

## CONTACT SENSITIVITY IN MICE

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FOR MANY YEARS, on the basis of results from some direct experimentation, mice have been thought to develop delayed hypersensitivity poorly, if at all. On the contrary, larger laboratory animals and man have been found to develop it quite readily, and, consequently, almost to complete exclusion of the mouse, these have been the subjects for experiments on this type of allergy. Nearly all information on delayed hypersensitivity and on a particular form of it, contact sensitivity, has been obtained by the use either of human volunteers or of guinea pigs; necessarily, it is biased toward occurrences in these two subjects specifically. Important species differences in the characteristics and, perhaps, the mechanisms of the general phenomenon of delayed hypersensitivity are known to exist.<sup>1-4</sup> For example, pure delayed-type skin hypersensitivity is induced readily in man or guinea pigs, but it is not easily induced in rabbits without the complication of a simultaneously occurring immediate hypersensitivity.<sup>1</sup> Apparently, contact sensitivity to simple chemicals, while easily provoked in man and guinea pigs, has never been elicited in rabbits.<sup>4</sup> In passive transfer experiments, many more lymphoid cells from hypersensitized donors are needed to transfer delayed hypersensitivity in the lower animals than in man.<sup>2</sup> A "transfer factor" capable of transferring delayed hypersensitivity from one human being to another can be leached from human leukocytes, but, to date, discouragingly little success has been achieved in utilizing anything but living cells for passive transfers in animals.<sup>3</sup> Even when induced, passively transferred delayed hypersensitivity in guinea pigs or rabbits seems to last only for a few days, whereas in man it lasts for months or years.<sup>2</sup> Species disparity also is clear regarding the effects of hormones on homograft reactions, themselves a form of delayed hypersensitivity; the abilities of ACTH and of cortisone to prolong the life of transplanted tissue, presumably by depressing allergic reactions, vary from species to species and are nil in human beings.<sup>5</sup> If, then, for no other reason than to broaden general information on delayed hypersensitivity, experiments performed directly on this type of allergy in the mouse are worthy. However, they also can be justified because they provide models for experiments which have the advantages of using an animal species

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This work was supported by U. S. Department of Health, Education, and Welfare Grant E-2689, and by the New York Tuberculosis and Health Association, Inc., through its James Alexander Miller Fellowship award made to the senior author for the period 1959-1960.

Received for publication Jan. 31, 1961.

of small size and ready proliferation available in several different highly inbred strains, which has been utilized so successfully for analyzing the homograft reaction. Considering these factors, then, and encouraged by our previous success in devising ways to study delayed hypersensitivity to protein allergens in mice,<sup>5-8</sup> we attempted to elicit in these animals contact sensitivity to a simple chemical and, having succeeded,<sup>9</sup> have begun to examine and characterize this allergy. Herein we present details from some of our experiments.

#### MATERIALS AND METHODS

*Mice.*—Female CF-1 white mice, 4 to 6 weeks old, purchased from Carworth Farms (New York) were used, the individual groups averaging 10 animals each. These mice were maintained on water and Rockland Mouse Diet.

*Drugs.*—1-Chloro-2,4-dinitrobenzene (Eastman Organic Chemical 101\*) served as chemical allergen; it was dissolved for both sensitization and challenge in reagent grade acetone. Four different drugs were studied for effects on mouse contact dermatitis. One is the antihistamine tripeleennamine hydrochloride.† Two others have potent antiserotonin activity. One, called SA-87,‡<sup>10</sup> is N-p-chloro-benzylhydryl-N'-methyl-homo-piperazine dihydrochloride. In addition to its antiserotonin activity, it also is a potent antihistamine, has mild anti-acetylcholine activity, bronchodilator activity, antifibrillatory and coronary dilator activity, and can exert a certain degree of central stimulation.<sup>10</sup> The second antiserotonin, UML-491,§ is 1-methyl-D-lysergie acid butanolamide tartrate.<sup>11</sup> Aside from its antiserotonin activity, it is said to enhance the effects of antihistaminics, although apparently not itself having antihistaminic activity. The fourth drug used in the present studies has many activities but among these, and of foremost interest to our experiments, is its proved depressing effect on delayed hypersensitivity skin reactions in mice.<sup>8</sup> This drug is cortisone acetate, marketed as the saline suspension, Cortone acetate.||

*Sensitization and Challenge.*—As a consequence of preliminary experimentation, we have adopted the following methods for sensitizing and challenging mice in our contact sensitivity experiments.

