STUDIES ON THE SENSITIZATION OF ANIMALS WITH SIMPLE CHEMICAL COMPOUNDS

XI. The Fate of Labeled Picryl Chloride and Dinitrochlorobenzene after Sensitizing Injections*,‡

By EGON MACHER,§ M.D., and MERRILL W. CHASE, Ph.D.

(From The Rockefeller University, New York 10021)

Delayed-type hypersensitivity to simple chemical compounds is still but little understood. Deposited onto or into the skin, the allergenic chemical results in the appearance of immunologically committed cells capable of recognizing a hitherto unknown chemical structure. It is not known how the cells acquire the information.

Apart from the use of artificial adjuvant systems, skin deposition offers the greatest chance for the simple chemical to be recognized as representing or forming a foreign substance. When administered via different routes, e.g. intraperitoneally, intravenously, or even subcutaneously, the simple chemical essentially does not sensitize. This suggests a local interaction between chemical and host tissue at the site of deposition as being essential for sensitization.

The role of the site of deposition was examined; the rate of dispersal of ^{14}C-picryl chloride and ^{14}C-dinitrochlorobenzene from the injection site and the mode of distribution are reported here. The governing idea was to secure sensitization with amounts approaching the minimal dose, applied by a single intradermal injection, to avoid excess determinant groups. The ear was chosen since injection can be made without physical losses due to back pressure, and the direction of venous flow and lymphatic drainage of the ear site are clearly defined. The following paper (1) analyzes the consequences of excision of the ear, made at varying times, on the immunological responses of the animals.

Several previous studies have been directed to tracing the fate of radiolabeled chemical allergens (2–6). These studies have utilized, usually, liberal contact applications of ^{14}C-dinitrochlorobenzene to the skin. In three of these studies (3–5), emphasis was placed on lymphatic drainage and alterations detected in

the regional lymph node. In another (6), ^{14}C-dinitrochlorobenzene was applied topically to sensitized gunea pigs, and various tissues were examined for radio-activity up to 12 hr by means of tissue combustion; the data do not bear directly on the question raised here.

Radioactivity present in the tissues was determined by liquid scintillation counting after combustion of tissue to CO_2 and H_2O. Both chemicals (perhaps also as hydrolysis products or soluble hapten-protein conjugates) commenced to leave the site quickly within minutes and hours. The pathway of escape turned out to be not the regional lymphatics but the local blood vessels. The chemical allergens at the local site could be traced in continually decreasing amounts through several weeks.

Materials and Methods

Animals.—Rockefeller University albino guinea pigs of both sexes, weighing 450–600 g, were used in all experiments.

Chemicals.—1-^{14}C picryl chloride (PCl*) and 1-^{14}C picric acid were synthesized by Volk Radiochemical Co. (Burbank, Calif.). Specific activity of both was 2 mc/mM; mp of PCl* 82.1–82.8.° 1-Chloro-2,4 dinitrobenzene-^{14}C (DNCB*), uniformly labeled, was purchased from Nuclear Chicago (Des Plaines, Ill.). Specific activity was 4.86 mc/mM. "Cold" picryl chloride and dinitrochlorobenzene were recrystallized (1).

Chemicals used for scintillation counting were almost uniformly of spectroquality (Matheson, Coleman, and Bell, Cincinnati, Ohio); some samples were ACS reagent grade. Phenethylamine was freshly distilled in high vacuum at 70°C and stored free from CO_2. Liquifluor (Pilot Chemicals, Inc., Watertown, Mass.), a premixed solution of PPO and POPOP, was used for preparation of liquid scintillator. The liquid scintillator was composed of 850 parts by volume of toluene, 50 parts of absolute ethyl alcohol, 50 parts of p-dioxane, and 50 parts of Liquifluor.

Solutions of PCl and DNCB* for Sensitizing.*—For injection of 0.25 μg/0.01 ml, a stock solution of 0.3% in absolute alcohol was prepared and kept at 4°C. By means of a 0.2 ml pipet clamped upright and connected to an Adams suction apparatus No. A-2473 (Clay-Adams Inc. New York), 0.02 ml of the stock was added slowly to 2.4 ml of physiological saline, with mixing. For injection of 5 μg/0.01 ml, a stock of 1.25% of DNCB* in absolute alcohol was made up and 0.063 ml thereof was added to 1.5 ml of saline.

Sensitization.—For the sensitizing injection, the animals were firmly fixed on a board (7), the ear having been freed of hair by means of an electric clipper. A 30 gauge hypodermic needle (regular point) attached to a Hamilton microliter syringe (No. 705, 0.05 ml) was introduced as superficially as possible and carefully advanced for not less than 5 mm. When in proper position, the tip of the needle can clearly be seen through the thin epithelium. Then a volume of 0.01 ml was slowly discharged forming a bleb of about 3 mm in diameter and 1 to 1½ mm in height. After a few seconds, the needle was withdrawn with a quick twist of about 180°, thus blocking the needle track; in this way, no fluid was ever seen flowing back. The exact location of the site was marked by means of an indelible pencil. (Injection was withheld if blood vessels of the ear were distended; to collapse local vessels, ears were sometimes cooled by sponging with 70% ethanol and evaporating with a gentle stream of air.)

Animals were housed usually in groups of 3 and 4. All guinea pigs in one experiment, including animals intended for later addition to the experiment, were randomized in advance: subgroups within each experiment were scattered among the cages.

Contact Test for Determination of Sensitivity.—Animals were subjected to a contact test on

day 14 after the sensitizing injection. One drop of a 1% solution of the cold chemical in olive oil was applied from a capillary pipet held at an angle of 45° on a freshly clipped site of the back and spread gently over a circular area of about 25 mm in diameter. The reactions were read blindly at 24 and 48 hr by one of us (MWC) and graded 0, negative; f.tr., faint trace; tr, very few faint pink spots; tr, few faint pink spots; ±, faint pink dots, or confluent very faint pink; ∔, faint pink but spotty; +, faint pink; + +, pale pink; + + +, pink, but either somewhat pale or macular; + + + +, pink and definitely thickened; + + + + +, bright pink, well thickened.

Preparation of Sample.—The injection sites were removed by excision of the entire ear at its base. The operation was performed under ether anesthesia and the wound closed by sutures of three-twist linen thread. When organs were desired for examination, the animals were
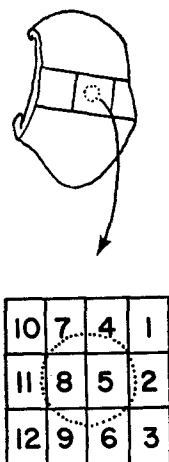

Fig. 1. Removal of injection site and surrounding tissue from right auricle. The area containing the ¹⁴C-labeled allergenic chemical was removed from the excised and frozen ear and subdivided into 12 pieces as shown (cf. Fig. 2) prior to ignition.

anesthetized, exsanguinated, and killed by overdose of chloroform. All tissues taken were immediately submerged in liquid nitrogen and kept frozen in the deepfreeze at −14°C.

