A STUDY OF THE DISTRIBUTION OF 2,4-DINITROBENZENE SENSITIZERS BETWEEN ISOLATED LYMPH NODE CELLS AND EXTRACELLULAR MEDIUM IN RELATION TO INDUCTION OF CONTACT SKIN SENSITIVITY*‡

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Many protein conjugates, prepared *in vitro* with reactive simple chemicals, are highly effective reagents for inducing the formation of antibodies which are specific for the simple chemical components of the conjugates (1). Antibodies may also be formed directly in response to injections of certain simple chemicals, providing the simple substances used are capable of reacting *in vivo* to form protein conjugates (2–4). These generalities are only partly applicable to the induction of contact skin sensitivity. In order for simple chemicals to induce the latter hypersensitive state, the *in vivo* formation of protein conjugates (6, 7) is also obligatory (5). However, with a few possible exceptions (8), protein conjugates prepared *in vitro* have little, if any, capacity to induce contact skin sensitivity (9). Part of the present work is devoted to the presentation of evidence which supports and extends older observations (8, 9) concerning the unexpected inability of protein conjugates, made *in vitro*, to induce contact skin sensitivity. The different conditions required for induction of antibody formation and for induction of contact skin sensitivity constitute an interesting distinction between these two inducible responses. But this difference introduces a problem in respect to induction of contact skin sensitivity: If the formation *in vivo* of protein conjugates is obligatory, why are "synthetic" conjugates, prepared in the test tube, ineffective?

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The present work, which is concerned primarily with a possible solution to this problem, is based on the assumption that an absolute requirement for induction of contact skin sensitivity is localization of protein conjugates in some unique intracellular sites. Simple chemical inducers and protein conjugates bearing the same simple chemical group have, obviously, different sizes, solubilities and charges, and may be expected, accordingly, to be distributed differently across semipermeable membranes and interfaces. Inasmuch as simple chemical inducers are all capable of forming protein derivatives, the injection of low molecular weight inducers may be visualized as leading to an ultimate localization of protein conjugates (formed *in vivo*) in sites that are inaccessible to the otherwise comparable conjugates which are prepared *in vitro* and then offered to the intact organism. In order to evaluate this concept, we have, in the present study, examined the distribution of some representative simple chemical inducers of contact skin sensitivity and their homologous protein conjugates between isolated lymph node cells and extracellular medium.

Methods and Materials

Animals.—Male, albino, Hartley-strain guinea pigs of 400 to 700 gm. body weight were used throughout. The animals were fed Purina guinea pig chow, supplemented three or four times a week with lettuce. Water was administered *ad libitum*.

Sensitization.—The procedures used for inducing sensitivity varied considerably and are described for individual experiments by appropriate footnotes to Tables II, III, and IV. When emulsions of the Freund type were used, they were prepared with 1 volume of arlacel A, 4 volumes of mineral oil (bayol F) containing 100 μg. air-dried *Mycobacterium butyricum* per ml., and 5 volumes of aqueous (0.15 M NaCl) solutions or suspensions of sensitizers (10).[1]

Assays for Hypersensitivity.—Contact skin sensitivity was elicited with approximately 50 μl. of 0.01 M DNCB[2] in 1:1 acetone–corn oil applied to skin on the dorsum after removing excess hair with a clipper. Test sites were depiliated 24 hours later. The responses summarized in Tables II, III, and IV are those recorded 48 hours after skin tests were applied. Responses were scored as follows:—

0, negative; no visible response.

±, ambiguous; exceedingly faint and irregular erythema, indistinguishable from the response seen in about 10 to 20 per cent of non-sensitized control animals.

1+, weakly positive; pale erythema.

2+, moderately to strongly positive; uniform and pronounced erythema, with hemorrhage and edema in the strongly positive reactions.

Intravenous injections of DNP-proteins were used to provoke anaphylaxis as a qualitative assay for the presence of anti-DNP antibody. When typical signs of anaphylaxis were observed, but animals failed to die, the results are scored as "non-fatal" in Tables II, III,

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[1] "Specially treated" arlacel A (mannide monooleate) and bayol F were generously supplied by the Atlas Powder Co., Wilmington, and the Penola Oil Co., New York.

[2] Abbreviations used are as follows: DNCB, 2,4-dinitrochlorobenzene; DNFB, 2,4-dinitrofluorobenzene; $DNBSO_3$, 2,4-dinitrobenzenesulfonic acid, Na or K salt; DNP, 2,4-dinitrophenyl group combined with proteins or amino acids; BSA, bovine serum albumin; BγG, bovine serum γ globulin; RSA, rabbit serum albumin; EA, chicken egg albumin.

and IV; all deaths from anaphylaxis recorded in these tables occurred within 2 to 10 minutes after injection.

Preparation of Lymph Node Cell Suspensions.—Animals were anesthetized by intraperitoneal injections of sodium pentobarbital (4.5 mg. per 100 gm. body weight), and exsanguinated by cardiac puncture. When non-sensitized animals were used, lymph nodes were taken from axillary, popliteal, inguinal, and cervical regions and from the mesentery. In some experiments cells from sensitized animals were used. In the latter cases, axillary, popliteal, and superficial inguinal lymph nodes were removed 10 to 42 days after footpad injections of sensitizers in emulsions of the Freund type. Cells from uninjected animals and from a variety of sensitized animals were the same as regards the distribution of dinitrobenzenes between cells and extracellular medium.[3]

Cells were combed with 50-mesh stainless steel wire screens (about 12 × 10 mm.) from lymph nodes which were supported on a disc of 50-mesh stainless steel wire screen, about 35 mm. diameter, mounted on a shallow stainless steel cylinder. The latter was immersed in sufficient medium to keep the tissue wet. The base of the cylinder was notched to allow free flow of medium, and the complete assembly was supported in a small dish surrounded by an ice-water mixture. Cells released from the nodes were collected by centrifugation at 140 *g* for 10 minutes at 4°C. The packed cells were then resuspended in fresh cold medium by gentle mixing with a glass rod, and filtered through a small Buchner funnel (1.5 cm. diameter) mounted in a test tube with side arm tubulature. The filtering surface was 325-mesh stainless steel wire cloth. Gentle suction was required at times to facilitate filtration. The concentration of cells in the final suspension was estimated by counting in a standard hemocytometer, or by determining the absorbance at 660 mμ in a Beckman spectrophotometer, model DU (11, 12).