Inconsistent results developed from simply allowing a small drop of 1-chloro-2,4-dinitrobenzene (CDNB) solution to fall upon the shaved skin of a mouse, either for sensitization or challenge, because such a drop will spread in an irregular and inconsistent pattern over skin areas of differing size from animal to animal. Consequently, we employ circular Plexiglas templates, such as shown in Fig. 1, both to sensitize and to challenge mice. When one of these is held firmly against shaved skin, as shown in Fig. 2, it limits the spread of CDNB solution to the area covered by its orifice, so that a sharply defined spot of skin, which is the same from mouse to mouse, is exposed to chemical.

\*Distillation Products Industries, Rochester, New York.

†Pyribenzamine, Ciba Pharmaceutical Products, Summit, New Jersey.

‡Supplied by Abbott Laboratories, Chicago, Illinois.

§Donated by Sandoz Pharmaceuticals Division, Hanover, New Jersey.

||Merck Sharp & Dohme, Philadelphia, Pennsylvania.

Hypersensitization proceeds as follows. Forty-eight hours before a mouse is treated with CDNB, a large area of its flank skin is clipped and then dry-shaved with a razor blade. This pre-shaving is desirable to allow the skin to recover from the irritation of shaving, since compounding this irritation with those of acetone swabbing and application of CDNB results in sealing of the skin. The mouse to be treated after pre-shaving is anesthetized lightly with ether vapor and taped to a flat surface to make the skin taut on its shaved flank<sup>8</sup> (Fig. 2). This shaved area is wiped once gently with a small acetone-soaked cotton swab to remove superficial body lipids and then air-dried. Next, the template is positioned in the center of the shaved area and held there firmly while three drops of 0.125 per cent acetone solution of CDNB are delivered into its orifice from a 20-gauge needle (Fig. 2); this volume is approximately

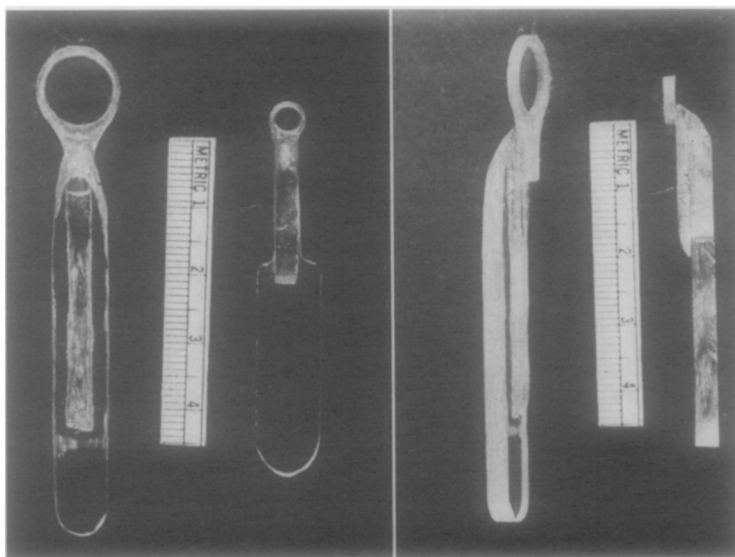


Fig. 1.—Top and side views of Plexiglas templates used to hypersensitize and to skin test mice with acetone solutions of CDNB. The template with the larger loop, seven times the area of the smaller, is employed for hypersensitization. Both types of loop are offset from handles, as shown in the side view, to prevent acetone solution of CDNB from running out from under them along the handles by allowing application of pressure with them to a more restricted portion of mouse skin.