The method to be used for combustion required samples not to exceed 3 mg dry weight. It was found not to be necessary to combust the entire ear, for all radioactivity was found localized in an area of 8 × 8 mm that surrounded and included the injection site (Fig. 2). Excised ears were handled throughout with equipment that was kept below the freezing point by being repeatedly immersed in liquid nitrogen. The ears were placed on a small plastic table and cut with a razor blade to free the piece of tissue that included the injection site. The site itself and the surrounding margin of tissue, while still frozen, was then divided into twelve samples, each of them measuring about 2.5 × 2 × 1 mm, and weighing up to 10 mg moist weight (Fig. 1). Each sample was placed inside a hollow cotton pellet, mounted on the platinum-iridium coil stand, and finally set in the liquid scintillator vial in which it was allowed to thaw.

Small lymph nodes such as the retroauricular nodes, up to 10 mg wet weight, were mounted uncut on the platinum-iridium coil together with one or two cotton pellets. Bigger nodes such as mesenteric lymph nodes had to be divided into a suitable number of samples. Thymus tissue was mounted either on one or two coil stands, according to the size of the lobes. Spleens were

cut crudely and 10 samples per spleen were taken at random, representative of both the red and white pulp.

Fluids such as whole blood, plasma, whole lymph, or urine were impregnated into the cotton pellet, mounted on the coil, in volumes known to contain up to about 3 mg of dry solids.

Combustion Procedure.—The combustion method introduced by Gupta (8) was used, in which combustion takes place in the scintillator vial that will be used for counting. Essentially, on a platinum-iridium coil stand within a scintillator vial capable of gas-tight closure, one or two cotton pellets, No. 4, are placed to serve as fuel and absorbent for liquids. The vials are flushed with pure oxygen and capped tightly. The sample and fuel are ignited from without the vial by the beam of a projection lamp with a self-contained reflector (DFC, 120 v, 150 w, General Electric) housed for convenience in a tubular aluminum casing with a handle.

Since uncombined PCl and DNCB both sublime rather readily, we modified the described procedure: (*a*) The tissue was dried within the sealed vial, the water vapor being allowed to condense on the walls, chiefly within the cap; (*b*) phenethylamine was not introduced into the vial prior to combustion (advice of G. N. Gupta, personal communication); (*c*) lens paper and the heat-collecting ink spot were omitted in favor of drying and raising the temperature of the tissue to the combustion point. Too rapid an onset of ignition led to incomplete combustion.

Sample Nos. 1–12 (Fig. 1) were mounted on the coil stands, the vials were flushed with oxygen and caps were tightened. The sample inside each vial was dried by focusing the light beam of the projection lamp on the cotton pellet. Slow turning of the vial was useful to raise the temperature equally throughout the cotton-tissue unit. The ignition temperature was reached within 20 sec and the sample burned to completion. After the combustion, the vial was plunged into liquid nitrogen up to its neck in order to freeze the CO_2 resulting from the combustion as well as water vapor. The vial cap was then opened briefly for introduction of a premixed cocktail containing 0.2 ml of phenethylamine, 0.6 ml of absolute ethyl alcohol, and 0.6 ml of p-dioxane, and then closed tightly again. A period of at least 30 min was allowed for the CO_2 to react with amine, with repeated vigorous shaking; the carbamate which formed dissolves readily at room temperature. When entirely defogged (the ethanol-dioxane mixture dissolves the water), the vial was opened a second time and a mixture of toluene (10 ml) and Liquifluor (0.6 ml) was added rapidly from an automatic pipette (Repipet, Labindustries, Berkeley, Calif.), thus completing the liquid scintillator. After thorough mixing of the contents, the vials were ready for radioassay in the liquid scintillation counter.

Counting Apparatus.—All measurements were performed in a three channel liquid scintillation spectrometer (Packard Tri-Carb Model No. 3003). Gains for ³H, ¹⁴C, and ³⁵S were set at 57.7, 10, and 8.6% respectively. Discriminators were set at 30–1000 on all three channels.

Collection of Lymph of the Cervical Duct.—For collecting lymph an operative approach was adopted from Reinhardt and Yoffey (9). Under ether anesthesia a midline incision was made through skin and fascia of the ventral part of the neck, separating the submandibular salivary glands and the sternohyoideus muscles. The left muscle was loosened by blunt dissection and drawn towards the midline by means of a blunt hook, exposing the cervical duct. Although very thin-walled and entirely transparent, the duct appeared to be rather plastic and distensible.

In preliminary studies, the exact site of the cervical duct was identified by injecting intradermally 0.01 ml of 0.5% buffered Evans Blue into the ear. The dye appeared in the lymph 5 min later, demonstrating the site of entrance of the cervical duct into the venous system at the jugular-subclavian angle. Polyethylene tubing size PE-60 (Clay-Adams) was siliconized with a commercial product (Beckman Desicote). The tapered tubing was inserted into the duct with a quick stab. Lymph fluid rushed immediately into the tubing. The junction of cannula and duct was then sealed by applying a small drop of Eastman 910 adhesive with a capillary pipette according to the method of Boak and Woodruff (10). The adhesive hardened within 20 sec. The tubing was then fixed to a suitable site on the chest wall by a further application of East-

man 910, thus preventing dislocation of the cannula during the time of lymph collection and permitting safe movement of the free end.

Further use of dye was abandoned as soon as sufficient familiarity had been attained with the operative and draining techniques. Lymph collection could be maintained up to 4 hr, with ether anesthesia kept as light as possible. The lymph fluid was allowed to drop from the end of the tubing into a siliconized calibrated 8 × 75 mm tube and was thoroughly mixed with 10% glycyl-glycine at pH 8.0–8.3 in equal volume by shaking (11). The yield per hour of collection ranged from 0.02–0.6 ml. Total protein concentration was about 2.5%. The glycyl-glycine lymph mixture was delivered into cotton pellets as described above, but the water was allowed to evaporate freely in the heat provided by the projection lamp. Picryl chloride combines readily with glycyl-glycine at pH 8.0 and the resultant picryl glycyl-glycine is not subject to sublimation during the drying. Oxygen was then introduced into the vial and combustion carried out in the usual way.

Collection of Blood (from the Draining Auricular Vein).—For collecting venous blood the retroauricular vein draining the ear was dissected out under ether anesthesia and slit by scissors. The blood flowing out was taken up into a siliconized glass syringe prefilled with 0.4 ml of 3.8% aqueous sodium citrate. Blood flow was sustained for 1–2 min; volumes of 0.5–1.5 ml of blood could be collected. A more effective alternate procedure consisted in nicking the central ear vein by a fine scalpel, about 6 mm away from the edge of the injection site. By use of one or two drops of 0.5% heparin in saline and slight mechanical disturbance of the vessel wall with the tip of the blunt collecting needle, a slow bleeding would be sustained for 6–13 min. Because of the smaller size of this vessel blood volumes did not exceed 0.7 ml.

When PCl* was to be traced, the citrated blood was mixed at once 1:1 with 10% glycyl-glycine (pH 8.0–8.3), and placed on 50 mg of absorbent cotton. The cotton was dried under the infrared lamp and combusted within a plastic bag according to a macromethod devised by Gupta and put at our disposal prior to its publication (12). In this way, radioactivity could be estimated in single combustions of 2 ml volumes representing up to 1.0 ml of whole blood. When the central ear vein was employed for collection, combustion of the entire yield was made as a series of single drops of 0.05 ml in separate scintillator vials.

In the case of DNCB, no glycyl-glycine was used in view of the low reactivity of DNCB. Single drops of 0.05 ml whole citrated blood were dried individually on cotton pellets in the open air. In one experiment, the cellular elements were sedimented, washed, and counted apart from the plasma.