The basic medium used was modified Krebs-Ringer solution. Its composition is given in Table I. Cells isolated by the foregoing procedures are capable of transferring contact skin sensitivity and have metabolic functions quantitatively like those of many normal tissues (11–13).

Differential counts of occasional cell suspensions revealed them to have approximately the following composition: 85 per cent lymphocytes, 5 to 10 per cent larger mononuclear cells, and 5 to 10 per cent erythrocytes.

Distribution of Penetrants between Extracellular Medium and Cells.—Cell suspensions were mixed with medium containing the substance whose distribution was to be studied (hereafter referred to as the penetrant), and the mixtures were incubated in air for varying periods at 37°C. or at room temperature. Tubes were shaken manually just often enough to prevent the cells from settling. Following incubation, suspensions were transferred to calibrated McNaught tubes,[4] centrifuged at 1300 *g* for 5 minutes, and packed cell volumes were determined (±5 μl). Supernatants were removed for analysis and the glass walls were rinsed

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[3] A possible exception was $DNBSO_3$. At an extracellular concentration of 3 to 3.5 × 10^{-3} M, the $DNBSO_3$ space, measured by elution, was significantly larger in lymph node cells of guinea pigs sensitized with DNP-BSA in Freund's adjuvant (51 ±2 per cent) than in lymph node cells of (*a*) guinea pigs sensitized with BSA in Freund's adjuvant (35 ±3 per cent), or (*b*) non-sensitized guinea pigs (32 ±3 per cent). In other experiments, however, cells from animals sensitized with DNP-BγG in Freund's adjuvant or with BγG in Freund's adjuvant were not different as regards the $DNBSO_3$ space. The $DNBSO_3$ space given in Table V was obtained with cells of non-sensitized guinea pigs over a wide range of extracellular concentrations of $DNBSO_3$.

[4] Obtained from the A. S. Aloe Co., St. Louis. The manufacturer's calibrations were found to be reliable.

twice by filling the tubes with ice-cold, penetrant-free medium. Penetrants in cell pellets were then analyzed by one of two general procedures:

(*a*) In some experiments the packed cells were resuspended in penetrant-free medium and incubated for 10 to 30 minutes at room temperature. After centrifugation at 1300 *g* for 5 minutes the supernates were analyzed. Penetrant recovered by this procedure is obviously only a fraction of the total content of the packed cells. Nevertheless, data obtained by this procedure were reproducible and were useful for the main purpose of the present study. Distribution values obtained by this procedure are referred to as "eluted spaces" (Table V).

TABLE I

*Medium for Isolation and Incubation of Lymph Node Cells**

Solute	Concentration
NaCl	120 mM/liter
KCl	6 mM/liter
$CaCl_2$	2 mM/liter
$MgSO_4$	1 mM/liter
Glutamine	2 mM/liter
Buffers:	
(*a*) Na_2HPO_4‡ or	10 mM/liter
(*b*) Na_2HPO_4‡	5 mM/liter
and 'tris'§	5 mM/liter
Streptomycin sulfate	50–100 μg./ml.

* Vitamins in amounts recommended by Eagle (39) were added. With phosphate as the only major buffer (0.01 M) the final pH is 7.3 and the solution becomes turbid after several hours at 4°C. and after 1 hour at 37°C. With phosphate + 'tris' (each at a final concentration of 0.005 M), the solution remains clear indefinitely at a final pH of 7.4. Water was twice distilled, the second time from glass. A dilute solution of $CaCl_2$ and $MgSO_4 \cdot 7H_2O$ (0.01 M and 0.005 M, respectively) was added after the other reagents had been mixed and diluted to near the final volume.

‡ Added as 0.1 M phosphate, adjusted to pH 7.4 with HCl.

§ Added as 1.0 M tris (hydroxymethyl) amino methane, adjusted to pH 7.4 with HCl.

(*b*) In other experiments, the entire cell pellet was dissolved by heating in 0.1 N NaOH for 30 to 60 minutes in a boiling water bath and analyzed for total penetrant content. Distribution values based on this procedure are referred to as "total spaces" (Table V).

In most experiments, penetrant concentrations permitted sample dilution to the point at which the "blank" contribution by the cells was negligible. Where sample dilution was not feasible, the "blank" contribution of the cells was determined and deducted. Packed cell pellet volumes were nearly all 100 to 200 μl. In this range, distribution values were independent of packed cell volume.

The distribution of a penetrant is expressed as a "space" which corresponds to that virtual volume of a packed cell pellet in which the penetrant is at the same concentration as in the extracellular medium. A space, expressed as per cent of the total packed cell pellet volume, is, therefore $a/cv \times 100$, in which a is the amount of penetrant recovered from a cell pellet of volume v, and c is the concentration of penetrant in the extracellular medium. In order to determine the amount of penetrant actually in the cells, it is necessary to cor-

rect for extracellular medium trapped in the cell pellet. Since the extracellular medium occupied about 45 per cent of the volume of cell pellets packed by centrifugation (1300 *g*, 5 minutes; see below), penetrant spaces within the cells actually correspond to

$$\frac{a - 0.45\,vc}{c(1 - 0.45)v} \times 100.$$

Distribution of Dinitrobenzenes between n-Amyl Alcohol and Water.—The solvents used for determining the distribution of dinitrobenzenes between water and a model immiscible organic solvent were: (*a*) 0.01 M phosphate in water, pH 7.4, saturated with *n*-amyl alcohol, and (*b*) *n*-amyl alcohol saturated with the aqueous phosphate solution. All measurements were carried out at room temperature (25°–28°C.). Five ml. of a solution of a dinitrobenzene were shaken intermittently for 15 minutes with an equal volume of the immiscible solvent. The alcohol phase was then analyzed for dinitrobenzenes by absorbance measurements, after dilution 10- to 100-fold with ethanol, and the aqueous phases were analyzed as described below. Distribution equilibrium was attained within 15 minutes, and recoveries for all substances were complete.

2,4-Dinitrobenzenes.—DNCB, DNFB, and $DNBSO_3$ were obtained from commercial sources. DNCB was recrystallized twice from ether, and $DNBSO_3$ was recrystallized four or five times from water as the potassium or sodium salt. The preparation and characterization of ε-DNP-lysine and of ε-DNP-aminocaproic acid were described previously (14, 15).

2,4-Dinitrophenyl-Proteins.—DNP-proteins were prepared so as to have DNP groups combined predominantly with ε-NH_2 groups of lysine residues (N-DNP-proteins) or combined predominantly with cysteine residues (S-DNP-proteins).