0.03 ml. Immediately afterward, a gentle jet of air is directed from an air hose upon the area to evaporate the acetone while the template still is held in place. Finally, the template is withdrawn, and the mouse is untaped and returned to its cage.

This sensitization procedure is meant to apply a maximum quantity of allergen over enough skin area so that it will cause very little damage to the skin, in comparison with the damage which results when a smaller area is utilized, and so that the animals will be more effectively sensitized. No particular portion of the treated skin will be exposed to more allergen than is used for the challenge (skin-test) dose of the same concentration, which itself was selected as the largest one which is tolerated by the skin without causing frequent false-positive reactions. Originally, we employed the same small

template for both challenge and sensitization, but, when we found that hypersensitivity occasionally was induced only poorly and somewhat variably after such treatment, we began using a larger template (Fig. 1) which exposes approximately 6.6 times as much skin area and consequently can be used with a correspondingly larger volume of CDNB solution.

Our skin-testing procedure is the same as that which we used for sensitization except that, as we have intimated, we employ the smaller template shown in Fig. 1. The orifice of this template exposes a circle of skin 4 mm. in diameter upon which 0.004 ml. of 0.125 per cent CDNB acetone solution is delivered as a single drop from a 30-gauge needle. Since the drop must be applied to unirritated healthy skin to avoid nonspecific inflammation, as in sensitization, we pre-shave mice to be tested 48 hours before testing. The shaving must be close, for hair bristles can impede the function of the template in limiting spread of the applied drop to a uniform skin area from mouse to mouse.

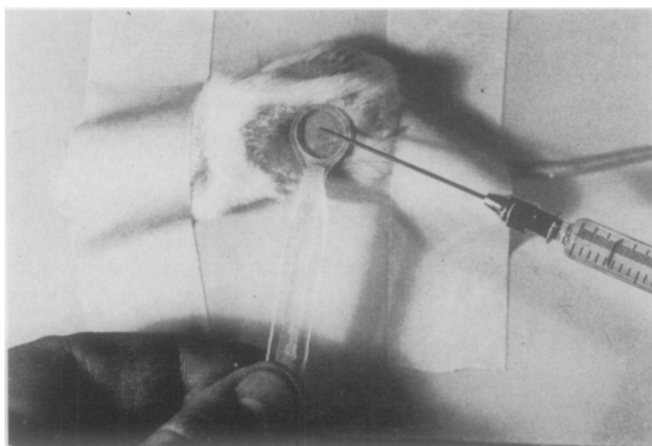


Fig. 2.—Method of applying CDNB solution to the skin of an immobilized mouse through the loop of a template.

mouse. Shaving is best accomplished by first removing most hair with electric clippers and then using a sharp razor blade against the direction of hair growth and on taut skin.

*Reaction Readings.*—Contact dermatitis reactions in mice almost never are accompanied by obvious erythema. Hence, our primary readings are measurements of the palpable diameter of skin inflammation (edema and/or induration) made along its longest axis. We use vernier calipers both for this measurement and for a second one of the thickness of skin which the reader picks up gently at the reaction center. The magnitude of skin reactions, judged from this second measurement, does not consistently parallel that assessed by diameter measurements, for mice can develop broad, flat reactions, or narrow, thick (deep) ones. Consequently, both measurements are worth making because of their somewhat different meanings. Our measurements of skin reaction diameter are made to the nearest millimeter,  $\pm 1$  mm., and our thickness measurements to the nearest tenth of a millimeter,  $\pm 0.3$  mm. Normal mouse

skin feels uniformly flat and, of course, provides no diameter reading. However, a measurement of thickness can be made. This ranges between 0.8 mm. in young mice to 1.4 mm. in mice 3 or 4 months old. Occasionally, undue irritation of the skin from shaving or small injuries from one cause or another will cause thickening of the skin which usually is temporary but occasionally is permanent, and which can lead to misinterpretation of results unless it is recognized.

