into account the solvent as well as concentration of solute. For DNCB and DNFB, 0.01 M in 1:1 acetone-corn oil or in methyl cellosolve (ethylene glycol monomethyl ether) are non-irritating solutions. In some of the histologic studies, concentrations five- to tenfold greater were used for expedience; the latter solutions produced slight degrees of inflammation in the skin of all animals, previously sensitized or not, *i.e.* are primary irritants.

fixed. Fixed pieces of skin were finally embedded in paraffin and sections were cut in a conventional manner.

Radioautography.—Paraffin sections, 10 μ thick, were mounted on nuclear track plates (Eastman Kodak Co., NTB type, emulsion 10 μ or 14 μ thick) and stored at 4°C. in light-tight boxes which contained a small amount of anhydrous calcium sulfate. At the end of exposure periods, which varied from 1 to 24 months, the slides were developed photographically and then stained with metanil yellow (11); several sections on each slide were also stained lightly with iron hematoxylin. Some sections were left unstained after photographic development for examination by phase contrast microscopy. After drying, the sections were cleared with xylol and coverslips were sealed over them with "permount."[6]

Hematoxylin staining was necessary to visualize tissue details by direct microscopy, but obscured reduced granules of emulsion, unless very lightly applied. Satisfactory visualization of tissue detail and emulsion granules was possible, on unstained sections, by phase contrast microscopy.

Histochemical Staining.—In order to increase color intensity the paraffin sections used were 25 μ thick. After removal of paraffin with xylol, the sections, which had been mounted on glass slides, were placed for a few minutes in 95 per cent ethanol, and then successively, for the stated time intervals, in each of the solutions listed below:[7]

1. Two per cent stannous chloride (W/V) in equal parts of 8 N HCl and absolute ethanol. (This solution was prepared freshly each day.) Fifteen minutes immersion at room temperature produced what appeared to be maximal reduction of the aromatic nitro groups as estimated from the color developed at the conclusion of the subsequent steps. At higher concentrations of HCl tissue sections were rapidly digested. Following reduction the sections were rinsed in water.

2. Aromatic amines produced by the foregoing treatment, were next diazotized by immersing the sections in 0.1 M nitrous acid for 5 minutes at about 5°C. Following this treatment the slides were washed by dipping in ice cold water.

3. Residual nitrous acid was removed by immersion for 5 minutes in cold 0.1 M ammonium sulfamate, following which the slides were again rinsed with cold water.

4. The diazonium salts formed by the above process were next permitted to couple at room temperature by immersing the slides for 15 minutes in a solution of any of several aromatic amines or phenols. Coupling with phenols, such as 1,8-dihydroxy, 3,5-disulfonic acid naphthalene was carried out in dilute K_2CO_3, and coupling with amines, such as α-naphthylamine was done at neutral pH. At the end of the coupling reaction, slides were rinsed in water, air dried, cleared with xylol, and coverslips were sealed in place with permount. Counter stains were not used; satisfactory distinction between epidermis and dermis was possible with the phase contrast microscope.

The most satisfactory results were obtained by coupling with *N*-1-naphthylethylenediamine dihydrochloride (0.4 per cent in water). The azo dye formed with the latter reagent had a relatively strongly acidic amino group: thus, when given a final rinse with 0.1 N HCl, colored areas in the sections were brilliant purple, but after rinsing with a neutral solution, or even with distilled water, the color was reddish brown. Sections coupled with this reagent were finally rinsed in dilute acid prior to drying, clearing in xylol, and covering. Color was stable for at least 1 year (see Fig. 2*a*).

If a tissue section contains aromatic nitro groups chemically combined with tissue constituents, *e.g.* protein, the foregoing process is expected to form a complex azo dye which is combined through primary chemical bonds to reactive groups in tissue, the aromatic nitro

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[6] Obtained from Fisher Scientific Co., Pittsburgh.

[7] Gell (12) and Danielli (27) have used a similar procedure for quantitative analysis of trinitrobenzenes and for cytochemical localization of proteins.

groups being converted into azo groups. From each sample of skin treated *in vivo* with dinitrobenzene sensitizers, control slides were prepared by processing skin sections in the foregoing manner, save that the initial reducing step was omitted. In no instance did such control sections show any color (see Fig. 2 *b*). It is inferred, therefore, that little, if any, reduction of nitro groups to amino groups occurs spontaneously in the living guinea pig skin.

As contrasted with radioautography, the staining procedure was simple, rapid, but less sensitive. For example, the lowest concentration of sensitizer which could be applied to the skin to furnish sections having color intensity great enough for study was 0.05 M, and then only in the case of 2,4-dinitrofluorobenzene.[5] By contrast, skin applications of 0.01 M DNCB* (0.39 millicuries per millimole) yielded satisfactory radioautographs, but after about 1 year's exposure on plates.

Preparation of Skin for Radiochemical Analysis.—In order to separate epidermis from corium, the excised skin was placed, corium side down, for 2 minutes on a shielded hot plate whose surface was at about 52°C. Promptly after removing the skin from the hot surface, epidermis was scraped cleanly from corium (13).

The tissues were immediately placed in weighed tubes containing 2:1 (V/V) ethanol-acetone or 3:1 (V/V) ethanol-ether, in both cases the solvents being acidified with HCl (4 and 1 ml. 6 N HCl per 100 ml. solvent, respectively). From 8 to 21 extractions with these solvents were next performed at 40°–50°C. on each sample, using about 10 ml. solvent per extraction.

The first two extractions removed about 90 to 95 per cent of the total organic solvent extractable C^{14}. The C^{14}-labelled material thus extracted (which is DNCB*, partially if not entirely) failed to combine with epidermis when added to the latter *in vitro* and immediately reextracted in the usual way. Hence, continued reaction between DNCB* and epidermal constituents did not occur during the washing procedure. On the other hand, complete extraction of organic solvent soluble material was only approached and never fully achieved. Thus, analysis of occasional extracts (after the fifth one) revealed 1 to 6 C.P.M. per ml., even in the twenty-first of a series of extracts.

In a few experiments all the ethanol-acetone washings from each sample were combined (referred to as fraction I below) and counted after removing traces of hair and fine shreds of tissue by centrifugation (see fraction I, Table IV).

After the final ethanol-ether or ethanol-acetone extraction, the tissues were extracted with acetone, dried at room temperature overnight, then at 100°C. for about an hour, weighed, and hydrolyzed in sealed tubes with 6 N HCl at 105°C., using about 1 ml. acid per 10 mg. tissue. After 20 to 24 hours of hydrolysis, hydrolysates were diluted and aliquots were counted to furnish a measure of the dinitrobenzene groups combined chemically with the tissue, subject to the reservation expressed above regarding contamination by trace amounts of organic solvent-extractable radioactivity. In some experiments, described in the section immediately following, aliquots were further fractionated for separation and quantitation of dinitrophenyl amino acids.

Analysis of C^{14}-Dinitrophenyl Amino Acids.—Epidermal hydrolysates, which contained about 100 times more chemically bound dinitrophenyl groups than did dermal hydrolysates (Table III), were analyzed chromatographically. In these experiments, just before sealing hydrolysis tubes, 200 μg. of non-radioactive DNP-amino acid was added as a "carrier." This procedure was adopted to permit compensation for the variable and often extensive, losses of DNP-amino acids which occur during hydrolysis and chromatography, as revealed by preliminary recovery experiments. For example, with ε-*N*-DNP lysine, recoveries ranged from 60 to 80 per cent. The latter was chosen as the "carrier" of choice because previous qualitative work had shown it to be the principle DNP-amino acid recovered from skin treated *in vivo* with 2,4-dinitrochlorobenzene (8). Since this lysine derivative was found to account for such a large fraction of total bound radioactivity (see below), additional DNP-amino

acids were not used as "carriers." In a typical experiment hydrolysates were diluted to 5 ml.; 0.5 ml. samples were counted to measure total radioactivity combined with epidermis, and 4 ml. samples were analyzed for DNP-amino acids. The latter aliquots were extracted three times with peroxide-free ether[8] to furnish for each sample an acid-soluble fraction and an ether-soluble fraction, referred to below as fraction II and fraction III, respectively.