N-DNP-proteins were prepared by minor modification of a procedure described previously (16). In a typical preparation of a soluble conjugate, 25 mg. quantities of BSA, K_2CO_3, and $DNBSO_3$ were shaken gently in 2 ml. water for 1 to 24 hours at room temperature. Low molecular weight substances were subsequently removed with an amberlite ion exchange resin (IRA-400, 20 to 50 mesh, chloride form). A single passage of the reaction mixture described above through a resin column about 1 × 18 cm. gave almost complete recovery of the DNP-protein (>90 per cent), and removed virtually all unconjugated $DNBSO_3$. The number of DNP groups substituted per protein molecule was estimated from absorbance at 360 and 290 mμ (when samples were diluted in 0.1 N NaOH; see reference 17), or at 360 and 278 mμ (when samples were in neutral or acidic solutions). Typical calculations for DNP-proteins dissolved in 0.1 N NaOH are given in reference 17. In neutral or acidic solutions, similar calculations apply, but in such solutions ε-DNP-lysine has a ratio of absorbances at 278 mμ/360 mμ of 0.361. In some preparations, N was measured by micro-Kjeldahl analyses.

All preparations of DNP-hair and DNP-epidermis, but one, were prepared with DNP groups on lysine residues (6) by reacting unwashed finely minced hair and freshly obtained epidermis (18) with approximately equal weights of $DNBSO_3$ and K_2CO_3 (0.2 to 0.5 M). In most experiments, the intensely yellow products formed were washed thoroughly with water, air-dried, and implanted subcutaneously (groups 1 to 5, Table IV). One sample of DNP-epidermis was prepared in the same manner but with a mixture of 1-C^{14}-$DNBSO_3$ and C^{12}-$DNBSO_3$ (Table IV, group 6). This preparation was homogenized, dialyzed for 4 days against water, and then injected in Freund's adjuvant.

A soluble conjugate of BSA with DNP groups on cysteine residues (Table III, S-DNP-BSA) was made by treating BSA with mercaptoethylamine (19), and by shaking the reduced protein for 16 hours under N_2 at pH 7.0–7.5 with an excess of $DNBSO_3$ ($C^{12}DNBSO_3$ mixed with 1-C^{14}-$DNBSO_3$). Unreacted $DNBSO_3$ was removed with resin (IRA-400). From the absorption spectrum and C^{14} content of the conjugate, it was estimated that 90 per cent

of the DNP groups were combined with cysteine residues, and the remainder with lysine residues. This estimate was based on absorption spectra of S-DNP-glutathione (20) and of ε-DNP-lysine.

One preparation of DNP-epidermis was made under conditions which also led to DNP groups being combined with cysteine residues (Table IV, S-DNP-epidermis, group 7). This conjugate was obtained by painting a mixture of C^{12} and 1-C^{14}-$DNBSO_3$ on the skin of 2 guinea pigs after depilation (7). The next day epidermis was removed (18) washed with water, homogenized, and dialyzed for 4 days against running tap water; it was finally injected in Freund's adjuvant.

With a number of C^{14}-DNP-conjugates, the extent of contamination with unreacted dinitrobenzenes was measured after precipitation of protein with 0.5 M perchloric acid at 5°C. (see footnote, Table IV).

Crystallized bovine serum albumin, bovine γ-globulin (fraction II of bovine plasma) and crystallized chicken ovalbumin, were obtained from Armour and Company. Rabbit serum albumin was prepared by ammonium sulfate precipitation (21). The molecular weights of these proteins were assumed to be 40,000 for ovalbumin, 70,000 for the serum albumins, and 160,000 for γ-globulin.

C^{14}-Labelled 2,4-Dinitrobenzenes.—1-C^{14}-DNCB and 1-C^{14}-$DNBSO_3$ were prepared from the same lot of 1-C^{14}-chlorobenzene (Tracerlab, Boston; 0.39 mc./mM). Some characteristics of the 1-C^{14}-DNCB were described previously (22). An additional indication of its radiopurity was obtained by determination of its equilibrium distribution between *n*-amyl alcohol and water (see Table VI).

1-C^{14}-$DNBSO_3$, K salt, was prepared by sulfonation of 1-C^{14}-DNCB (Tracerlab, Boston). Radiochemical purity of 1-C^{14}-$DNBSO_3$ was established by its equilibrium distribution between *n*-amyl alcohol and dilute phosphate buffer (see Table VI). From the partition coefficients between these solvents of authentic DNCB and $DNBSO_3$ (K salt), and the respective 1-C^{14}-labelled substances, it was shown that the 1-C^{14}-$DNBSO_3$ (K salt) was free of 1-C^{14}-DNCB (less than 0.01 per cent).

C^{14}-DNP proteins were prepared with 1-C^{14}-$DNBSO_3$. The number of DNP groups in the labelled proteins determined by absorbance measurements and by C^{14} counting agreed to within 10 per cent.

Analytical Procedures.—ε-DNP-lysine, ε-DNP-aminocaproic acid and DNP-proteins were measured by determination of absorbance at 360 mμ (molecular extinction coefficient for the amino-substituted dinitrophenyl group is 17,400) or by C^{14} counting.

DNFB, DNCB, and $DNBSO_3$ were quantitatively converted to the sodium salt of 2,4-dinitrophenol by heating for 1 hour in 1 N NaOH at 80°C. 2,4-dinitrophenylate was measured spectrophotometrically at 360 mμ (molecular extinction coefficient, 14,600). When glucose was present, it interfered with the quantitative hydrolysis of the dinitrobenzenes and was, therefore, removed with glucose oxidase (23).

C^{14}-labelled substances were counted in a windowless gas flow counter, and the counting rates were corrected for self-absorption (22). 1-C^{14}-DNCB was counted after conversion to 1-C^{14}-2,4-dinitrophenol, sodium salt (22). For all the C^{14}-substances used, the specific activity was 235,000 C.P.M. per μmole DNP.

Inulin and raffinose were measured with resorcinol (24). In some experiments (Table V, "total" spaces), inulin was measured with skatol (25).

RESULTS

Induction of Sensitivity.—DNFB and DNCB induce contact skin sensitivity in virtually all guinea pigs when 0.1 to 0.2 μmoles are injected in Freund's

adjuvant. Typical results are given in Table II. $DNBSO_3$ reacts much more slowly with proteins in general and, as expected, larger quantities are required to induce contact skin sensitivity (2 to 3 μmoles, Table II).