Collection of Urine.—Urine was collected from individual guinea pigs in metabolism cages (Acme guinea pig metabolism cage, AC-5362). Animals were acclimatized to these cages over a period of 5 days prior to injection of radiolabeled allergen. Urine was collected under toluol for 24 hr in one or more samples. In early experiments, the urine was evaporated to a syrup and extracted with ethanol, which was separated and evaporated sufficiently to allow combustion in a series of cotton pellets. In another experiment toluene and ethanol were used in turn as solvents, evaporated to a jelly, and likewise combusted separately in a series of cotton pellets.

RESULTS

The over-all efficiency with which ^{14}C could be traced by the combustion methods used was evaluated closely. It may be stated at the outset that the over-all efficiency in tracing ^{14}C was found to be 56% of the actual radioactivity (76% machine efficiency times 90% counting efficiency times 82% recovery).

Critical Evaluation of Methodology in Counting

Counting Efficiency.—The *machine efficiency* of the liquid scintillation counter was 76% for ^{14}C in repeated checking throughout the experiments, i.e., counts per minute

$\Sigma = 2380$ cpm

FIG. 2. Distribution of radioactive counts in segments of ear tissue. Adequacy of the chosen area of Fig. 1 for estimating total radioactivity is shown. PCl was injected intradermally, 0.25 μg of 1-^{14}C-PCl (specific activity, 2 mc/mM) in 0.01 ml volume. Counts in peripheral segments represent the respective proportional parts of the original bleb volumes; areas still more peripheral were always negative.

TABLE I

Replication Counts, Combustions of Guinea Pig Ear Tissue

	Solutions			
	No. I	No. II	No. III	No. IV
cpm/ear	2315 2494 2685	2378 2479	2380 2589	2246 2301 2338
Parameters*	$\bar{x} = \dfrac{\Sigma x}{N} = 2420;\quad s^2 = \dfrac{\Sigma(x - \bar{x})^2}{N - 1} = 19{,}254$ Confidence limits, $100(1 - 2\alpha) = 95\%$ $\mu = \bar{x} \pm k_2 \cdot s = 2420 \pm 100$ cpm			

Actual countings done over 10 min periods of time following injection of 1-^{14}C PC1 into the ear as stated in the text. The values shown are summations of counts of ^{14}CO$_2$ resulting from the separate combustions of 12 bits of each ear within scintillation vials by the method of Gupta (8).

* x, observed values; $\bar{x}$, mean; s^2, estimate of variance in the sample; μ, mean of the population; k_2, factor for 95% confidence limits with value of 0.7154 for $N = 10$.

(cpm) from a standard commercial ^{14}C preparation divided by its known disintegration per minute (dpm). The *counting efficiency* for our standard dose, namely 0.25 μg of PCl* or of DNCB*/0.01 ml of solvent (saline with 0.83% ethanol) was 90%. Counting efficiency is expressed here as counts of the experimental dose introduced directly

into scintillator fluid divided by the calculated cpm of this standard (without any allowance for quenching). Quenching due to solvents and solutes in the scintillation counting medium amounted to 10%.

When the experimental standard amount of allergen in saline was combusted in a vial following application to one cotton pellet, 85% of the radioactivity was recovered by liquid scintillation counting. (The percentage of recovery is expressed as counts of the combusted sample divided by direct counts of the sample added to scintillator fluid
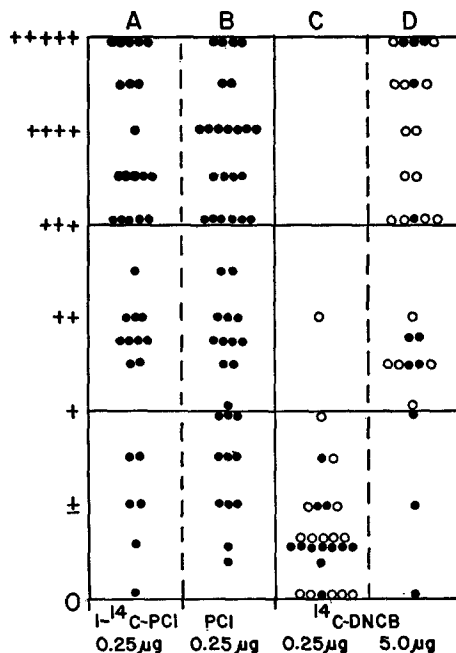

FIG. 3. Contact reactivity attained by single injections of chemical allergens into the ear. The Rockefeller University strain of albino guinea pigs was injected intradermally into the ear in doses of 0.01 ml, containing allergens as shown. Entries in open circles refer to additional animals injected with cold DNCB. Contact tests (1 drop of 1% solutions of the cold allergens in olive oil) were applied 14 days later and readings were made at 24 and 48 hr. The results of the contact tests are shown in the left-hand margin, graded as described under Materials and Methods.

as above.) Evidently, chemical and/or color quenching is somewhat greater after combustion of the sample largely owing to excess of oxygen, water, and other combustion products in the liquid scintillator.

The experimental standard dose, injected into excised control ears, gave a recovery of 82% (counts of all combusted ear sites divided by cpm obtained upon direct counting of the experimental dose). Ears of normal guinea pigs were removed and injected 3-5 min later when circulatory effects had ceased. The central areas of the ears were cut into 12 samples each and combusted (Fig. 1). The counts of the 12 samples were then totalled (Fig. 2). The recovery of radioactivity from combustion on cotton

shows that loss of radioactivity through the procedure employed for preparing tissue samples lies in the range of 3%.

Reproducibility.—To determine *pipetting and injection errors*, ears of 10 normal guinea pigs were excised and injected in groups of 2 or 3 with 0.25 μg PCl* from solutions set up at different times (Table I). Mean and confidence limits were calculated as shown, indicating 2420 ± 100 cpm/ear. These figures were taken as the 100% value for the actual experiments, which dealt with the rate of dispersal of the allergen from the injection site.
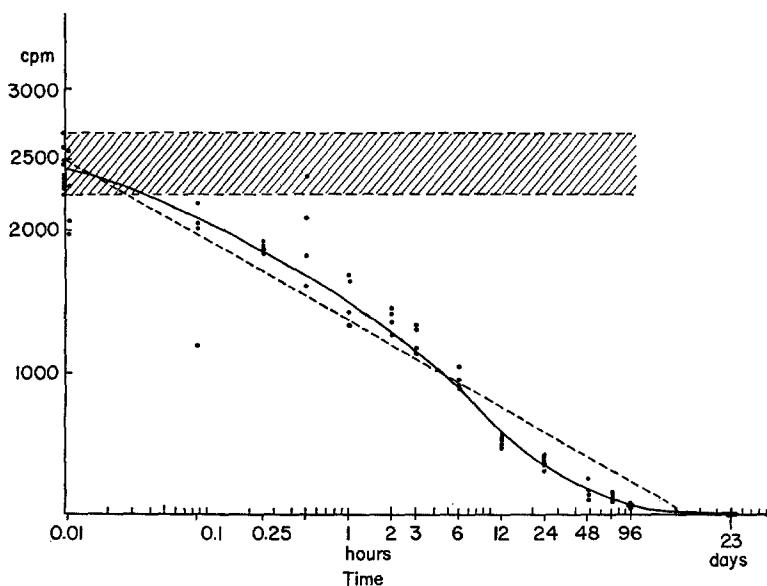

Fig. 4. Radioactivity counts of ear tissue following single injections of 0.25 μg 1-¹⁴C-PCl in 0.01 ml. The central areas of the ears were divided into 12 segments as described (Figs. 1 and 2), combusted separately, and counted for 10-min periods. The results are expressed as cpm/ear. The range of counts in 10 control ears ("0" time) established the dashed norm, varying between 2246 and 2685 cpm for a 95% confidence limit of 2420 ± 100 cpm. 57 ears were excised after injection at times varying from 30 sec to 23 days.