In addition to measurements of reaction diameter and thickness, we also record the presence or absence of skin necrosis. This third reading more often correlates with the second than the first, in that it is most likely to accompany deep reactions rather than flat ones. Usually, this necrosis is superficial. The affected skin becomes stiff, turns brown, and eventually flakes off, generally without leaving an open sore (see illustration, reference 9, p. 132).

#### EXPERIMENTS AND RESULTS

*Development of the Skin Reaction.*—Mice which have been hypersensitized to CDNB and then are skin tested with it appear to develop both immediate and delayed types of dermal reactions, that is, reactions due to humoral and to cellular antibodies, respectively. Differentiating these two types of reaction in the mouse seems to us to be in some ways more difficult than comparable differentiation of immediate and delayed types of dermatitis induced by protein allergens.<sup>7, 8</sup> The skin reaction in mice with both types of hypersensitivity to a protein appears first as an edematous (soft) swelling, beginning to be distinguishable from the bleb of injection, and nonspecific swelling that this causes, within 1 to 2 hours after the skin test has been performed. This edematous swelling, due to reaction between antigen and humoral antibodies, reaches a maximum in approximately 3 hours, and then quickly recedes. Approximately 6 hours after intradermal antigen injection, affected skin begins to develop an indurated swelling which is apparently due to reaction between antigen and cellular antibodies. This inflammation broadens and deepens slowly, attaining a maximum diameter 8 to 12 hours after the skin test. Central necrosis begins to develop about this time, but its incidence in a given group of mice usually is greatest about 48 hours after skin testing. The immediate hypersensitivity phase of these reactions to protein allergens in mice often is severe enough to result in petechiae, and when necrosis develops it usually is deep enough to be visible as much as a week after intradermal injection of the antigen. Erythema is not seen.

The following experiment shows that although the over-all tempo of development of contact dermatitis reactions in mice hypersensitized to CDNB very closely resembles that of the skin reaction in mice hypersensitive to proteins, differences exist, perhaps reflecting contrasts in the way allergen is applied to the skin and in the depth and breadth of skin affected. For example, no clear temporal break occurs between the appearance of immediate (edematous) and delayed (indurated) reactions to CDNB in mice, these apparently

blending into one another. Petechiae never have been observed in hypersensitive mice tested with CDNB. Necrosis is more superficial in the contact dermatitis reaction than in tuberculin or similar protein-induced reactions.

Mice employed for this experiment were hypersensitized to CDNB with two exposures, through the large Plexiglas template, given a week apart and on the same side of the animal, so that tests could be applied to an area of skin not previously exposed to chemical. Skin tests were performed on 10 of these mice as well as on 10 unsensitized control mice 14 days after the second sensitizing treatment, and dermal reactions were read and averaged at various intervals as shown in Fig. 3.