TABLE II

Sensitivity Induced by Simple 2,4-Dinitrobenzenes

Group*	Sensitized with		Contact skin sensitivity					Anaphylaxis‖		
	Substance	Total quantity given	Time tested‡	Results§				None	Not fatal	Fatal
				0	±	1+	2+			
		μmoles	*days*							
1	DNFB	0.01	12	4	2	4	0			
2	DNFB	0.2	10	0	0	10	90			
3	DNCB	0.15	10	0	2	5	7			
4	$DNBSO_3$	0.2	7	2	1	2	0			
5	$DNBSO_3$	1.0	6	1	1	1	2	3	2	0
6	$DNBSO_3$	2.0	6–9	2	0	0	6			
7	$DNBSO_3$	0.003	14	4	0	1	0	5	0	0
8	$DNBSO_3$	0.03	14	3	1	1	0	5	0	0
9	$DNBSO_3$	0.3	14	4	0	1	0	5	0	0
10	$DNBSO_3$	3.0	14	0	1	3	1	3	1	1
11	$DNBSO_3$	500.0	6	0	1	0	4	3	2	0

* Groups 1, 2, 3, and 7 to 10 were injected once in their footpads with sensitizers incorporated in Freund's adjuvant, each animal receiving 0.4 ml. containing 16 μg. *M. butyricum*.

Groups 4, 5, and 6 were injected with $DNBSO_3$ in 0.15 M NaCl. Groups 4 and 5 were given 10 intradermal injections over a 14 day period. Group 6 was injected once in the footpads.

Group 11 was given 10 percutaneous applications over a 14 day period of 0.5 M $DNBSO_3$ in a mixed solvent (ethylene glycol monomethyl ether–water–tween 80 in a volume ratio of 5:5:1).

‡ Time between final sensitizing injection and skin testing with 0.01 M DNCB.

§ Number of animals giving responses of the designated intensity. See Methods for scoring of responses. The results given for group 2 are estimates based on experience with several hundred animals. For earlier results obtained with DNFB and DNCB see references 5, 40.

‖ Evoked by an intravenous injection 25 to 30 days after the final sensitizing injection. Group 5 was given DNP-sheep serum (about 14 mg. protein), and groups 7 to 11 were given DNP-guinea pig serum (about 30 mg. protein, 2.5 μmoles DNP).

Under appropriate conditions $DNBSO_3$, DNCB, and DNFB all react with proteins *in vitro* to give essentially the same conjugates (6, 16, 26). $DNBSO_3$ was chosen for the preparation of conjugates in the present work for two reasons: First, it is very soluble in water and removal of unreacted reagent can be more nearly complete than in the case of the halogenated dinitrobenzenes. Second, and more important, unreacted $DNBSO_3$ as a trace contaminant is

TABLE III

Sensitivity Induced by DNP-Conjugates Prepared with Serum Proteins and Ovalbumin

Group*	Sensitized with			Contact skin sensitivity					Anaphylaxis¶		
	Substance‡	Total quantity given		Time§	Results‖						
		as DNP	as protein		0	±	1+	2+	None	Not fatal	Fatal
		μmoles	*mg.*	*days*							
1	DNP-BγG	0.0004–0.01	0.001–0.03	11	13	2	0	0			
2	DNP-BγG	0.5	1.2–3.0	21	12	1	0	0			
3	DNP-BγG	4.0	9.6	13	5	0	0	0	0	1	4
4	DNP-BγG	20.0	48.0	13	3	1	0	0	0	0	4
5	DNP-BSA	0.3	1.6	14	18	5	0	0			
6	DNP-RSA	0.2	1.2	13	8	1	0	0			
7	S-DNP-BSA	0.9	2.0	11	12	3	0	0	0	0	15
8	DNP-guinea pig serum	0.8	2.4	10	2	2	1	0	0	0	5
9	DNP-EA	0.5	1.5	7	4	1	0	0	0	1	4
10	DNP-EA	0.5	1.5	7	5	0	0	0	0	0	5
11	DNP-EA	0.5	1.5	7	4	0	1	0	0	2	3
12	DNP-EA	0.6	2.4	20	3	1	0	0	0	0	4

* Groups 1, 2, 3, 5, and 6 were injected once in footpads with DNP-proteins in Freund's adjuvant, each animal receiving 0.4 ml. containing 16 μg. *M. butyricum*.

Groups 4 and 7 were injected once with DNP-proteins in Freund's adjuvant, each animal receiving 2.0 ml., containing 80 μg. *M. butyricum*, distributed among footpads, muscle, and subcutaneous and intradermal sites.

Group 8 received DNP-proteins in 0.15 M NaCl, 10 intradermal injections being given over a 12 day period.

Groups 9 to 11 received a single preparation of DNP-EA in 10 intradermal injections over a 14 day period: group 9 was given a solution of this protein in 0.15 M NaCl, group 10 was given the protein in alum-precipitated form, and group 11 was given the protein after it had been made insoluble by treatment with sodium dodecyl benzenesulfonate and acidified ammonium sulfate (28). The results obtained with DNP-EA were given elsewhere in preliminary form (41).

Group 12 was injected intraperitoneally once with the protein in 1.0 ml. of Freund's adjuvant (40 μg. *M. butyricum*).

‡ All preparations had DNP on lysine residues, except for S-DNP-BSA which had DNP combined with cysteine residues (see Methods).

§ Time between final sensitizing injection and skin testing with 0.01 M DNCB.

‖ Number of animals giving responses of the designated intensity.

¶ Evoked by an intravenous injection 25 to 42 days after the final sensitizing injection. Groups 3, 4, and 7 were given DNP-guinea pig serum (about 30 mg. protein, 2.5 μmoles DNP), group 8 was given DNP-EA (18 mg. protein, 5.7 μmoles DNP), and groups 9 to 12 were given DNP-sheep serum (about 10 mg. protein, 1 μmole DNP).

less likely to introduce ambiguities than DNFB or DNCB since it is a much less potent inducer of contact skin sensitivity (Table II).

In contrast to the effectiveness of low molecular weight dinitrobenzenes,

DNP-proteins were remarkably ineffectual in inducing contact sensitivity. Thus, out of 155 guinea pigs given a variety of conjugates, 148 animals were not sensitized and 7 gave only weakly positive responses (Tables III and IV). DNP-proteins can, however, induce the formation of DNP-specific antibodies (17), and, as expected, they induce DNP-specific anaphylactic sensitivity (Tables III and IV).