Acid-soluble fractions were evaporated to dryness *in vacuo* at 45°C. The residues were extracted four times with a 2:1 (V/V) mixture of methylethylketone-ether[9] saturated with water (hereafter referred to as M66), and the combined extracts were again dried; the final residue was redissolved in a small volume (about 0.5 ml.) of M66 and was chromatographed on a silica-gel column[10] (1 cm. diameter, about 15 cm. high) with 1 N HCl as a stationary phase and M66 as the developing solvent (14). The yellow ϵ-*N*-DNP lysine fraction was collected, dried, and made up to a known volume with 1 N HCl. Aliquots were counted, and the concentration of ϵ-*N*-DNP lysine was determined from the optical density at 390 mμ in a Beckman spectrophotometer, Model DU. The total amount of ϵ-*N*-DNP lysine formed *in vivo* was calculated from the C^{14} count, after correcting for recovery of unlabelled "carrier." The contribution of C^{14}-ϵ-*N*-DNP lysine (formed *in vivo*) to the ultraviolet absorption of the non-radioactive "carrier" was negligible.

The ether-soluble fractions of the hydrolysates were taken to dryness, the residues dissolved in acetone, and counted. Aliquots, after drying, were taken up in chloroform (saturated with water) and chromatographed on silica-gel with chloroform or chloroform-butanol (14).

Validation of the Carrier Method.—The validity of the carrier method used above for analysis of ϵ-*N*-DNP lysine rests on the assumption that decomposition of this amino acid is the same when incorporated in protein and when free in the form of added "carrier".

To test this assumption, DNP-bovine gamma globulin (DNP-BGG) was prepared (15), and the number of lysyl residue amino groups substituted by DNP was estimated spectrophotometrically (16). DNP-BGG was then hydrolyzed with C^{14}-ϵ-*N*-DNP lysine. Dried, defatted epidermis was added to simulate analytical conditions. The hydrolysates were extracted with ether, and chromatographed on silica-gel with M66. The recovery of non-radioactive ϵ-*N*-DNP lysine (*i.e.* originally incorporated in the DNP-BGG) was calculated by subtracting from the total ϵ-*N*-DNP lysine recovered (determined spectrophotometrically) the amount of carrier C^{14}-ϵ-*N*-DNP lysine which was present in the final eluate.

The results of two experiments, shown in Table I, indicate that there occurs during acid hydrolysis only slightly more destruction of protein-incorporated ϵ-*N*-DNP lysine than of the corresponding free amino acid. Since the over-all error in the determination of the lysine derivative is estimated to be ±5 per cent, the analytical results were not adjusted for the slightly greater recovery of "carrier" than of the corresponding protein-incorporated derivative. The

[8] Unless the ether is first freed of peroxides, exceptionally large losses of ϵ-*N*-DNP lysine are incurred. Peroxides were removed by extracting the ether twice with an acidified aqueous solution of ferrous sulfate (3 per cent).

[9] Methylethylketone was purified by redistillation. The fraction boiling at 80–81°C. was used.

[10] Mallinckrodt's silicic acid, 100 mesh, marked "specifically prepared for chromatography by the method of Ramsey and Patterson."

values for ϵ-*N*-DNP lysine formed *in vivo* are, accordingly, probably one or two per cent higher than are shown in Table V.

TABLE I

Recovery of ϵ-DNP Lysine Residues from a DNP-Protein and of C^{14}-ϵ-DNP Lysine after Acid Hydrolysis‡

Experiment No.	Recovery of total ϵ-DNP lysine§	Recovery of carrier C^{14}-ϵ-DNP lysine	Recovery of ϵ-DNP lysine from DNP-protein ‖
	per cent	*per cent*	*per cent*
1	67	69	65
2	76	78	73

‡ 1.5 mg. DNP-bovine gamma globulin (containing the equivalent of 66 μg. ϵ-DNP lysine) mixed with 85 μg. "carrier" C^{14}-ϵ-DNP lysine (6680 C.P.M.) and with 25 mg. dried, ethanol-extracted epidermis; mixture was hydrolyzed with 6 N HCl, 105°C., 20 hours, and the hydrolysate was extracted with peroxide-free ether and chromatographed on silica-gel (see Methods). One yellow band (Rf 0.15; ϵ-DNP lysine) was noted; it was eluted for analyses.

§ Determined from optical density of eluate at 390 mμ.

‖ Difference between total recovered and C^{14}-labelled "carrier" recovered.

Change in Amount of Bound Dinitrobenzene Groups Within Epidermis in Respect to Time.—At varying times after application of DNCB* to skin sites,[11] guinea pigs were injected with sodium pentobarbital and several minutes later, when the animals were comatose, the sites were carefully clipped to remove hair tips that had grown out since the initial clipping and application, and the sites were then gently wiped with thin absorbent paper. When multiple sites had been prepared in one animal (as many as five sites at different times prior to sacrifice), clipping and wiping were carried out first on an untreated site (control) and then on the treated sites in order of decreasing age. Skin was then excised, and epidermis was removed and extracted with ethanol-acetone or ethanol-ether, dried at 100°C., weighed, hydrolyzed, and counted. Untreated epidermal samples from the same animals were treated likewise to furnish blank values. The results, shown in Text-fig. 2, and Table VI, were not corrected for blanks as these were low and variable, *e.g.* 0, 1, 2, and 7 C.P.M. per mg. epidermis in four animals.

RESULTS

Localization of Bound Dinitrobenzene Groups in Skin.—The results of the histologic studies are given in Table II, summarized schematically in Text-fig. 1, and some of the more revealing sections are shown in Fig. 1. That free, *i.e.* unreacted, dinitrobenzene was not present in sufficient amount to influence the results may be assumed from the "zero" time experiment (Table II, 0.05 M DNFB), and from the absence of detectable radioactivity in the final change of fixative fluid in the case of skin treated with DNCB*. As a consequence, the

[11] Time was dated, in these experiments, as the interval between skin application and excision of skin. About 10 minutes elapsed between excision of skin and completion of the first organic solvent extraction.

TABLE II

Distribution of Bound Dinitrobenzene Groups in Skin at Varying Times after Surface Application of 2,4-Dinitrobenzene Sensitizers

Applied		Animal's status	Time on skin prior to excision	Appearance	
Substance	Concentration (and solvent‡)			Epidermis§	Corium
	moles/liter		*days*		
DCNB*	0.12(A)	Normal	0.1	C^{14} predominantly in outer half; also in hair shafts (cortex) and outer sheath cells of hair follicles.	Faint C^{14} in uppermost one-third.
			0.27	C^{14} spread diffusely.	Faint C^{14} in subepidermal region only.
			1.17	Same as above	Faint trace C^{14} in subepidermal region only.
			1.9	More C^{14} in outer half than in Malpighian layers; slight hyperplasia.	No C^{14}
			3	C^{14} only in outer cornified layers (stratum corneum); much hyperplasia.	No C^{14}
			4	C^{14} as above; much hyperplasia	No. C^{14}
DNCB*	0.012(B)	Normal	1	C^{14} spread diffusely, but slightly more in corneum than in Malpighian layers.	Questionable trace C^{14} in subepidermal region.
			3	C^{14} only in stratum corneum; slight inflammation.	No C^{14}
			7	C^{14} only in patches of stratum corneum; in hair shafts at level of epidermis.	No C^{14}
			13	C^{14} only in superficial patches of corneum; intense C^{14} concentration in some hairs above surface of epidermis.	No. C^{14}
DNCB*	0.012(B)	Sensitized	1	C^{14} in all levels; more C^{14} in stratum corneum than in Malpighian layers; inflammatory exudate intense.	Questionable trace C^{14} in subepidermal area; inflammatory response evident.
			3	Intense inflammatory exudate at epidermis-corium junction, separating these layers; epidermis necrotic; C^{14} through all levels of epidermis possibly some C^{14} in exudate separating epidermis and corium.	No C^{14}
			7	Striking hyperplasia; C^{14} only in topmost cornified layer.	No C^{14}
			13	Hyperplasia slight; C^{14} only in rare patch of cornified layer; intense C^{14} in a few hairs above surface of skin.	No C^{14}