A typical diagram of one control ear is shown in Fig. 2, cpm being entered in the respective areas of tissue samples 1–12. All counts on ears were diagrammed in this way; the curves shown below are based on such records.

The *counting error* of the liquid scintillation counter was determined by repeatedly counting vials set up with dilutions intended to give 3 cpm and 6 cpm per vial. Each vial was counted 10 times for 10 min. Average counts above background in the second series were 56, twice the average count of 27 in the first series. Radioactivity even as low as 3 cpm above background is therefore recognized when counting is done for a 10 min period. Accordingly, we chose to count for 10 min as standard procedure. As a rule of thumb, we considered 5 cpm over background to be the lowest radioactivity that is distinguishable with certainty. In terms of weight, 5 cpm equals 0.5 × 10⁻⁹ g of 1-¹⁴C

PCl or about 0.2% of the amount injected into one ear. With regard to ^{14}C DNCB and its higher specific activity, 5 cpm equals 0.16×10^{-9} g in weight or about 0.06% of the amount injected into one ear. When 5 μg of ^{14}C DNCB was injected, 5 cpm represents 0.003% of the amount applied for sensitization.

Selection of PCl and DNCB Doses for Sensitization and Radioassay

A dose was required which would permit ready detection of depressions, whenever encountered, in response to experimental sensitization (1). This was attained by a single intradermal injection of 0.25 μg 1-^{14}C PCl (1 nmole) in 0.01 ml of saline with 0.83% alcohol, made into the right ear of 35 animals. Contact tests on day 14 revealed moderate to high contact reactivity in about 50% of the animals injected, low to moderate (but definite) reactivity in another 25–30%, and practically no sensitivity in the rest of the group (Fig. 3, column A). This over-all result is in accord with former experience in the use of "cold" PCl in this sensitizing technique (Fig. 3, column B).

The same dose (0.25 μg) of ^{14}C-labeled DNCB failed to sensitize any of the 12 guinea pigs (Fig. 3, column C), but a single injection of 5 μg (25 nmoles) effected about the same over-all degree of sensitization (Fig. 3, column D), as did one-twentieth this amount of PCl. Sensitization with these same amounts of cold DNCB gave identical results (Fig. 3, columns C and D).

Escape of Allergen from Ear Sites

With 0.25 μg of 1-^{14}C PCl/0.01 ml injected intradermally into the right ear, the rate of dispersal from the site followed approximately an exponential curve (Fig. 4). Individual animals showed considerable variation during the 1st hr. The chemical started to leave the site almost immediately after its deposition; about 20% had escaped by 30 min, 50% by 3 hr, and 75% by 12 hr. The proportion remaining at the site had declined to 16% at 24 hr, to 7% at 48 hr, and 3% at 4 days. Despite continuing loss, local traces were still present at 3 wk, being then equivalent to 1–3 ng of the chemical originally injected.

The same dose of ^{14}C-labeled DNCB escaped even more rapidly, more than 90% of the applied chemical disappearing within the 1st hr after the injection (Fig. 5). Thereafter, the rate of dispersal slowed down markedly; but only 3 ng were left in the site at 12 hr, 1 ng at 24 hr, and about 0.5 ng at 4 days.

Upon increasing the dose of ^{14}C DNCB to 5 μg in the same 0.01 ml volume, the same pattern was observed (Fig. 6). About 90% left the site within 1 hr, seemingly with an initial outflow even greater than that with the smaller dose. Apart from the first 15–30 min, the local concentration of the chemical was approximately 20–40 times higher at all times during the observation period than with the smaller dose: about 60 ng were found at 12 hr, 40 ng at 24 hr, 25 ng at 4 days, while amounts equivalent to 1–2 ng could still be traced at 6 and 10 wk after the sensitizing injection.

The curves of Figs. 4–6 are plotted as per cent of counts of control ears in Fig. 7. The fundamental difference in disappearance rate of the two chemical allergens accords with the reaction constants of the two compounds (reference 13, p. 195). PCl* remains at the site in greater proportion and longer than does DNCB*. Seemingly, PCl is bound to local tissue components earlier and/or more firmly. If this explanation is correct, picric acid, which results from hydrol-
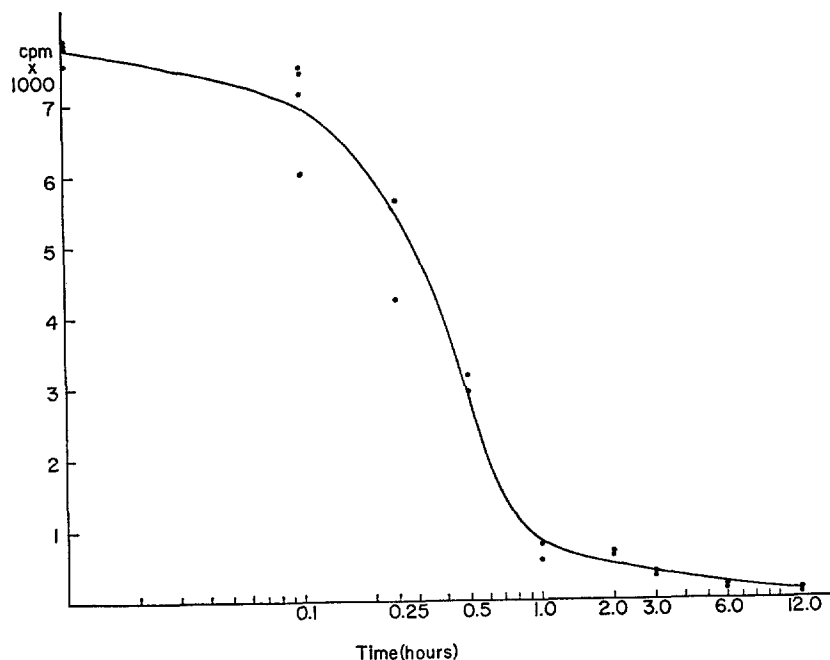

Time(hours)

FIG. 5. Radioactivity counts of ear tissue following single injections of 0.25 µg ¹⁴C-DNCB in 0.01 ml. The procedure follows that of Fig. 4. Four control ears ("0" time) established the norm (7553–7956 cpm with a mean value of 7833). 24 ears were excised after injection at times varying from 6 min to 4 days: 18 determinations within the first 12 hr are plotted. Radioactivity in ears excised later are as follows: 1 day (40, 23 cpm); 2 days (33, 28); 4 days (18, 9).

ysis of picryl chloride and which hardly interacts with proteins, should show a high outflow rate. A few animals were injected with 0.25 µg of 1-¹⁴C picric acid (2 mc/mm) contained in 0.01 ml, and indeed the dispersal was found to be as expected (Fig. 7). By 1 min after application, almost 30% had left the site and after 3 hr only 5% remained. The latter is exactly the same as was found at 3 hr with ¹⁴C DNCB.