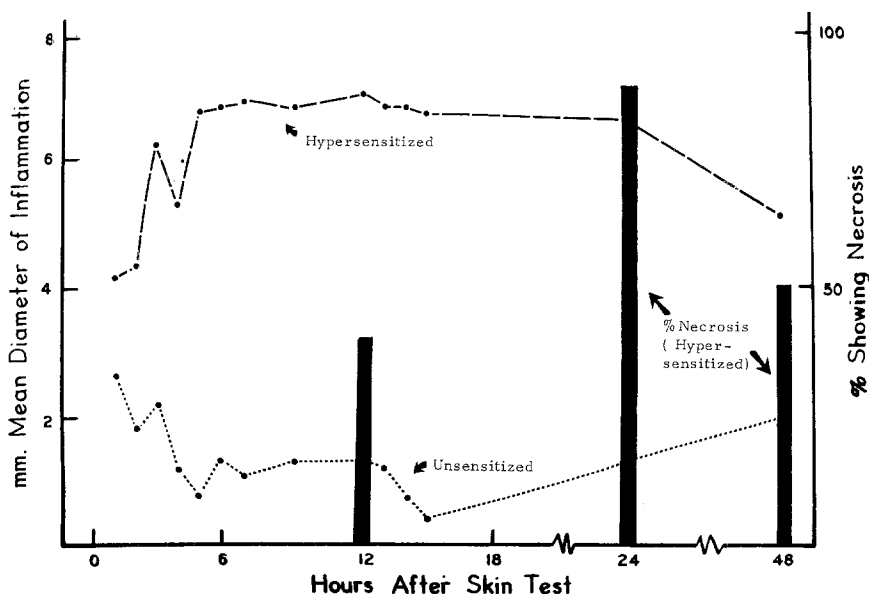


Fig. 3.—Tempo of development of the CDNB-induced contact dermatitis reaction in mice. Hypersensitized and normal (unsensitized) mice were skin tested at the same time, and then their skins were observed for reactions at intervals indicated in the graph. Bars in this graph indicate percentage of hypersensitized mice showing skin necrosis 12, 24, and 48 hours after skin testing.

The results in this figure show that some specific reaction to CDNB can be distinguished in hypersensitized mice as early as 1 hour after skin testing, and that this immediate hypersensitivity reaction rises abruptly and probably peaks about 3 hours after testing. This reaction is predominantly edematous but also seems to be accompanied by some induration. The delayed hypersensitivity phase of dermatitis observed in this experiment arose more quickly than we expected from our previous experiments with mouse delayed hypersensitivity to proteins, and thereby seems to have obscured the phase of diminished reaction which we commonly regard in our experiments with protein antigen to signify the ending of immediate and beginning of delayed reaction. Thus, although a small dip in mean reaction diameter is evident at the 4-hour reading, numerically this is quite small and is followed rapidly by the initial stages of the delayed hypersensitivity reaction; the edema of the first reaction blends

into the induration of the second. This second reaction reaches its maximum within 2 or 3 more hours, at approximately 6 hours after skin testing. Thereafter, induration diameter decreases very little until after the 24-hour reading; by the 48-hour reading it is considerably smaller and, although not shown in Fig. 3, is likely to have become undetectable 3 days after testing. As the bar graph portion of Fig. 3 shows, whereas induration peaks in less than 12 hours after testing, the incidence of necrosis reaches a maximum later, by the 24-hour reading. That it is so superficial is documented by the abrupt fall in this incidence of detectable necrosis within the following 24 hours.

For the delayed hypersensitivity aspects of our research on mouse contact sensitivity, we most often employ the 24-hour reading. To indicate how individual mice in a group react at this interval after skin testing, we supply, in Table I, readings made for both hypersensitized and normal control mice at 24 hours for the experiment shown in Fig. 3. These are typical readings; thus, we do not find it unusual to encounter 1 or 2 mice in a group of 10 unsensitized control animals that show a reaction indistinguishable from those seen in allergic mice, but, on the other hand, seldom do more than 1 or 2 unsensitized animals react in this manner. Reaction diameter among individual hypersensitive mice varies less in these CDNB experiments than it has in our experiments with protein allergens, especially regarding reaction maximums. No reaction in Table I exceeded 7 mm. in diameter and, among the 10 allergic mice employed, 8 had exactly this reading. We attribute this uniformity to use of a template for applying the skin test; this not only increases the reproducibility of our testing procedure but, as we shall explain more fully below, probably sets an upper limit on potential reaction diameter.