TABLE II—*Concluded*

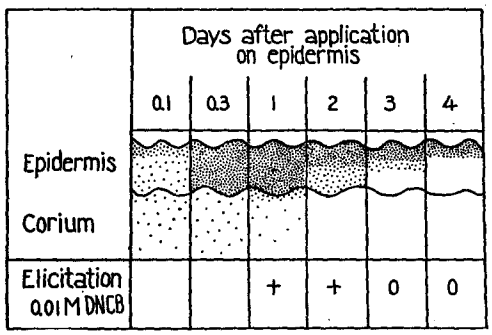
Applied		Animal's status	Time on skin prior to excision	Appearance	
Substance	Concentration (and solvent‡)			Epidermis§	Corium
	moles/liter		*days*		
DNFB	0.05(A)	Normal	0‖	Very rare, faint patch of dye in corneum.	No dye
			0.1	Much dye in corneum; little dye in Malpighian layers.	No dye
			0.3	Much dye in corneum; faint dye in Malpighian layers.	No dye
			0.7	Much dye uniformly distributed about equally intense in stratum corneum and Malpighian layers. Basal cell nuclei stand out as unstained objects.	No dye
			1	Dye diffuse through all levels; corneum and Malpighian layers are about equally colored; basal cell nuclei unstained.	No dye
			2	Dye mostly in corneum; very little in Malpighian layers.	No dye
			3	Dye light in corneum, none in Malpighian layer.	No dye
			4	As on day 3.	No dye
DNFB	0.05(A)	Sensitized	0‖	Very rare, faint patch of dye in corneum.	No dye
			0.1	Faint dye in corneum; very faint in Malpighian layers; slight dye in cortex of hairs and in outer root sheath of follicles.	No dye
			0.3	More intense than 0.1 day; more dye in corneum than Malpighian layer; nuclei of basal cells stand out as unstained objects.	No dye
			0.7	Dye intense at all levels; corneum and Malpighian layers about equally colored; basal cell nuclei have no dye.	No dye
			1	Dye at all levels; equal in corneum and Malpighian layers; basal cell nuclei not stained.	No dye
			2	Less dye than day 1; much more dye in corneum than in Malpighian layers.	No dye
			3	Hyperplastic; dye in corneum, none in Malpighian layers.	No dye
			4	Hyperplastic; corneum dyed; no dye in Malpighian layers.	No dye

‡ Solvent A is 1:1 acetone-corn oil; Solvent B is ethylene glycol monomethyl ether. Sections of skin treated with DNCB* were examined radioautographically; sections of skin treated with DNFB were stained (see Methods).

§ Epidermal hyperplasia was noted in normal (*i.e.* non-sensitized) animals 3 and 4 days after treatment with irritant concentrations, *i.e.* 0.12 M DNCB* and 0.05 M DNFB. In the normal animal treated with 0.012 M DNCB* 13, 7, 3, and 1 day prior to sacrifice, the third site of application in this series showed epidermal hyperplasia, probably ascribable to the animal's having become sensitized in response to the preceding skin applications (10 and 4 days previously).

‖ Actually about 5 minutes elapsed between applying sensitizer and placing excised skin in ethanol-formalin (see Methods).

sections may be regarded as revealing only those dinitrobenzene groups that are combined chemically with tissue constituents. Such bound groups are conspicuously concentrated in epidermis. Very small amounts, however, were noted in the corium, particularly when (1) highly concentrated solutions were applied to the skin, and (2) sections were removed within a few hours after such applications.



TEXT-FIG. 1. Schematic summary of cutaneous distribution of 2,4-dinitrophenyl (DNP) groups combined with skin components at different times after surface application of 2,4-dinitrochlorobenzene (DNCB) or 2,4-dinitrofluorobenzene (DNFB). Dots represent DNP groups, not extractable with organic solvents, localized by means of radioautography and specific stains (see Methods). The diagram is based chiefly on examinations of skin sites which had been treated *in vivo* with irritant concentrations, *e.g.*, 0.5 M DNFB and 0.12 M DNCB*. The results were essentially the same when 0.05 M DNFB or 0.012 M DNCB* were applied to skin, but the corium had either no detectable combined DNP groups, or, at most, faint traces of these groups were detected in the subepidermal zone of the corium. For the significance of the lowest line (elicitation with 0.01 M DNCB) see Tables VII and VIII and text.

When concentrations at, or approaching, the non-irritant levels ordinarily used for eliciting allergic reactions[5] were applied to the skin, bound dinitrobenzene groups were recognized in the corium only with great difficulty: with 0.05 M DNFB, no color was seen in the dermis (Table II); with 0.012 M DNCB* a faint trace of radioactivity was made out in the subepidermal layer of skin excised 24 hours after the initial application, and exposed to radiographic plates for 18 months.

As expected from their concentration in epidermis, dinitrobenzene groups were also present in hair follicles. The latter were involved principally at the level of the epidermis; on occasion the upper one-tenth of the dermal projection of the follicles contained some dinitrobenzene. Within hair follicles, dinitrobenzene groups were noted in epidermal outer root sheath cells and in the outer (cortical) zone of hairs.

Sensitive and non-sensitive animals yielded substantially the same results with respect to distribution.

Distribution between Epidermis and Corium Determined by Counting C^{14}.—Twenty-four hours after applying DNCB* to the skin surface of a non-sensitive guinea pig, the treated area of skin and a nearby untreated area were excised, and from each piece of skin epidermis was

separated from corium. The four samples thus obtained were analyzed after extraction with hot ethanol-acetone and hydrolysis. The results are given in Table III.

The small amount of radioactivity present in the corium of treated skin amounted only to about 1 per cent of the total bound to the skin and to about 0.02 per cent of the amount initially applied to the surface. As far as can be determined by histologic examination the method used for separating epidermis from corium (13) furnishes an epidermal fraction which is uncontaminated by corium, and a corium fraction which contains (1) only rare, very small patches of epidermis and (2) some of the deeper dermal projections of the hair follicles. It is possible that slight epidermal contamination accounts for the very small amount of radioactivity found in the corium.

TABLE III

Distribution of Bound Dinitrobenzene Groups in the Skin 24 Hours after Surface Application‡

Sample	C^{14} in tissue hydrolysate§	
	Total	Specific activity
	c.p.m.	*c.p.m. per mg. dry weight*
Control skin		
Epidermis	37	1.2
Corium	0	0
Treated skin		
Epidermis	4,360	47.0
Corium	47	0.4

‡ Applied 0.1 ml. of 0.01 M DNCB* in 1:1 actone-corn oil. Control skin refers to an area about 7 $cm.^2$, adjacent to the site treated with DNCB*. The guinea pig used in this experiment had not been sensitized.

§ Results expressed as counts per minute (C.P.M.), and C.M.P. per mg. lipide-free dry weight tissue.

Determination of the Extent to which Dinitrobenzene Groups Bound in Epidermis are Chemically Combined with Protein.—Inasmuch as about 99 per cent of the dinitrobenzene groups bound to the skin is present within epidermis, subsequent analyses were limited to this tissue. The analytical results obtained are most conveniently summarized by distinguishing between three fractions obtained with each sample of epidermis: Fraction I is the combined ethanol-ether and ethanol-acetone extracts of whole epidermis; it contains free, unreacted, DNCB*, and perhaps such DNP derivatives as those of basic phospholipides (17). Fractions II and III are, respectively, the acid-soluble and ether-soluble fractions of acid hydrolysates of extracted epidermis.