Fig. 8 expresses the data of Fig. 7 as amounts still present in the site at various times, plotted against the logarithm of time. The curves of dispersal of the two chemicals are fairly parallel when the amounts injected were 0.25 µg

for PCl and 5.0 μg for DNCB respectively. Several changes in output rates were found.

Allergens present in the local site between 1 and 4 days, as is shown in the following paper, constitute the sensitizing stimulus since ear excisions made prior to 24 hr nearly obliterate sensitivity (reference 1, Fig. 2) and those made after 4 days do not lessen either frequency or intensity of sensitivity.
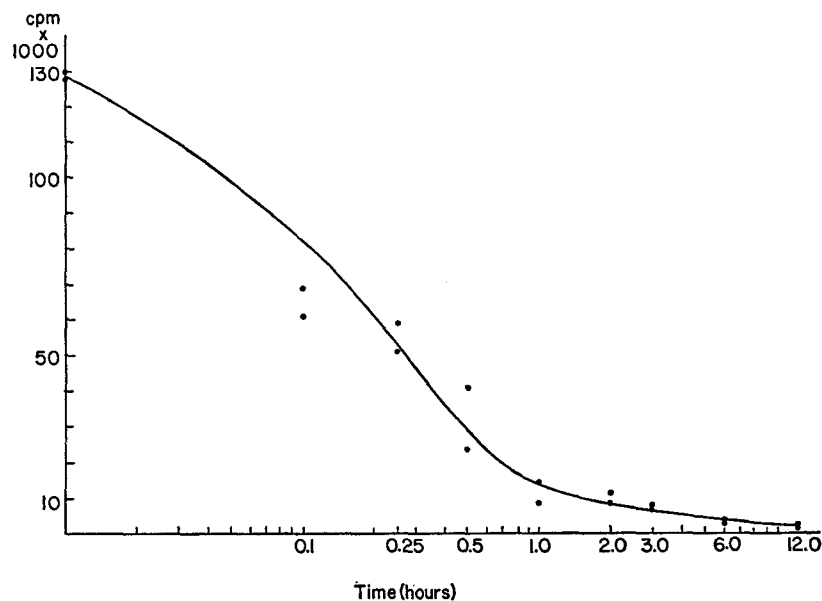

Time (hours)

FIG. 6. Radioactivity counts in ear tissue following single injections of 5 μg ^{14}C-DNCB in 0.01 ml. The procedure follows that of Fig. 4. Two control ears (0 time) yielded duplicate counts of 127,393 and 130,646 to establish the norm. 38 ears were excised after injection at times varying from 6 min to 70 days, of which 16 determinations through 12 hr are plotted on the graph. Radioactivity in ears excised later are as follows: 1 day (1602, 1182 cpm); 2 days (1223, 1130) 3 days (1013, 836) 4 days (793, 446); 5 days (990, 332); 7 days (327, 307); 17 days (477, 208); 21 days (571, 302); 28 days (53, 46); 42 days (54, 10); 70 days (112, 5).

When escape rates are calculated as "half-lives" of allergen with time, it is found that 0.25 μg PCl shows a sequence of half-lives, namely, 2.5 hr during 6 hr, then 28 hr up to 2 days, and finally 43 hr up to 4 days. Similarly, 5.0 μg DNCB leaves the site in a series of half-lives, namely, 1.8 hr during 8 hr, then 29 hr up to 2 days, and finally a much lengthened half-life (72–88 hr?). Material leaving the site with a slow rate might represent, in part at least, hydrolytic cleavage of the ^{14}C chemicals from protein carriers.

In contrast, when only 0.25 μg of DNCB is injected, the escape is so great that during the induction phase of hypersensitivity practically no local depot is formed.
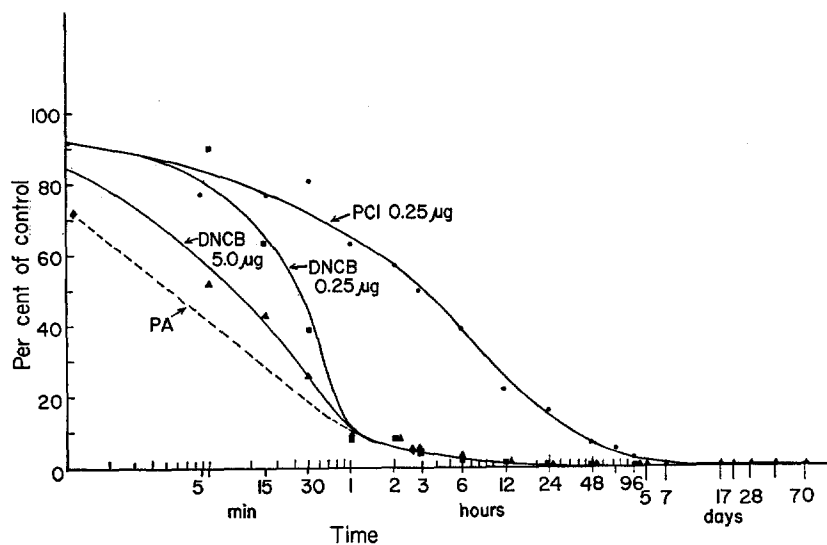
FIG. 7. Comparative escape of ¹⁴C-labeled allergens from ear tissue. Data of Figs. 4–6 are plotted against time, as per cent of the values of control ears. In addition, the escape of 1-¹⁴C-picric acid (PA) from ear tissue is shown, 0.25 μg in 0.01 ml being injected (see text); the dotted line indicates that no determinations of picric acid were made between the 1 min and 60 min values.
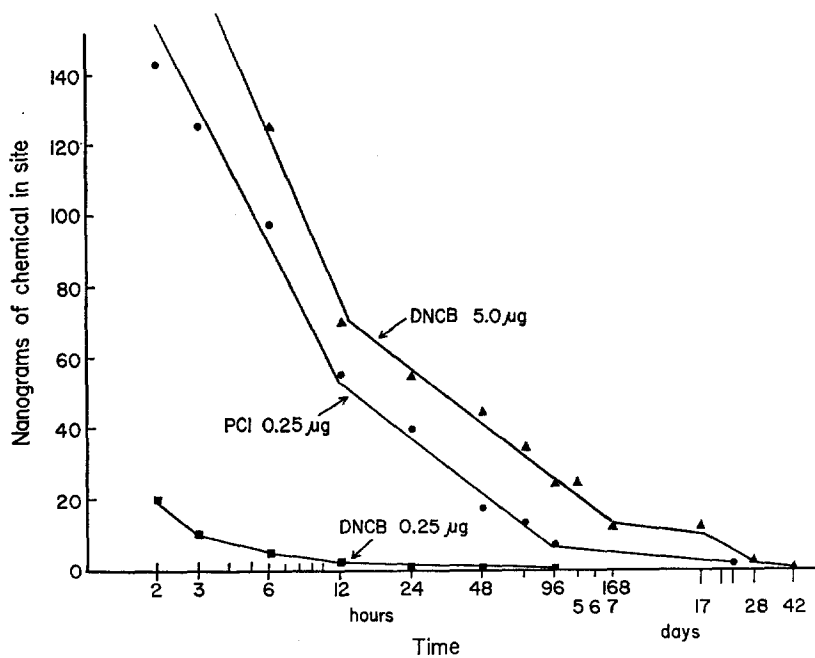

FIG. 8. Allergenic chemical retained in ear site at different times. The data of Fig. 7 are replotted on the basis of the actual ¹⁴C introduced into the ear, to show absolute amounts of chemical remaining in the ear. The smaller dose of DNCB is not retained and does not lead to sensitization.