TABLE IV

Sensitivity Induced by DNP-Conjugates Prepared with Epidermis and Hair

Group	Sensitized with			Contact skin sensitivity‡				Anaphylaxis‖		
	Substance*	Total quantity given		Results§				None	Not fatal	Fatal
		As DNP	As dry weight	0	±	1+	2+			
		μmoles	*mg.*							
1	DNP-hair	(3)	25	6	0	0	0			
2	DNP-hair	(4)	35	4	1	0	0	4	1	0
3	DNP-hair	(2)	20	4	0	0	0			
4	DNP-epidermis	(2)	20	5	3	1	0	1	6	1
5	DNP-epidermis	(2)	20	3	1	1	0	0	0	5
6	DNP-epidermis	3.2	10	7	1	1	0	0	3	6
7	S-DNP-epidermis	0.23	13	5	2	2	0	9	0	0

* Rabbit hair and epidermis were used to make the conjugates given to groups 3 and 5. For all other groups the hair and epidermal samples used were from guinea pigs.

Groups 1 to 5 had conjugates implanted subcutaneously. Groups 6 and 7 received homogenized material in Freund's adjuvant, each animal being injected once with 2.0 ml., containing 80 μg. *M. butyricum*, distributed among muscle, intradermal and subcutaneous sites.

In group 7, DNP-epidermis was made *in vivo* by applying $DNBSO_3$ on living guinea pigs' skin (see Methods); hence DNP was combined with cysteine residues (7). In groups 1 to 6, conjugates had DNP combined with lysine residues (see Methods and Materials).

For groups 6 and 7, conjugates were made with mixtures of C^{12} and 1-C^{14}-$DNBSO_3$; the amount of C^{14} not precipitated by 0.5 M perchloric acid was 1 per cent in the case of group 6 and 3 per cent in the case of group 7.

For groups 6 and 7, the amount of DNP given was determined by C^{14} counting (235,000 C.P.M. per μmole DNP). For groups 1 to 5 the amounts of DNP given were rough estimates (given in parentheses) based on the lysine content of hair and epidermis (42), and on the assumption that 50 per cent of the lysine residues were substituted.

‡ Elicited with 0.01 M DNCB 9 to 21 days after final sensitizing injection.

§ Number of animals giving responses of the designated intensity.

‖ Evoked by an intravenous injection 25 to 30 days after the final sensitizing injection. Groups 2 and 4 received DNP-EA (18 mg. protein, 5 μmoles DNP), group 5 received DNP-sheep serum (about 10 mg. protein), and groups 6 and 7 were given DNP-guinea pig serum (about 30 mg. protein, 2.5 μmoles DNP).

DNP-hair was virtually inert, but of 32 animals given DNP-epidermis, 5 gave weakly positive responses (Table IV). The latter results cannot be clearly interpreted because of the presence of trace amounts of unconjugated dinitrobenzenes.[5] It is possible that with some of the DNP-epidermal conjugates, induction by unconjugated DNP contaminants was potentiated by (*a*) the unusually large amount of *M. butyricum* used (80 μg. as compared with 16 μg. used otherwise; *cf.* footnotes of Tables II and IV) or (*b*) by an adjuvant-like effect of the epidermal homogenates. It is clear, in any event, that the most suggestive results obtained with DNP-epidermis conjugates were far inferior to the results obtained with comparable quantities of the more potent simple dinitrobenzene sensitizers (*cf.* Tables II and IV).

Some years ago Gell suggested that while soluble conjugates are unable to induce contact skin sensitivity (9), particulate conjugates might be competent in this respect (8). Similarly, Mayer has recently proposed that conjugates made *in vitro* from fibrous proteins may have a unique capability for inducing contact skin sensitivity (27). The results given in Tables III and IV do not support these suggestions. Thus, a number of DNP conjugates made from hair and epidermis did not induce contact skin sensitivity (Table IV, groups 1 to 5). Moreover when soluble DNP-EA was converted into an extremely insoluble form by treatment with a procedure which converts soluble EA into fibers (dodecyl benzenesulfonate and acidified ammonium sulfate, see reference 28), this material was likewise incapable of inducing contact skin sensitivity (Table III, group 11). Finally, the DNP–BγG preparations listed in Table III had about 60 DNP groups per BγG molecule and were moderately insoluble in 0.15 M NaCl at neutral pH; these preparations also did not induce contact skin sensitivity.

Extracellular Volume in Packed Lymph Node Cell Pellets.—Measurements of the distribution of penetrants between cells and extracellular medium require careful evaluation of the extracellular space in cell pellets. Inulin, which has been widely used to determine extracellular space, occupied 44 per cent of the volume of packed lymph node cell pellets (see Total Space, Table V). Because this value seemed extraordinarily large, additional procedures were used to evaluate the extracellular space: (*a*) raffinose, which has also been used for this purpose (29), occupied a space which amounted to 50 per cent of pellet volume (Table V). (*b*) The extracellular space was also estimated from the mean diameter of the cells and from the number of cells in pellets of known volume. In nine experiments, counting 250 cells per experiment, the mean diameter of

[5] The most suggestive evidence that protein conjugates induce contact skin sensitivity has been obtained with conjugates prepared from picryl chloride or DNFB and guinea pig erythrocyte stromata (8). The stromal conjugates, however, lost the capacity to induce contact skin sensitivity after refluxing in hot ethanol (31), suggesting that they might, likewise, have been contaminated by traces of free picryl chloride or DNFB.

lymph node cells isolated from non-sensitized guinea pigs was determined in the phase contrast microscope to be 7.2 ± 0.1 μ. Assuming the cells to be spherical and not to be deformed by centrifugation and packing, this value corresponds to a mean cell volume of 196 μ^3. Since there are, on the average,

TABLE V

*Distribution of 2,4-Dinitrobenzenes between Isolated Lymph Node Cells and Extracellular Medium**

Substance‡	Eluted space			Total space			Ratio concentration in cells: concentration in medium**
	n§	Uncorrected‖	Corrected¶	n§	Uncorrected‖	Corrected¶	
		per cent	*per cent*		*per cent*	*per cent*	
DNFB	4	520 ± 50	870 ± 90	4	13,900 ± 1600	25,400 ± 2900	330 ± 40
DNCB	5	260 ± 15	390 ± 30	11‡‡	1,240 ± 120	2,180 ± 170	29 ± 2
ϵ-DNP-aminocaproate	8	140 ± 3	173 ± 6	—	—	—	—
ϵ-DNP-lysine	13	123 ± 3	142 ± 5	—	—	—	—
$DNBSO_3$	21	64 ± 1	34 ± 2§§	6‡‡	102 ± 4	104 ± 7	1.4 ± 0.1
DNP-BSA	5	29 ± 1	0	14‡‡	39 ± 1	0	0
Inulin	13	41 ± 1	0	10	44 ± 1‖‖	0	0
Raffinose				13	50 ± 2‖‖		

* Results are mean values ± standard error of the mean.