As shown in Table IV, the ratio of chemically combined DNP groups (fraction II + III) to organic solvent-extracted C^{14} (fraction I) is higher in those skin sites which showed an allergic response than in those sites which did not[12]. Possibly, this difference is a consequence of increased blood flow through sites of

[12] Dr. Victor H. Witten presented compatible findings before the Society for Investigative Dermatology, June 8, 1957, New York City.

inflammation leading to augmented removal of small, diffusible molecules. It may be noted in this connection that while gross inspection of positive allergic reactions in intact animals reveals only erythema, and edema (and hemorrhage in severe reactions), inspection of the under surface of the hide during excision of the skin reveals such sites to have extraordinarily intense vascular engorgement.

TABLE IV

Ethanol-Acetone Extractable and Non-Extractable Dinitrobenzene Groups in Epidermis‡

Skin responses at time of sacrifice§	DNCB* applied		C^{14} extracted by ethanol-acetone (Fraction I)		C^{14} combined with epidermis (Fractions II + III)		Ratio Non-extracted C^{14}: Extracted C^{14}
	Concentration	Total C^{14}	Total	Part of amount applied	Total	Part of amount applied	
	moles/liter	*c.p.m.* $\times 10^{-6}$	*c.p.m.*	*per cent*	*c.p.m.*	*per cent*	
0‖	0.019	2.23	71,500	3.2	24,800	1.1	0.35
0¶	0.019	2.23	190,000	8.4	34,000	1.5	0.18
0	0.010	1.18	44,000	3.7	8,300	0.70	0.19
±	0.010	1.18	27,000	2.3	7,100	0.60	0.26
1+	0.012	1.41	51,500	3.7	24,900	1.8	0.48
2+	0.011	1.06	28,400	2.7	21,000	2.0	0.74
3+	0.012	1.41	7,410	0.53	13,000	0.92	1.8
3+	0.012	1.41	14,500	1.0	16,400	1.2	1.1

‡ Applied 0.1 ml. of DNCB* to each skin site, using 4 or 5 sites per guinea pig. Animals sacrificed 24 hours after application. Solvent was 1:1 acetone-corn oil. C^{14} expressed as counts per minute (C.P.M.); one μmole DNCB* has 235,000 C.P.M.

§ Grading of skin responses as in reference (8). In non-sensitized guinea pigs, responses were negative (upper four lines); responses in the sensitized animals (lower four lines) recorded as an impression of the average response in the four or five sites in each animal.

‖ 440,000 C.P.M. voided in urine during the 24-hour period (20 per cent of amount applied).

¶ 400,000 C.P.M. voided in urine during the 24-hour period (18 per cent of amount applied).

In regard to the diffusion of dinitrobenzene groups through the skin, it should be noted that about 20 per cent of the C^{14} applied to normal animals' skin was excreted in the urine during the 24 hours following application (footnote, Table IV)[13].

As shown in Table V, the epidermal C^{14} which is not extractable with organic solvents is predominantly acid-soluble (fraction II), and about 85 per cent of it can be accounted for as ε-*N*-DNP lysine.

The fraction of epidermis-bound dinitrophenyl groups which is extractable by ether from epidermal hydrolysates is very small (fraction III, Table V). Most of the C^{14} in this fraction moved rapidly on silica-gel columns developed with

[13] During the 2nd day after surface application of DNCB*, the urine contained one-tenth the C^{14} excreted in urine during the first 24 hours after application.

chloroform saturated with water, and probably was dinitroaniline and, or, dinitrophenol (18). The rest of this fraction, part of which was eluted with chloroform-butanol, was not characterized further owing to the very small

TABLE V

Analyses of Epidermis-Combined Dinitrobenzene Groups‡

Experiment No.	Skin response at time of excision	Fraction of total combined DNP (fraction II + III) recovered as ϵ-DNP lysine§	Fraction of total combined DNP (fractions II + III) recovered in fraction III¶
		per cent	*per cent*
1	0‖	85	—
2	0	84	—
3	0	96	4.5
4	0	81(A)	5.6
		85(B)	4.1
5	0	74	—
6	0	74	—
7	±	89(A)	5.6
		87(B)	—
8	1+	92(A)	4.4
		92(B)	—
9	2+	78(A)	4.1
		74(B)	—
10	3+	91	6.0
11	3+	71	5.7

‡ Applied to each skin site 0.1 ml. of 0.01 M DNCB* in 1:1 acetone-corn oil (235,000 C.P.M.). Skin sites excised 24 hours later; the appearance at time of excision is recorded in column 2. Negative responses in non-sensitized guinea pigs; positive responses in sensitized animals. Epidermis-combined dinitrobenzene groups are defined as those not extractable with ethanol-acetone or ethanol-ether. After extraction with the latter solvents, epidermis was hydrolyzed (with "carrier" ϵ-DNP lysine) and hydrolysates were extracted with ether to give acid-soluble (II) and ether-soluble (III) fractions. Fraction II analyzed for ϵ-DNP lysine, correcting for recovery of "carrier" (see Methods and Table I).

§ Isolated chromatographically from fraction II. Designations (A) and (B) refer to paired epidermis samples from a given skin site; one sample (A) treated in usual manner, and the other sample (B) extracted prior to hydrolysis with 5 per cent trichloroacetic acid at 95–100°C.

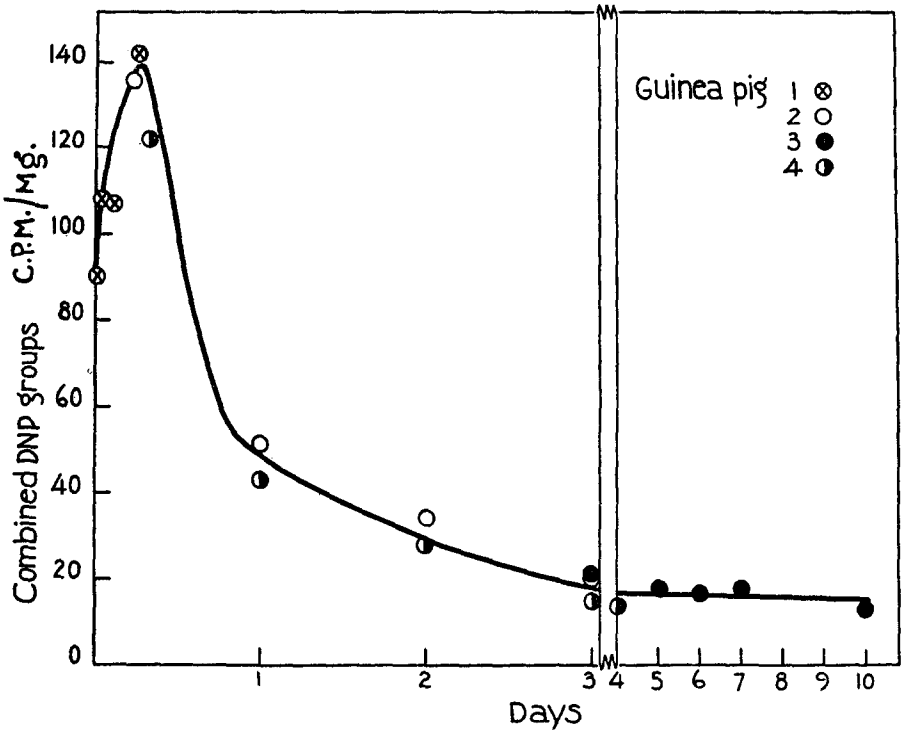
¶ Dash means no analysis performed.

‖ In this experiment, treated skin excised about 10 minutes after DNCB* application; specific activity after ethanol-ether extraction was 22 C.P.M. per mg. lipide-free dry weight epidermis (compare with Text-fig. 2).

amount of radioactivity available, and to the expectation—based, in part, on Middlebrook's analysis of *N*-terminal amino acids of wool (19)—that it was divided amongst several different α-*N*-DNP-amino acids. There is, finally, the probability that traces of free DNCB* are present in this fraction.