Role of Regional Lymph Nodes.—The regional lymph node draining the ear (ca. 1 × 1.5 × 2.5 mm and normally weighing 7–15 mg) is located near the ventral margin of the ear at its base and is easily approached by retroauricular incision. Upon injecting 0.01 ml of buffered 0.5% Evans Blue dye intradermally into the ear, the node becomes stained a dark blue within a few seconds, even if the animal is dead. Three further nodes along the chain are stained in turn by the same dye injection, often decreasing in color and sometimes spotty, namely the Lymphonodus parotideus, Lymphonodus cervicalis cranialis superficialis, and Lymphonodus cervicalis cranialis profundus (14). From the last node, the dye flows directly into the cervical duct which, together with the subclavian duct, both paired, constitute the major lymphatic trunks in the anterior part of the body (9).

In marked contrast to drainage of dyes and suspended particles, no evidence was secured for substantial uptake of ^{14}C-labeled PCl and DNCB into regional lymph nodes. When 0.25 μg PCl* was injected intradermally, the regional node from a single individual, only, excised $\frac{1}{2}$ min later, contained about 1% of the injected dose, and some other measurements made between 5 and 60 min did not exceed 0.3–0.5%. (Many other nodes combusted at these various times showed no radioactivity at all.) At 3 hr, only 1 out of 15 retroauricular nodes showed slight radioactivity. Radioactivity was never thereafter detected. The three nodes deeper in the chain were consistently negative.

With intradermal injections of 5 μg DNCB*, the highest value ever obtained was 0.1% of the entire dose at 30 min. Some other nodes taken between 6 min and 2 hr after injection contained less radioactivity, ranging from 0.05–0.005% of the injected amount. Many nodes lacked radioactivity, sampled at various times. At stages from 3 hr through 17 days following the injection, no radioactivity was ever detectable.

Cervical Duct Lymph.—Lymph was collected from the cervical duct to check whether allergen passes via the lymphatics even if it is not detected within the node.

An animal was injected intradermally with 0.25 μg PCl* into one ear following insertion of the cannula into the cervical duct. During collection over a 4 hr period, 2.4 ml was secured, as eight portions of 0.3 ml each. The ear was then excised and the injection site combusted. Since 900 cpm was still present at the site, 1500 cpm must have escaped over the 4 hr period (about 63% of the injected PCl dose). Each of the eight collected portions of cervical duct lymph were mixed with 0.3 ml of 10% glycyl-glycine at pH 8.0 in order to trap free picryl chloride; these portions, combusted separately, could not be distinguished from the background count. If small amounts of radioactivity had been lost technically by subdivision of yield into smaller portions, even a liberal estimate would not exceed 35–50 cpm totally. Thus, maximally 2 or 3% of the entire amount might have taken the lymphatic route; but probably the true percentage was even smaller.

Another animal injected with the same dose of PCl* was cannulated in the same way. Lymph collection was obtained for 170 min before death by overdose of ether. Lymph production was much smaller in this animal, only 0.24 ml was collected. The ear site taken immediately after the removal of the cannula yielded 1367 cpm, therefore it could be assumed that at least 1000 cpm had left the site during the time of lymph collection. No radioactivity was measurable in this specimen of lymph (mixed with glycyl-glycine as in the previous experiment). This negative result was even more striking than the first one because the smaller volume collected from the cervical duct eliminated the disadvantage of having to divide the yield into smaller portions suitable for examination.

To test the sensitivity of the methods employed here, a known radioactivity of only

TABLE II

Escape of ¹⁴C-Labeled Picryl Chloride from Ear into Regional Blood Vessels

Allergen used	Guinea pig No.	Blood sample			Radioactivity		
		Vessel	Time	Volume	Estimated escape	Recovery	Estimated recovery
			min	*ml*	*cpm*	*cpm*	*%*
0.25 μg PCl*,	1	Retroauricular vein	1.5	1.0	50	26	50
	2	"	5	1.5	140	108	75
	3	"	0.5	0.5	25	10	40
	4	"	0.5	0.5	25	26	100
0.25 μg DNCB*,	1	Central ear vein	13	0.6	2200	2004	90
	2	"	15	0.4	2100	1350	65
	3	"	7	0.35	1000	970	95
	4	"	6	0.35	550	360	65

The two chemical allergens differed in specific activity (¹⁴C) and in rate of loss from the ear following an injection of 0.01 ml containing the amount shown. Technical procedures are described in the Materials and Methods section.

20 cpm per vial was set up as a "control lymph." Precisely this amount was recovered, attesting to the significance of the negative results of the experiments described above.

Role of Regional Blood Vessels.—The discrepancy between demonstrated rapid escape of labeled allergen from the site and insignificant uptake into the lymphatic system led to the presumption that escape of the major proportion of the chemical deposited intradermally might occur through local blood vessels.

Attempts to cannulate the small draining veins were thwarted by premature clotting of blood. We then slit a vessel wall longitudinally and drew the blood into a syringe containing 0.4 ml of 3.8% sodium citrate. In a first experiment with 0.25 μg PCl* injected into the ear, the retroauricular vein was chosen as a likely vessel for blood samples. Positive results were obtained in four out of four animals (Table II). The

measured radioactivity, though rather low, was in fair agreement with rough estimates of the proportion that should have escaped.

In a second experiment with 0.25 µg DNCB* injected intradermally, the counts recovered from blood samples were considerably higher because of the higher specific activity of this allergen and its faster disappearance from the site. An alternate procedure for blood collecting proved superior. The central ear vein, the vessel closest to the site, was slit on the auricular surface close to the angle between ear and skull. With slow bleeding sustained for periods up to 15 min as described under Materials and Methods, small blood volumes were harvested which possessed highly concentrated radioactivity. Countings on four animals confirmed the previous results (Table II, bottom half) and proved that the venous route was the principal pathway for escape of chemical allergens. Once, red and white blood cells were separated from plasma taken after 5 min, washed, and counted separately. The radioactivity originally counted in whole blood was recovered in the plasma; the cells showed no radioactivity at this time.

Further Examinations for Distribution of Chemical Allergens in Vivo

Urine.—Three guinea pigs injected with 0.25 µg PCl* were held in metabolism cages for 24 hr. With extraction methods aimed at qualitative rather than quantitative analysis, all urines were found to be radioactive, the total counts representing 5–20% of the amount actually injected.

A dose of 0.25 µg DNCB* was injected into three additional animals, and urine was collected in fractions during 24 hr. The first fractions, obtained 3.5–4.5 hr after the injection, contained more radioactivity than later fractions. In one sample, initial extraction with toluene yielded no radioactivity, but sequential ethanol and saline extracts both gave positive results.