‡ Final extracellular concentrations were in the following ranges (moles/liter × 10^{-5}): DNFB, 0.5 to 0.6; DNCB, 1.5 to 60; ϵ-DNP-aminocaproic acid, 75 to 120; ϵ-DNP-lysine, 2 to 176; $DNBSO_3$, 1.2 to 350; DNP-BSA, 1 to 120 (for the DNP group, and 0.17 to 6.5 mg./ml. for BSA). Other extracellular concentrations were (mg./ml.): inulin, 1.3 to 4.0; raffinose, 3.8 to 4.4.

§ Number of individual observations.

‖ Calculated as $a/cv \times 100$ (see Methods and Materials).

¶ Calculated as $a - (0.45)vc/c(1 - 0.45)v \times 100$ (see Methods and Materials).

** Calculations based on "total space" values, corrected for extracellular volume, and on the assumption that intracellular penetrant was uniformly distributed in total cell water (78 per cent of cell volume).

‡‡ Observations obtained with 1-C^{14}-labelled 2,4-dinitrobenzenes. 235,000 C.P.M./μmole DNP.

§§ See text—footnote 3.

‖‖ Cell pellet analyses performed with protein-free filtrates which were prepared with $Ba(OH)_2$-$ZnSO_4$ (for raffinose) or with tungstate or NaOH-$ZnSO_4$ (for inulin); see reference 13.

about 3 × 10^8 cells in a 100 μl. packed cell pellet, the extracellular volume in pellets formed under standardized conditions may be estimated to be about 41 per cent of the pellet volume.[6]

[6] In the case of cells isolated from regional lymph nodes 2 to 6 weeks after footpad injections of Freund's adjuvant (with or without the addition of other antigens), there are about 2.6 × 10^8 cells per 100 μl. packed cells, and the inulin space in cell pellets of this type was

The extracellular space, is therefore, between 40 and 50 per cent of packed cell pellet volume. A value of 45 per cent, essentially that given by inulin, was used to calculate penetrant spaces.[6] The validity of this estimate is supported by the finding that in lymph node cells the volume occupied by several non-utilized hexoses and pentoses, estimated on the basis of the inulin space as a measure of extracellular volume, never exceeded the cells' water content (78 per cent; see reference 13).

Dinitrobenzene Spaces in Isolated Lymph Node Cells.—Distribution values obtained by eluting penetrants from cells and by total pellet analyses are given in Table V. Within experimental error (± 10 per cent), the spaces measured were constant over the range of penetrant concentrations used.

For inulin, DNP-BSA, ε-DNP-lysine, and ε-DNP-aminocaproic acid the spaces shown represent steady-state values which were attained virtually instantaneously and did not change with prolonged incubation (up to 90 minutes).

For DNFB, DNCB, and $DNBSO_3$, which form protein conjugates and hydrolyze to 2,4-dinitrophenol at alkaline pH values, two issues must be considered: (*a*) the side reactions are expected to increase in extent with time; (*b*) analytical methods developed for the original penetrants may not distinguish between these and probable derivatives. For DNCB and $DNBSO_3$, which are only very slowly reactive at pH 7.4, these complications appear not to have influenced their distribution values since there was no appreciable change in space with incubation over a 90 minute period. For DNFB, which is much more reactive at pH 7.4, only 10 minutes was allowed for incubation with cells, and an equally short time for subsequent elution, in order to minimize complicating side reactions.

All of the low molecular weight dinitrobenzenes, including $DNBSO_3$, were concentrated by the cells. DNP-BSA, however, was confined to a volume which was slightly less than the extracellular volume as measured by inulin. The data given for C^{14}-DNP-BSA (Table V) were obtained with 4 different conjugates having 2 to 8 DNP groups per BSA molecule.

It is likely that extensively denatured proteins which adhere to glass surfaces may also be bound to cell surfaces. Such proteins would be expected to have larger distribution values than were obtained with DNP-BSA. One preparation of C^{14}-DNP-BγG which was visibly aggregated had a total space of 130 to 155 per cent (not corrected for extracellular volume). Another preparation of C^{14}-DNP-BγG had a total space of 55 per cent (not corrected for extracellular volume). The latter protein was detectably bound to glass, and when its apparent space was corrected for glass binding (2 per cent of the C^{14} concentration in extracellular medium), the space obtained was 46 per cent. In contrast to DNP-BγG preparations, DNP-serum albumin conjugates, of the kind shown in Table V (up to 10 groups per

found to be 49 per cent (12, 13). Since virtually all the distribution data of Table V were obtained with cells from normal guinea pigs, the extracellular volume used for calculation in the present study was 45 per cent.

protein molecule), have optical rotation values that are not significantly different from that of unreacted native serum albumin.[7] It may be assumed, therefore, that such DNP-BSA preparations have hardly any alteration in structure as compared with native BSA.

Distribution of Dinitrobenzenes between n-Amyl Alcohol and Water.—Partition values are shown in Table VI. DNFB undergoes slight hydrolysis to 2,4-dinitrophenol under the conditions in which these experiments were performed. Since the latter has a partition coefficient between the solvents used of 1.23 the value given in Table VI for DNFB is a minimum. Except for DNFB, there is an orderly agreement among the dinitrobenzenes as regards

TABLE VI

*Partition of Dinitrobenzenes between n-Amyl Alcohol and Water (0.01 M Phosphate, pH 7.4)**

Substance	Equilibrium Ratio: Concentration in alcohol phase / Concentration in aqueous phase
DNFB‡	>25
DNCB	84§
ϵ-DNP-aminocaproate	1.56
ϵ-DNP-lysine	1.10
$DNBSO_3$, K salt	0.066‖
C^{14}-DNP-BSA	<0.001¶

* At room temperature (25°–28°C.). Distribution equilibrium was achieved within 15 minutes.

‡ DNFB undergoes slight hydrolysis to 2,4-dinitrophenol under the conditions used. The latter has a partition ratio with the solvent pair used of 1.23 (concentration in alcohol phase/concentration in aqueous phase). Hence the value given for DNFB is minimal.