The analyses shown in Table V leave less than 10 per cent of the C^{14} com-

bined with epidermis unaccounted for either as ϵ-DNP lysine or as falling in Fraction III. This residual activity has not been identified. Owing to the reactivity of 2,4-dinitrochlorobenzene with sulfhydryl groups (20), we suggest that it may be, at least in part, *S*-DNP-cysteine. The latter is insoluble in M66, but some of its decomposition products, formed during hydrolysis, are



TEXT-FIG. 2. Time-course followed by 2,4-dinitrophenyl (DNP) groups combined with epidermis. Four or five skin sites were prepared in each guinea pig at different times before sacrifice by applying to each area 0.1 ml. of 0.01 M DNCB* (235,000 counts/minute/μmole). Epidermal samples were thoroughly extracted with warm ethanol-ether prior to hydrolysis and counting. Ordinate is counts per minute per mg. lipide-free dry weight epidermis.

soluble in this solvent and either fail to move on the columns used, or move (Rf about 0.4) appreciably faster than ϵ-*N*-DNP lysine (9). Other acid-soluble DNP-amino acids might also have been present in amounts too small for detection.

In a few experiments, the effect of removing nucleic acids by hot trichloroacetic acid (TCA) extraction was examined. After repeated organic solvent extractions, the tissues were dried and then extracted with 5 per cent TCA in a boiling water bath for 30 minutes. The insoluble residue was then washed with

ethanol-ether, dried, and analyzed for distribution of radioactivity. No significant change in respect to C^{14}-ϵ-DNP-lysine was observed, as shown in Table V. The extraction with hot TCA removed 10 per cent by weight of the lipide-free epidermis and only 3 per cent of its C^{14} activity.

Changes in DNP-Protein Conjugates in Epidermis in Respect to Time.—As indicated in Text-fig. 2, the reaction of DNCB* with epidermal protein was evident within 1 hour after applying the simple chemical on the skin surface (see also Table VI, and Table V, experiment No. 1). The amount of bound dinitrobenzene per mg. epidermis was maximal 3 to 6 hours after skin application, and fell rapidly during the following day. On the 3rd day and beyond, when histologic examination by radiography and staining had demonstrated confine-

TABLE VI

Changes in Epidermis-Combined Dinitrobenzene Groups in Sensitized and Non-Sensitized Guinea Pigs

Time after surface application‡	Normal guineapigs§	Sensitized guinea pigs§, ¶
hrs.	*c.p.m. per mg.*	*c.p.m. per mg.*
1	56	57
3	88	104
5	—	95
7	88	80
15	38	57
24	41	46

‡ Each application consisted of 0.08 ml. 0.01 M DNCB* in 1:1 acetone-corn oil.

§ Results expressed as C.P.M. per mg. lipide-free, dry weight epidermis. 235,000 C.P.M. per μmole 2,4-dinitrobenzene.

¶ Animals sensitized about 2 weeks previously by injecting each in the foot pads with a total of 20 μg. 2,4-dinitrofluorobenzene in Freund's complete adjuvant.

ment of the bound groups to the cornified layer of epidermis, disappearance was exceedingly slow and probably reflected the rate of desquamation of epidermal scales from the surface. The more rapid disappearance, noted particularly during the 1st day after application, may be ascribed to movement of DNP-protein conjugates, or their breakdown products, into internally situated sites.

At the time of greatest accumulation of conjugates in epidermis, about 4 per cent of the DNCB* initially applied was present in epidermis in chemically bound form. Owing to the rapid disappearance of conjugates from the viable layers of epidermis, a larger fraction of the applied DNCB* actually combined with epidermal protein.

As shown in Table VI, the time-course followed by conjugated DNP groups in epidermis is essentially the same in guinea pigs with contact skin sensitivity for 2,4-dinitrobenzenes and in non-sensitized guinea pigs.

Correlation between Distribution of Combined Dinitrobenzene and the Ability to Elicit the Allergic Skin Response.—In order to challenge sensitive animals under conditions in which dinitrophenyl conjugates occupied various positions within the skin, advantage was taken of the 5-day interval following initial exposure to the sensitizer. During this period the capacity to respond to a contact skin test is not yet developed. Accordingly, skin tests were performed in a standard manner at varying times during the 5 days following induction of sensitivity by a single application of 2,4-dinitrofluorobenzene (Tables VII and VIII).

TABLE VII

Responses to Skin Tests Made Daily Beginning at Time of Induction‡

Guinea pig No.	Days after initial exposure when skin test applied					
	0	1	2	3	4	5
	Appearance of test sites on 6th day					
1	0	0	0	0	±	1+
2	0	0	0	0	±	±
3	0	0	0	0	1+	2+
4	0	0	0	0	±	±

‡ Sensitization induced by a single application on abdomen of 0.1 ml. 0.5 M 2,4-dinitrofluorobenzene in 1:1 acetone-corn oil. Skin tests made on dorsum with 0.07 ml. 0.01 M DNCB per site; each animal tested daily, for a total of six test applications per guinea pig. Results refer to appearance of depilated sites on the dorsum on the 6th day after initial application (grading as in reference (8)). The negative responses had not been positive prior to the time when recorded readings were made.

In one set of experiments, skin tests were performed at 24-hour intervals in each animal for 6 days, beginning at the time of initial application of sensitizer. The sites were inspected daily, and on the 6th day after induction animals were depilated so as to permit more detailed examination. The readings made 2 to 3 hours after depilation are shown in Table VII.

The foregoing experiment was repeated in modified form in order to avoid multiple tests in each animal. Fifteen animals were sensitized as above, and at 24-hour intervals, beginning 24 hours after induction, groups of three animals were tested. Each animal was tested once. Test sites were depilated 24 hours after they were prepared, and read 2 to 3 hours later. Sites were examined daily thereafter and depilation was repeated at 2 to 3 day intervals subsequently to permit careful scrutiny throughout the duration of the experiment. The results, which are shown in Table VIII, are in agreement with those of the preceding experiment.

Owing to the characteristic delay between application of a skin test in an already sensitive animal and appearance of the allergic response, it is not possible to define the time of acquisition of responsiveness more closely than that it begins to be manifest during the 5th day and is quite regularly manifest on the 6th day after induction.

If one defines the test sites made at the end of the 5th day after induction as

being 1 day old when the positive skin responses are evident at the end of the 6th day, it follows from the data of Tables VII and VIII that 2-day old sites are irregularly effective, and sites which are 3 days old, or older, lack entirely the capacity to elicit the allergic response. These results are in agreement with those obtained by Chase, who induced sensitization in guinea pigs both by intradermal injection of picryl chloride and by transfer of lymph node cells from sensitive donors (2, 21).

TABLE VIII

Responses to Skin Tests Made at Various Intervals after Induction‡

Guinea pig No.	Days after initial exposure						
	1	2	3	4	5	6	7
1	T		0	0	0	0	0
2	T		0	0	0	0	0
3	T		0	0	0	0	0
4		T	0	0	0	0	0
5		T	0	0	0	0	0
6		T	0	0	0	0	0
7			T	0	0	0	0
8			T	0	0	0	0
9			T	0	0	0	0
10				T	0	0	0
11				T	0	1+	1+
12				T	2+	2+	2+
13					T	3+	3+
14					T	2+	3+
15					T	2+	2+

‡ Sensitization induced as in footnote of Table VII. A single skin test was made in each guinea pig with 0.07 ml. 0.01 M DNCB. Time of application of skin test indicated by T; responses were recorded daily beginning on 3rd day, and are graded as in reference (8). The responses to DNCB skin tests recorded above were much more intense than in the experiment of Table VII, probably because in the present experiment a *single* test was made in each animal, whereas in Table VII the animals were each tested daily for a total of six test applications per guinea pig.