Thymus, Spleen, and Mesenteric Lymph Nodes.—No radioactivity was detected in thymus, spleen, and mesenteric lymph nodes following the deposition of either PCl* or DNCB*, when taken at ½, 5, 15, and 30 min; 1, 2, 3, 6, 12, and 24 hr; 2, 3, 4, 5, 7, and 17 days after the intradermal application of the allergen.

DISCUSSION

[14]C-labeled picryl chloride (PCl*) or 2,4-dinitrochlorobenzene (DNCB*) was injected intradermally into one ear in an amount approaching the minimal sensitizing dose. With The Rockefeller University albino guinea pigs, the dose adequate to induce quite satisfactory sensitization of two-thirds of the animals (Fig. 3) was 0.25 µg (ca. 1 nmole) of picryl chloride or 5 µg (25 nmoles) of DNCB. At varying times, the auricle was excised, frozen in liquid nitrogen, and subsequently subdivided into segments (Figs. 1, 2) which were combusted to [14]CO$_2$ and water within scintillation vials. The counts given by the individual segments were totalled. Accuracy and reproducibility of the method were high, as detailed above (Table I). 1-^{14}C-picryl chloride left the ears in three phases or stages (Figs. 4, 8) connoting escape in more than one form. The stages can be detected in principle also with ^{14}C-DNCB (Figs. 5, 6, 8).

At first, the chemicals are resorbed quickly—about 50% of PCl is gone after

3 hr, nearly 90% of DNCB in 60 min (Fig. 7). If the capability of the respective sensitizers to unite with proteins by covalent bonds at body pH applies here (reference 13, p. 195), this first fraction probably represents free chemical still uncoupled, its low molecular weight and perhaps its lipid solubility allowing it to pass easily through blood vessel walls (Table II). Then, apparently in connection with increasing dominance of the coupling reaction and formation of products of high molecular weight, the speed of resorption is slowed (Fig. 8). Finally, a small fraction, interpreted as representing insoluble coupling products such as epidermal conjugates, stays locally for a long time, possibly not at all subjected to resorption.

Obviously, DNCB* escaped to a greater extent than PCl* (Fig. 7) and less chemical is fixed at the site regardless of whether 0.25 μg or 5 μg DNCB* has been injected. This suggests that the much lower reaction constant of DNCB* applies to coupling in vivo.[1] Coupling in the skin has been shown clearly by Eisen and Tabachnick (2) who extracted skin proteins and isolated ^{14}C-dinitrophenylated amino acids from guinea pigs which had been painted over a broad skin area. After painting with ^{14}C-DNCB, the authors cited found less than 2% present as conjugate in the epidermis at 24 hr, and only traces in the corium. Hence, very little coupling to collagen could have taken place.

^{14}C chemicals actually present in the site of deposition between 12 and 96 hr, expressed as weight units of the original chemical allergens, are shown in Fig. 8. This period is the critical phase of the induction period (12 hr to 4 days). When 0.25 μg PCl* is injected intradermally, the local depot decreases with a "half-life" of 28 hr between 6 and 48 hr, then of 43 hr up to 4 days, whereas DNCB* in the same dose almost completely escaped during the first 12 hr and left only bare traces. This probably explains the failure to sensitize with a single injection of 0.25 μg DNCB*. When the dose of DNCB* was increased 20-fold to 5 μg, then roughly the same degree of sensitization was secured as with 0.25 μg PCl*. Indeed, the parallel lines in Fig. 8 show close resemblance respecting the depots established at ear sites.

These results in general suggest that the product which sensitizes can not be represented by the fraction which escapes earliest. The actual sensitizing fraction, as will be shown (1), must stay at the local site for more than 12 hr. (The part which escapes earliest plays a quite different role.)

The principal pathway for escape proved to be the regional blood vessels, although we had anticipated spread via the lymphatics. Blood samples taken from the retroauricular vein or the central ear vein regularly carried radio-

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[1] Picryl chloride is easily stripped of its halogen grouping by sodium methylate, the reaction constant being high, certainly above 1000 at 15°C; in comparison, the constant for removal of the halogen atom from DNCB is only 3.25 at 15°C with use of sodium ethylate (13). The two chemicals show corresponding differences in conjugation in vitro with aniline, with glycylglycine (13), and with proteins.

activity. No evidence was found of any competitive pathway, either lymphatics or lateral spreading in the ear tissues. Counts at the original injection site remained confined at all times to the same small area (Fig. 2). Traces of radio-activity that could be counted in the regional lymph nodes for a brief time, only, after the injection are likely to represent injection fluid squeezed passively into the lymphatics owing to an unphysiologically high pressure in the local ear tissue at the time of the injection. (We have brought evidence [1] that any irregular and transient passage of this small amount can not sensitize.) Since the regional lymph nodes lost this early radioactivity rapidly and never became radioactive again at later stages, an active transport that carries the allergen into or through the regional lymph nodes is unlikely. Further evidence for lack of such a mechanism was proved by direct examination of lymph fluid collected from the cervical duct draining the ear.

There was positive evidence of radioactive chemical travelling with the blood in the early stages. Admittedly, the concept of major and apparently exclusive transport via the blood vessels is supported more strongly by findings after the earlier excisions than later on. Negative findings in lymph and lymph nodes at 3 hr after injection natuarlly carry more weight than at 96 hr or later, when traceable radioactivity becomes dispersed or lost. Nevertheless, the negative results should not be underestimated, since the sensitivity of the methods used is such as to detect $\frac{1}{2}$ ng of 1-^{14}C-PCl per combustion vial or 0.2% of the amount injected, and $\frac{1}{6}$ ng of ^{14}C-DNCB per vial, which equals 0.06% of the small dose (0.25 μg) and 0.003% of the larger dose (5 μg) used for sensitization. Even if one makes liberal allowance for losses in detecting radioactivity due to technical inadequacies, about 1 ng of PCl* and 0.3 ng of DNCB* would be detectable in small lymph nodes that were combusted within a single vial, the usual practice with the regional nodes. (Had 0.3 ng of ^{14}C-dinitrophenyl groupings remained undetected in the regional lymph nodes, of course a very large number of groups (9 × 10^{11}) would be represented. If these molecules were all free DNCB, which is unlikely, it is difficult to attribute a role in sensitization to them in view of the earliest passage, but entirely ineffectual in sensitizing, of perhaps 5 ng of DNCB.)

Since the critical period commences around 12 hr, and only about 45–70 ng of allergen are then retained (Fig. 8), especial importance must be given to its fate during the next 3$\frac{1}{2}$ days, when traceable allergen in the local site drops to values of 10–25 ng. Not even a measurable part of this approximate difference of 40 ng accumulates in the regional lymph nodes. This statement carries even more weight when the specific activity of the preparations used for radioassay is taken into account. The specific activity of 1-^{14}C-PCl was 2 mc/mM, which means that 1 out of 30 molecules were labeled; the uniformly labeled ^{14}C-DNCB had a specific activity roughly three times higher, namely 4.86 mc/mM, which would mean that every 12th molecule, on the average, was labeled. Therefore,

substantial proportions of the chemical allergen or its coupling products could hardly be missed in the event that they had settled in the regional lymph nodes.