§ 1-C^{14}-DNCB had a partition ratio of 81.

‖ 1-C^{14}-$DNBSO_3$, K salt, had a partition ratio of 0.064.

¶ No C^{14} was detectable in the alcohol phase, even after standing 20 hours at room temperature, while the aqueous phase had 4700 C.P.M./ml. (2×10^{-5} M in respect to DNP; 0.18 mg. protein/ml.).

their partition between water and an immiscible organic solvent and their spaces in lymph node cells (DNCB > ϵ-DNP-aminocaproic acid > ϵ-DNP-lysine > $DNBSO_3 \gg$ DNP-BSA).

DISCUSSION

The experiments summarized in Tables III and IV demonstrate that a number of different DNP-protein conjugates, given in a variety of media and

[7] Unconjugated purified rabbit serum albumin has an optical rotation value, $(\alpha)_D$, of −62.5° (25°–26°C., pH 7.6). DNP-RSA with 2, 5 and 10 DNP groups per protein molecule gave, under these conditions, $(\alpha)_D$ values of −62.7°, −63.6°, and −60° respectively (Eisen, H. N., and Penefsky, H. S., unpublished observations).

over a 50,000-fold range as regards the quantity of protein and of conjugated DNP groups, are incapable of inducing DNP-specific contact skin sensitivity. These results confirm and extend previous observations by Gell (9) and by Landsteiner and Chase (8). Some evidence to the contrary has, however, been described by Landsteiner and Chase who used conjugates prepared from guinea pig red blood cell stromata. In animals sensitized with DNP-stromata, delayed allergic responses were elicited by DNFB applied on the skin and by intradermal injections of DNP-serum conjugates, but DNCB applied on the skin evoked little or no response (8, 31). DNFB reacts very much more rapidly than DNCB with proteins, and an application of DNFB could simulate an intradermal injection of a DNP-protein conjugate. The question arises, therefore, whether the animals sensitized with DNP-stromata (8) did not give "Arthus" reactions to percutaneous applications of DNFB; *i.e.*, that the exceptional responses in question were dependent on serum antibodies. This suggestion seems quite plausible because guinea pigs with contact skin sensitivity induced by DNFB respond with *equal* intensity to percutaneous applications of DNFB and DNCB, and react little, if at all, to intracutaneous injections of DNP-protein conjugates.[8] Although DNFB reacts much more rapidly with proteins than does DNCB, contact skin responses to both of these simple sensitizers are actually expected to be the same since the formation of protein conjugates in skin is not rate-limiting for the evolution of the allergic contact skin response (22).

The inability of DNP-protein conjugates to induce contact skin sensitivity might be interpreted to mean that the antigenic groupings of the conjugates that form *in vivo*, when DNCB is applied percutaneously to elicit the allergic skin response, are different from those present in the inducing conjugates which were prepared *in vitro*. When DNCB is applied to skin *in vivo* it combines with lysine ϵ-NH_2 groups and probably to a slight extent with cysteine residues (6, 7, 20, 22). Conjugates in which DNP groups were combined *in vitro* with lysine ϵ-NH_2, and those in which cysteine residues were substituted, were, however, equally incompetent as regards induction of contact sensitivity (Table III). Hence, if significant differences exist between the antigenic determinants of conjugates made *in vitro* and those that form *in vivo*, these differences must reside in amino acid residues in the immediate proximity of ϵ-DNP-lysine residues or S-DNP-cysteine residues. This possibility seems improbable since

[8] Guinea pigs sensitized by footpad injections of 0.21 μmoles DNFB in Freund's adjuvant were skin tested at the same time in 2 sites with corn oil solutions of DNCB and DNFB which varied in concentration from 1.0 to 0.01 per cent (*W/V*). At equivalent concentrations, DNCB and DNFB elicited indistinguishable reactions. In guinea pigs sensitized in the same manner, intradermal injections of DNP-BγG (3 μg. protein, 0.3 mμmoles DNP) produce small red papules (up to 4 to 5 mm. diameter at 24 hours) which are not larger than the responses to the same material in non-sensitized guinea pigs.

conjugates made from epidermis under "physiological" conditions failed to induce anything more than low grade contact sensitivity in 2 out of 9 animals (group 7, Table IV); as pointed out above, it is questionable whether even this small degree of induction is ascribable to conjugates in the complete mixture used. A further argument against the possibility under discussion is based on the following considerations concerning the probable size of antigenic determinants.

The dimensions of antigenic determinants can be discussed at present only in reference to serum antibodies. At a concentration of about 5×10^{-6}M, ε-DNP-lysine inhibits by 50 per cent the precipitation of anti-DNP antibodies by the antigen used for immunization (32), and the association constant for the reversible binding of this hapten by anti-DNP antibodies is about 2×10^5 (33). These data demonstrate the high affinity of ε-DNP-lysine for anti-DNP serum antibodies, and are consistent with the studies of Karush (34) who concluded that the combining region of antibodies is "probably not larger than that necessary to make contact with" the phenyl(*p*-azobenzoylamino) acetate group. This dye and ε-DNP-lysine have about the same molecular weight. Although Kabat (35) has inferred that the combining site of anti-dextran antibodies can be so large as to be complementary for a group with dimensions at least as great as that of isomaltohexaose, his data show that isomaltotriose has 75 to 90 per cent the affinity of the hexasaccharide for the antibody. Extrapolation from serum antibody studies to contact skin sensitivity is obviously of uncertain validity. Nevertheless, the only information relevant to the question under discussion has been obtained with serum antibodies. This information suggests that the antigenic determinants involved in DNP-specific contact skin sensitivity are probably not significantly larger than ε-DNP-lysine or S-DNP-cysteine. It does not seem likely, therefore, that the failure of DNP-proteins (prepared *in vitro*) to induce contact skin sensitivity for DNCB can be ascribed to differences in antigenic determinants between inducing and eliciting substances.

A more probable explanation for the failure of well defined DNP-protein conjugates to induce contact skin sensitivity may be found in the distribution data given in Table V. The inducers DNFB, DNCB, and $DNBSO_3$, were concentrated by lymph node cells, but DNP-BSA was not found in these cells to a detectable extent. ε-DNP-lysine and ε-DNP-aminocaproic acid were also concentrated by lymph node cells, but neither forms protein conjugates and neither is an inducer (30). These observations are consistent with the proposition that induction of contact skin sensitivity requires the concentration within cells of antigenic determinants conjugated to protein. This requirement seems to be met only by simple substances, such as DNFB, etc., which are (*a*) concentrated by lymphoid cells, and (*b*) have the capacity to form protein conjugates. According to this interpretation, DNP-proteins,

made *in vitro,* are incompetent inducers of contact skin sensitivity because of insufficient access to intracellular sites of lymph node cells.