Three days after a standard skin test with DNCB, bound dinitrobenzene groups are not only confined to the cornified layers of epidermis (Table II, Text-fig. 1, Fig. 1), but the total amount in epidermis is relatively low (Text-fig. 2; about 20 C.P.M. or 9×10^{-5} μmoles DNP per mg. dry weight epidermis). In order to determine whether absence of elicitatory capacity of test sites which are 3 days old is ascribable to the special localization of determinant groups at this time or to the small quantity of conjugates in sites of this age, the following experiment was performed:

Fifteen guinea pigs were sensitized by a single administration of 2,4-dini-

trofluorobenzene. Three days later, five animals were tested with 0.01 M DNCB. Five days after induction five other animals were tested with 0.003 M DNCB, and five were tested with 0.01 M DNCB. The results of the skin tests are given in Table IX. As noted earlier, 0.01 M DNCB applied 3 days after induction fails to evoke an allergic response even when generalized cutaneous reactivity is fully developed on the 6th day after induction. On the other hand, a skin site prepared with 0.003 M DNCB 1 day before generalized reactivity is

TABLE IX

Responses to Skin Tests with 2,4-Dinitrochlorobenzene at Varying Concentrations and at Different Times after Induction of Skin Sensitivity

Experiment No. ‡	Test procedure		
	Concentration of DNCB§	Time after induction when test solution applied	Results¶
	moles/liter	*days*	
I	0.01	3	0, 0, 0
	0.01	5	2+, 2+, 2+
	0.003	5	1+, ±, 0
II	0.01	3	0, 0
	0.01	5	2+, 2+
	0.003	5	1+, ±

‡ Induction of sensitivity as follows: In experiment I, with a single application of 2 drops 0.5 M 2,4-dinitrofluorobenzene in 1:1 acetone-corn oil on clipped skin of belly; experiment II, with 20 μg. 2,4-dinitrofluorobenzene in Freund's adjuvant (50 μg. *Mycobacterium butyricum* per ml. in complete emulsion) injected at one time in four divided doses of 0.1 ml. each into the paws.

§ Solvent for test solutions 1:1 acetone-corn oil.

¶ Readings made 1 day after test solution applied and about 3 hours after depilation. In the case of tests made with 0.01 M DNCB 3 days after induction, the readings remained negative for the duration of the experiment (7 days). Each value refers to a single guinea pig. Each guinea pig received a single skin test.

acquired, does produce an inflammatory reaction, although weakly and not in all animals.

That the amount of bound dinitrobenzene groups in epidermis 1 day after applying 0.003 M DNCB is about the same as 3 days after applying 0.01 M DNCB was shown with DNCB*. Twenty-four hours after applying 0.003 M DNCB* on the skin of a normal guinea pig, epidermal sites were analyzed as described above. Twenty-four C.P.M. (1×10^{-4} μmoles) bound dinitrobenzene per mg. dry, lipide-free epidermis was found, as compared to about 20 C.P.M./mg. 3 days after applying 0.01 M DNCB* (Text-fig. 2). Accordingly, the foregoing experiments indicate that the ineffectualness of 3-day-old test sites prepared in the standard manner with 0.01 M DNCB may be ascribed to the

sequestration of bound dinitrobenzene groups in the outer cornified layers of epidermis and to the absence of an adequate number of such groups from the deeper layers of skin, particularly near the epidermal-dermal junction.

DISCUSSION

In earlier studies it was suggested that DNP-protein conjugates which form in the skin following surface application of dinitrobenzene sensitizers are formed and concentrated predominantly in epidermis (8, 9). This suggestion was based, firstly, on the detection of DNP-amino acids in hydrolysates of epidermis, but not in hydrolysates of underlying corium, and secondly, on the observation that reactions of certain dinitrobenzene sensitizers with skin proteins are apparently limited to substitution in -SH and -S-S- groups; the latter are unusually abundant and reactive in epidermal proteins.

In the present work, using more direct and sensitive methods, the inference regarding epidermal localization has been confirmed. A trace of bound dinitrobenzene has, however, been noted in the corium. The analytical methods used could not be sufficiently well controlled to evaluate rigorously the possibility that bound dinitrobenzene in the corium might be an artefact arising from experimental manipulation. A rather large fraction of the DNCB* applied to the skin surface was, however, excreted in urine. This finding alone, even had there not been a trace in corium, demonstrates that dinitrobenzene groups, conjugated or unconjugated, pass through epidermis into corium and beyond.

Since collagen, per unit weight, has more lysine residues than keratins,[14] it might be expected that free DNCB diffusing from the skin surface would react as readily with proteins of the corium as with epidermal proteins. It appears, however, that this is not the case. Thus, when DNCB* was injected intradermally (*i.e.* into the corium) and the injected sites were excised 24 hours later, 90 per cent of the dinitrobenzene groups bound by the skin were in epidermis and only 10 per cent were in the corium.[15] Corroboration of these results was furnished by radiographs which demonstrated in such intradermally injected sites intense localization of C^{14} in hair follicles, much localization in epidermis diffusely, and only slight C^{14} in the corium (Fig. 2 *c*). The low reactivity of

[14] Moles of lysine in 10^5 gm. of protein are 32 for collagen and 18 for wool keratin (22). Collagen in the form of "purified hide powder" reacts readily *in vitro* with 2,4-dinitrofluorobenzene to yield an intensely yellow DNP-protein (unpublished work with Dr. Mary E. Carsten).

[15] Each of eight skin sites on a normal guinea pig was injected intradermally with 0.1 ml. of 0.15 M NaCl, 1 per cent ethanol, containing 11.3 μg. DNCB* and 13,150 C.P.M. The sites were excised the next day, and epidermis was separated from dermis. The corresponding tissues from the several sites were combined and extracted with ethanol-acetone and hydrolyzed. Total radioactivity in each of the hydrolysates were: epidermis 2,120 C.P.M., and corium 251 C.P.M., corresponding, respectively, to 32 and 0.8 C.P.M. per mg. dry, lipide-free tissue (see Fig. 2 *c*).

corium proteins with DNCB* *in vivo* is surprising.[14] Possibly, in intact skin interaction between anionic mucopolysaccharides of ground substance and NH_2 groups of collagen render the latter relatively inaccessible for reaction with DNCB. In view of the much lower reactivity of corium than of epidermis *in vivo*, it is possible that the trace of bound dinitrobenzene groups observed in corium following surface application of DNCB* represents conjugates which formed initially in epidermis and passed subsequently into corium.

About 85 per cent of the dinitrobenzene groups combined with epidermis are substituted in ϵ-amino groups of lysine residues. Accordingly, it is apparent that the bound groups are predominantly, if not entirely, combined through primary chemical bonds with protein.[16] While the remaining bound groups have not been characterized they probably are mostly, if not entirely, combined in similar fashion with a few other amino acid residues; cysteine (20) and *N*-terminal amino acids may be noted as particularly good possibilities.

In regard to the level in the skin at which the conjugates exert their specific effect, the experiments indicate that the allergic response is elicitable when bound dinitrobenzene groups are present in the vicinity of the epidermal-dermal junction, principally in the Malpighian layer of epidermis. It must be emphasized, however, that conjugates in the corium immediately subjacent to epidermis were only present in trace amounts, and may have originated in epidermis. When the conjugates are absent from these areas, and confined to the superficial half of the epidermis, they are ineffectual.

From the results summarized in Text-figs. 1 and 2, it appears that conjugates within the cornified layer of epidermis disappear very slowly. It is reasonable to assume that their loss is to the exterior, as a consequence of the shedding of epidermal scales. These findings, and the interpretation offered, provide an experimental basis for the common clinical observation that amongst sensitized humans who handle non-volatile allergenic materials it is usual to find lesions of allergic contact dermatitis not on the palms, but rather on the eyelids, face, dorsum of hands, etc. In the latter areas, exposure of skin surface would not be expected to be as great as on the palms, but the cornified layer of epidermis in these secondarily contaminated areas is much thinner. One may, accordingly, regard the cornified layer of skin as a barrier to the entry of many reactive substances, its effectiveness arising from dense packing of highly reactive side chain

[16] The ϵ-*N*-DNP-lysine recovered in these experiments must have been derived from DNP-proteins, or at least DNP-polypeptides. Had substitution in free lysine occurred, the extensive organic solvent extraction, prior to hydrolysis, would have removed ϵ-*N*-DNP-lysine which is significantly soluble in oganic solvents. (At pH 7 this DNP-amino acid has a distribution ratio between *n*-amyl alcohol and water of 1.23 (25)). Moreover, reaction of DNCB with free lysine would be expected to yield, besides the above derivative, also α-*N*-DNP-lysine and, or, bis-DNP-lysine, neither of which was detected in previous work (8).

groups and the gradual shedding of this layer to the exterior. It is likely that reactive simple chemicals which combine with protein of the stratum corneum are largely prevented from deeper penetration, and are eventually shed in desquamating epidermal scales.