The negative findings given above appear to accord with the concept of peripheral sensitization as first suggested by Medawar (15, 16, cf. 5). Antigens which are retained, for whatever reason, at the site of their deposition might be recognized as foreign matter by patrolling blood-borne lymphocytes at the local depot, i.e., peripherally as opposed to "central" recognition within the lymphatic organs. Having picked up the information at the site some of the cells, now stimulated, could travel "home" via the lymphatics into the regional lymph node where multiplication into a larger number of cells could take place.

Peripheral sensitization has been proved by ingenious experiments to occur in the case of transplantation immunity to renal homografts in rats (17). Analogously, in contact hypersensitivity to simple chemical compounds the "sessile" antigen (18) would be the products of local coupling which are held back at the site for several hours and days, i.e., in our experiments, material(s) retained longer than 12 hr (Fig. 8). When no such local depot was found to have become established, as was true after a single injection of 0.25 μg DNCB*, the injection did not suffice to provoke sensitization.

The regional lymph nodes remained free of detectable radioactivity throughout the induction period of sensitization after injection of small amounts of allergen. Therefore no significant amount of the antigenic material fixed locally passed directly through the lymphatics or was carried to the lymph nodes by migrating cells. One would have to conclude that peripheral stimulation of lymphocytes is associated rather with "information" gained from contacts made with foreign matter at the local site than in the node. In our findings, the absence of any retained radioactivity within the draining auricular node speaks against a mechanism of establishing contact sensitivity by intracellular incorporation of hapten-protein complex by a process of pinocytosis and transport into the node.

Well-documented studies clearly indicate the direct stimulation of lymph nodes by soluble or particulate antigens, with resultant initiation of antibody synthesis within the regional nodes (19–27). If antigen is injected subcutaneously, the phagocytosed antigen enters the draining node promptly.

Less clear-cut are studies on initiation of delayed hypersensitivity by drainage of allergen or soluble hapten-protein conjugates into the node.

The draining lymph nodes are known to give rise to immunocytes capable of transferring delayed hypersensitivity. Histological changes have been found in the regional lymph nodes after chemical allergens have been applied, studied particularly after epicutaneous application of allergen (3–5, 28–30). Whether the node stimulation seen by the workers cited represents the effect of passage via the lymphatic vessels of epicutaneously applied allergen seems rather unsure in light of the present report. The application of relatively large amounts of

DNFB or DNCB or oxazolone to one ear (3–5) probably did more than to stimulate delayed sensitivity, since plasma cells appeared in the node starting with the 6th day. Indeed, anti-dinitrophenyl antibody, in our experience, is synthesized fairly regularly when one drop of 2% DNCB in alcohol is applied to the same area of the skin on two successive days for the purpose of initiating contact sensitivity.[2]

The thymus was free of detectable radioactivity at all times, signifying the absence of a particular accumulation of allergen (distributed by the blood stream). The absence of radioactivity in the lymph nodes, apart from early occasional transient passage of tiny amounts, means that the occurrence of lymphatic drainage can be excluded. While the negative findings in these two organs have different meanings they both are highly significant in view of the sensitivity of the methods. Also, these tissues were small enough to combust within single scintillation vials.

Failure to detect radioactivity in spleen and mesenteric nodes does not have equal significance. The size of these latter tissues poses a methodological obstacle in detecting very low concentrations of radioactivity, assuming uniform distribution throughout the organ, since these soft tissues were divided into small pieces for examination and differences from background counts *per vial* would be minimized. In light of these factors, a liberal estimation would allow that amounts up to 10–20 ng of PCl* per organ or 5–10 ng of DNCB* could have been missed.

CONCLUSIONS

The two allergens were observed to disseminate from ear tissue with extremely great rapidity, the pathway unexpectedly proving to be direct entry into draining blood vessels, not the lymphatics. Indeed, no competitive pathway for escape from the ear site was detectable; radioactivity did not accumulate at any time in the lymph nodes apart from sporadic traces in the first 2 hr, which were not retained.

The actual sensitizing fraction is taken to be a relatively small proportion of the entire amount injected initially. With the two sensitizers employed, about equal absolute quantities of 40–50 ng are required to be present at the site between 12 and 96 hr after the sensitizing injection, if sensitization is to occur. For this requirement to be fulfilled with both chemicals, different amounts of each allergen have to be deposited initially since their respective reaction constants with protein differ widely. This retained minor fraction, which appears to play the sensitizing role, largely escapes from the site later, perhaps as inactive picric acid or dinitrophenol or hydrolysis products, but at a lessened rate. Its fate could not be traced with the methods used, but gradual accumulation in the regional lymph nodes was excluded.

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[2] Chase, M. W. 1955. Unpublished observations.

A third fraction probably stays at the site for weeks, escaping at a very low rate and still being present after delayed sensitivity has been established. It is not known whether this fraction is essential for sensitization, but the hapten-protein conjugate appears to differ from the above-mentioned haptenic complex which escapes sluggishly.

SUMMARY

The fate of ^{14}C-labeled allergens injected intradermally into guinea pigs, namely picryl chloride (PCl*) and 2:4 dinitrochlorobenzene (DNCB*), was followed during the induction period of delayed hypersensitivity. Both chemicals were applied in a single injection into one ear in amounts that approached their minimal sensitizing doses (PCl, 0.25 μg; DNCB, 5.0 μg). Radioactivity in the various tissues was determined by liquid scintillation counting after combustion of tissues to CO_2 and H_2O.

The injected allergens seemed to leave the injection site in three phases. A large proportion of allergen escaped rapidly from the ear, about 50% within 3 hr in the case of PCl, within 15 min for DNCB, the difference probably reflecting their unequal reaction constants. Initially there was a "half-life" escape in 2.5 hr with injected dosage of 0.25 μg PCl and in 1.8 hr for 5.0 μg DNCB. This escape occurred via the regional veins and not via the lymphatics. Radioactive decomposition products of the allergens were already present in the urine within 3–4 hr.

After 6–8 hr, the half-life time of escape lengthened to approximately 28 hr for both allergens used in their respective initial dosages, holding up to 2 days after which there occurred still further slowing; between 2 and 4 days the time was about 43 hr for PCl, much longer (72–88 hr?) for DNCB, apparently reflecting different physicochemical properties of this second fraction. Sensitization seemed to be connected with the portion that was present between 12 hr and 4 days of the induction period. It is not known how far the escape of radioactivity during this period may represent gradual hydrolysis of attached picryl and dinitrophenyl groupings, respectively, to form picric acid and dinitrophenol. Gradual accumulation of the second fraction in the regional lymph nodes could definitely be excluded. It was noted that no hypersensitivity arose and essentially no depot of radioactivity existed between 12 hr and 4 days when DNCB was injected in a dose of 0.25 μg, owing to its ready escape from the ear; but 20 times as much DNCB caused sensitization and provided about the same fixed depot as 0.25 μg of picryl chloride.

After delayed hypersensitivity had been established, traces of radioactivity were still measurable at the site. This third fraction, probably representing a different coupling product, escaped at a very low rate and was traceable up through several weeks.

No demonstrable radioactivity could be detected in thymus, spleen, and mesenteric nodes when examined at short intervals between $\frac{1}{2}$ min and 17 days.

In analogy with findings on transplantation "immunity" and with studies reported in the following paper, the induction of delayed hypersensitivity can be explained by encounters between lymphoid cells and the hapten complex which is found present in the local site for 4 days, in agreement with Medawar's concept of peripheral sensitization.

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