Since the foregoing viewpoint relies on the fact that DNP-BSA was not found in lymph node cells, it is necessary to consider the amount of protein which could have entered the cells and escaped detection. From the sensitivity of the methods used, and from the extracellular concentrations of C^{14}-DNP-BSA employed, it may be calculated that possibly 30,000 protein molecules (4×10^{-9} μg.) could have been present, per cell, and not detected.[9] Even if some protein did enter some of the cells it is obvious, however, that at all reasonable extracellular concentrations of penetrants the simple sensitizers gain access to intracellular sites far more readily than do protein conjugates. The demonstration by Coons' fluorescent antibody technique (37) of antigenically active materials within plasma cells of animals injected with protein antigens is not necessarily in contradiction to the main argument of the present discussion since the fluorescent-antibody method may not discriminate between an intact protein and antigenically active polypeptides derived from it; moreover, the amounts of antigenically active material (per cell) detected by fluorescence methods have not yet been defined.

Reconciliation of the foregoing views with the well known effectiveness of proteins in the induction of antibody formation and in the induction of certain kinds of delayed allergic skin responses (36) is certainly desirable. In particular, it will eventually be necessary to reconcile the inability of DNP-protein conjugates (prepared *in vitro*) to induce contact skin sensitivity with the well established capacity of the *same* conjugates to induce the formation of DNP-specific serum antibodies (17, 32) (Table III). Possibly, induction of serum antibody formation is much more efficient than induction of contact skin sensitivity, requiring perhaps only a few protein molecules, or even only a few polypeptide fragments, per cell. Further speculation along these and other lines is, however, not likely to be profitable now since virtually nothing is known at present of the molecular transformations involved in the induction of either of these adaptive responses.

The differences between simple 2,4-dinitrobenzenes and DNP-proteins have been discussed in the preceding sections only from the viewpoint of induction of contact skin sensitivity. Similar differences probably apply also to elicitation of responses in previously sensitized individuals. Thus, guinea

[9] Given a packed cell volume of 150 μl. (4.5×10^8 cells), and an extracellular concentration of C^{14}-DNP-BSA (with 10 DNP groups per protein molecule) of 0.10 mg. protein/ml. (3500 c.p.m./ml.), it may be calculated that if the cell pellet contained, in excess of trapped extracellular medium, 10^3 or 10^4 or 10^5 or 10^6 molecules of C^{14}-DNP-BSA, per cell, the total space observed would amount, respectively, to 40, 43, 74, and 377 per cent of the pellet volume. The total space actually occupied by C^{14}-DNP-BSA was certainly less than 50 per cent (*i.e.*, less than 30,000 molecules DNP-BSA/cell; *cf.* the observed total space of 39 $\pm$1 per cent in Table V).

pigs and human subjects with DNP-specific contact skin sensitivity (elicited by DNCB, etc.), do not ordinarily respond to DNP-proteins injected intradermally.[8, 10]

In view of the importance ascribed to permeation of inducers into cells, the mechanisms by which low molecular weight dinitrobenzenes are concentrated by lymph node cells are of some interest. Since the spaces occupied by these substances were constant within experimental error (±10 per cent) over the range of concentrations studied, their concentration by lymph node cells seems not to be due to binding by cell constituents. Concentration appears, instead, to be primarily a consequence of unequal partition between two separate phases, one cellular, the other extracellular.[11] This inference is in accord with the fact that, among the dinitrobenzenes examined, the order of distribution between cells and extracellular medium (Table V) followed fairly well the order of the partition coefficients (Table VI) between water and an immiscible organic solvent (DNCB > ε-DNP-aminocaproic acid > ε-DNP-lysine > $DNBSO_3$ ≫ DNP-BSA; DNFB data are only approximations). Similar parallelisms between permeation into cells, on the one hand, and distribution between water and immiscible solvents, on the other hand, have, of course, long been known to apply to many low molecular weight substances (38).

Although a rather broad argument has been constructed from the observation that DNP-BSA does not enter isolated lymph node cells to a detectable extent, it is obvious that such limited data may not apply to all proteins and all cells. Accordingly, the data presented cannot exclude the possibility that by proper selection of some rather unique protein a chemically defined conjugate, made *in vitro*, will induce contact skin sensitivity. For such a hypothetical result to be meaningful and useful, the conjugate would have to be not only an inducer, but effective in smaller quantities than conventional low molecular weight inducers of contact skin sensitivity.

SUMMARY

Although induction of contact skin sensitivity by low molecular weight 2,4-dinitrobenzenes requires the formation *in vivo* of 2,4-dinitrophenyl-proteins,

[10] For example, 10 human subjects with contact skin sensitivity to DNCB (induced with DNFB) were injected with DNP-human serum albumin (2.2 μg. protein, 1 mμmole DNP). One individual gave a prompt wheal-and-erythema response, but none gave delayed inflammatory responses. (Unpublished observations with Dr. Fuad S. Farah whom we wish to thank for permission to cite these results.)

[11] If reversible binding made a significant contribution, penetrant spaces would be dependent on the concentrations of penetrants in extracellular medium. The distinction between (*a*) binding and (*b*) a partition process (analogous to that between a pair of immiscible solvents) is, however, not feasible when distribution is determined under conditions when the maximum binding capacity is far from being saturated.

analogous protein conjugates prepared *in vitro* are unable to induce this hypersensitive state. Low molecular weight 2,4-dinitrobenzenes are concentrated by isolated lymph node cells, but a representative 2,4-dinitrophenyl-protein conjugate (2,4-dinitrophenyl–bovine serum albumin) was not taken up to a detectable extent by these cells. It is inferred that there exist large quantitative differences in the extent to which dinitrophenyl-proteins are localized within cells following the administration to an intact animal of (*a*) those simple dinitrobenzenes which are both concentrated by lymph node cells and have the capacity to form protein conjugates *in vivo*, and (*b*) 2,4-dinitrophenyl-protein conjugates prepared *in vitro*. It is suggested that this difference could account for the fact that a variety of 2,4-dinitrophenyl-proteins prepared *in vitro* are unable to induce contact skin sensitivity.

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