In contrast to the slow disappearance of conjugates from the superficial cornified layers of epidermis, the conjugates in the deeper layers of epidermis disappear relatively rapidly. It must be inferred that the latter rapid loss reflects penetration to internally situated sites. This inference is consistent with the large urinary excretion of C^{14} during the first 24 hours after surface application of DNCB*. The total recovery of applied dinitrobenzene at the end of this time interval is about 25 per cent: about 5 per cent from skin, and 15 to 20 per cent from urine. Hence about 75 per cent of applied material may be assumed to be distributed in non-cutaneous tissues. These values, although crude, are of interest in relation to the induction of sensitivity when accomplished by a surface application of sensitizer. There has been, for example, speculation as to whether the piece of skin whose surface is initially exposed to sensitizer is responsible for the synthesis of the hypothetical antibodies which are widely assumed to mediate allergic contact dermatitis (23). The results obtained in the present work make it gratuitous to ascribe any special inductive function to the conjugates which are held in the skin in view of the relatively large amount of sensitizer which perfuses internal organs, including, in all probability, regional lymph nodes.

In contrast to induction, it appears that for elicitation of the allergic response (in the already sensitized host) the conjugates which are fixed in the skin are of paramount importance. This conclusion is warranted because of (*a*) the high correlation between eliciting capacity and ability to form conjugates with skin protein (8, 9) and (*b*) the topographical correspondence between the position occupied by functional conjugates and the major site of the inflammatory response (centered about basal layers of epidermis and immediately underlying corium).

In view of the decisive role in elicitation accredited to protein conjugates in the skin, it is important to note that when DNP-protein conjugates, prepared *in vitro*, are injected intradermally in human subjects with contact skin sensitivity specific for 2,4-dinitrobenzenes, delayed inflammatory responses are *not* obtained (24). Such experiences indicate the existence of additional, as yet unknown, requirements for elicitation. It is possible, for example, that conjugates prepared *in vitro* and then injected intradermally have only little, if any, access to critical intracellular sites; low molecular weight sensitizers, on the other hand, readily penetrate into cells, and form intracellular conjugates (25).

In accord with the earlier work of Chase (2, 21) it has been found in the present work that, in guinea pig skin, test sites 3 days old, or older, are incapable of eliciting the response of allergic contact dermatitis. This situation

stands in sharp contrast with observations commonly made in man of skin test sites which are negative, and remain free of gross inflammation for 1 to 2 weeks, lighting up only then with the characteristic lesion of contact dermatitis. At such time, human subjects displaying this sequence appear to have become sensitized, since they react with typically positive responses in 1 to 2 days on repetition of the initial test. This sequence, commonly referred to as "flare up," demonstrates that in man non-irritant skin tests furnish products which remain effective eliciting agents for as long as 7 to 14 days after skin testing. This species difference may be ascribed to the well known circumstance that humans with contact skin sensitivity commonly react to much lesser amounts of sensitizer than do sensitized guinea pigs (8). Hence, a trace of sensitizer-protein conjugate in the basilar epidermal layers, not detectable by the means used in the present study, might suffice to evoke a response in sensitized man, but be insufficient in the sensitized guinea pig. An alternative possibility, for which there is now no evidence, is that disappearance of sensitizer-protein conjugates may be far slower in human than in guinea pig epidermis.

In immediate type allergic responses, such as anaphylaxis, it has been possible to define, in weight units of antigen, the minimum antigenic stimulus required for elicitation (26). For delayed type allergic responses, on the other hand, it would seem necessary to add to the quantitative definition of an antigenic stimulus the dimension of time. This requirement is brought out in Text-fig. 2, which shows large changes in concentration of epidermal conjugates during the 24-hour period over which the inflammatory response of allergic contact dermatitis evolves. From Text-fig. 2 the antigenic stimulus (in the form of protein-conjugated DNP groups) delivered over a 24-hour period following a standard test with 70 μl. 0.01 M DNCB could be estimated to be 0.003 μmole-hours per mg. dry, lipide-free, epidermis. This estimate is actually too high, perhaps by an order of magnitude, since most of the conjugated dinitrobenzene groups plotted in Text-fig. 2 are present in cornified layers of epidermis and considered, for reasons given above, not to be functional.

Combination of DNCB* with epidermal protein is rapid and the amount of conjugate present in the skin is maximal many hours before the allergic inflammatory response is grossly well developed. Combination with protein is, accordingly, not the rate-limiting step in elicitation of allergic contact dermatitis by dinitrochlorobenzene.

SUMMARY

When one or two drops of a dilute, non-irritating solution of 2,4-dinitrochlorobenzene (DNCB) is applied to a small area of skin of the intact guinea pig, about 20 per cent of the applied material, or some derivative of it, is soon excreted in urine. In normal, as well as in specifically sensitized guinea pigs, DNCB at the site of local application becomes rapidly bound to skin protein

through primary chemical bonds. Twenty-four hours after application roughly half of the material present at the local skin site is still extractable with organic solvents. Of the non-extractable dinitrophenyl groups, about 99 per cent are in epidermis, and about 85 per cent are substituted in ϵ-NH_2 groups of lysine residues. Only traces of bound dinitrophenyl groups were observed in the corium. It is uncertain whether these are formed *in situ*, or are experimental contaminants, or are migratory epidermally formed conjugates. Even when DNCB is injected intradermally it combines predominantly with overlying epidermis and with epidermal components of hair follicles, but only slightly with corium.

The 2,4-dinitrophenyl conjugates which are localized in the deeper, viable half of the epidermis, close to the epidermal-dermal junction, are inferred to be the agents responsible for specifically evoking the allergic response in sensitized animals. Conjugates which are situated in the outer, cornified half of the epidermis are shown to be incapable of eliciting the allergic response. The results furnish a basis for interpreting a common pattern of lesions in allergic contact dermatitis as it occurs spontaneously in man.

We wish to acknowledge with gratitude the capable assistance of Mrs. Elizabeth Byrnes Parnell.

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EXPLANATION OF PLATES

PLATE 49

FIG. 1. Radiographs of skin removed at different times after application of DNCB* on skin surface. × 430.

FIG. 1 *a*. One day after 0.012 M DNCB* (in ethylene glycol monomethyl ether) was applied to the skin of a guinea pig previously sensitized with DNFB. Radiographic plate exposed 5 months. Reduced emulsion granules are distributed uniformly over the area corresponding to epidermis.

FIG. 1 *b*. Two days after 0.12 M DNCB* (in 1:1 acetone-corn oil) was applied to the skin of a non-sensitized guinea pig. Radiographic plate exposed 3.5 months. Reduced granules are distributed diffusely over the area of epidermis, but are slightly more concentrated in the outer half of this layer.

FIG. 1 *c*. Three days after 0.12 M DNCB* (in 1:1 acetone-corn oil) was applied to the skin of a non-sensitized guinea pig. Radiographic plate exposed 3.5 months. Reduced granules are detected only in the cornified outer part of epidermis.

FIG. 1 *d*. Four days after 0.12 M DNCB* (in 1:1 acetone-corn oil) was applied to the skin of a non-sensitized guinea pig. Radiographic plate exposed 3.5 months. Reduced granules were confined to the cornified outer part of epidermis.

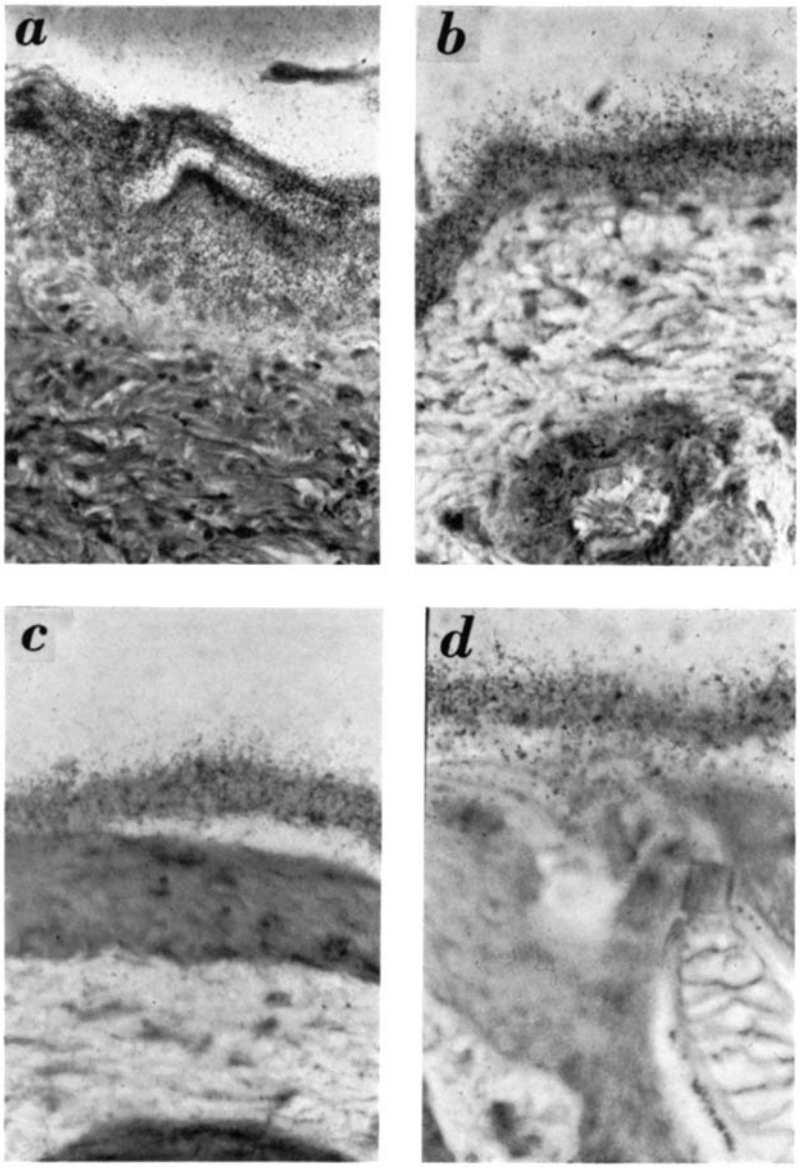

FIG. 1

(Eisen and Tabachnick: Allergic contact dermatitis)

PLATE 50

FIGS. 2 *a* and 2 *b*. Photomicrographs of skin of a non-sensitized guinea pig removed 1 day after placing on it one drop of 0.5 M DNFB (in 1:1 acetone-corn oil). Paraffin sections, 25 μ thick, were stained to demonstrate nitrobenzenes combined with skin (see Methods). The section shown in Fig. 2 *a* developed an intensely and diffuse red-purple epidermis, indicated by cross-hatched lines. The section shown in Fig. 2 *b* failed to develop any color; this section had been treated exactly as had been the section in Fig. 2 *a*, except that the initial reducing step was omitted. The sections of Figs. 2 *a* and 2 *b* were adjacent to each other in serial sections of excised skin. About $\times$ 430.

FIG. 2 *c*. Radioautograph of skin removed one day after intradermal injection of 11.3 μg. DNCB* in 0.1 ml. 0.15 M NaCl, 1 per cent in respect to ethanol. Radiographic plate exposed 3 months. Note relative concentration of reduced granules in epidermis and in hair follicles (arrows); for C^{14} content of skin layers in this experiment see text footnote 15. About $\times$ 100.

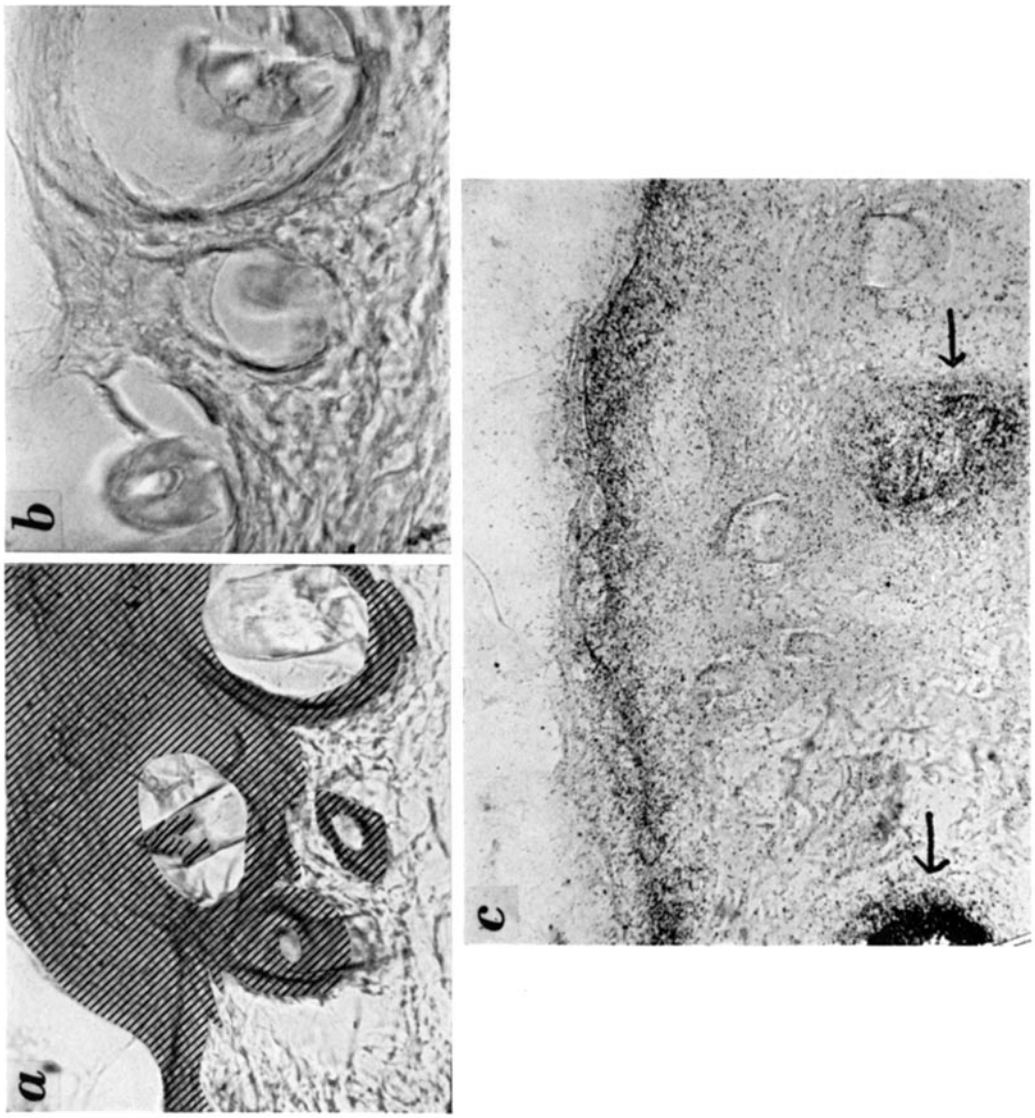

FIG. 2

(Eisen and Tabachnick: Allergic contact dermatitis)