TABLE I. CONTACT DERMATITIS REACTIONS IN INDIVIDUAL MICE 24 HR. AFTER EXPOSURE TO THE ALLERGEN CDNB

| HYPERSENSITIZED   |                    |          | NORMAL            |                    |          |
|-------------------|--------------------|----------|-------------------|--------------------|----------|
| DIAMETER<br>(MM.) | THICKNESS<br>(MM.) | NECROSIS | DIAMETER<br>(MM.) | THICKNESS<br>(MM.) | NECROSIS |
| 7                 | 1.8                | +        | —                 | n*                 | —        |
| 7                 | 1.7                | +        | 7                 | 1.7                | —        |
| 5                 | 1.3                | ±        | —                 | n                  | —        |
| 7                 | 3.3                | +        | —                 | n                  | —        |
| 6                 | 1.5                | +        | —                 | n                  | —        |
| 7                 | 1.6                | +        | —                 | n                  | —        |
| 7                 | 2.4                | +        | —                 | n                  | —        |
| 7                 | 3.0                | +        | —                 | n                  | —        |
| 7                 | 2.2                | +        | —                 | n                  | —        |
| 7                 | 2.4                | +        | 6                 | 2.0                | +        |

\*Normal skin thickness is indicated by the letter "n"; in mice of the age employed in this experiment, normal skin thickness ranges between 0.9 and 1.2 mm.

*Drug Experiments.*—In previous experiments with delayed and immediate hypersensitivity to tuberculo-protein, we have shown that reactions caused by these two types of allergy can be differentiated in mice, among other ways, by their respective sensitivities to depression by the drugs cortisone and tripele-namine. Thus, while cortisone depresses both types of reaction, tripele-namine, an antihistaminic, depresses only that caused by immediate hypersensitivity.

Although this differentiation by drug action is largely empirical, at present, our experience in other unpublished mouse experiments has been that it is reliable.

Serotonin (5-hydroxytryptamine) probably plays an important causative role in the immediate hypersensitivity reactions in the mouse, apparently being more of a force in this than histamine.<sup>12, 13</sup> Its connection, if any, with the mechanisms of delayed hypersensitivity skin reactions is unknown, not only for mice but also for other species of animals. Therefore, the following experiments are meant not only to test the effects of four drugs against mouse contact dermatitis, but also to find which is effective in obtaining basic information on the mechanisms of both immediate and delayed hypersensitivity reactions to drugs in mice.

Tables II and III summarize data from five experiments of this sort. Test mice for these experiments were hypersensitized to CDNB in essentially the same way as described for the preceding experiment, and all were verified allergic by preliminary skin testing. The drugs cortisone and tripeleennamine were administered according to the protocol used previously with success in mouse experiments on tuberculin allergy.<sup>8</sup> That is, 0.14 mg. of tripeleennamine, in 0.1 ml. physiologic phosphate buffer at pH 7.4, was injected subcutaneously 30 minutes before skin testing and then 16, 24, and 40 hours later, while 1 mg. of cortisone was administered intraperitoneally in 0.2 ml. of buffer 30 minutes before skin testing and once again immediately after the 24-hour reading. During experiments using these two drugs, the pharmacologic activity of batches employed was verified in mice with tuberculin allergy and skin tested with tuberculinoprotein. For Experiment I of Table II, 10 animals from each group of two cages of hypersensitive test mice were subdivided into groups of 5 mice each, 1 treated and the other not. However, for Experiment II and in the experiments of Table III, each group of animals consisted of 10 individuals.

TABLE II. EFFECTS OF TRIPELENNAMINE AND OF CORTISONE ON THE CONTACT DERMATITIS REACTION IN MICE TO CDNB

| EXPERIMENT | GROUP            | DRUG TREATMENT  | MEAN REACTION DIAMETER (MM.) |        |
|------------|------------------|-----------------|------------------------------|--------|
|            |                  |                 | 2 HR.                        | 24 HR. |
| I          | Hypersensitive—1 | Cortisone       | 5.0                          | 1.4    |
|            | Hypersensitive—1 | None            | 7.4                          | 8.8    |
|            | Hypersensitive—2 | Tripeleennamine | 6.2                          | 11.6   |
|            | Hypersensitive—2 | None            | 6.8                          | 11.4   |
|            | Unsensitized     | None            | 1.6                          | 0.5    |
| II         | Hypersensitive—3 | Cortisone       | 5.1                          | 4.1    |
|            | Hypersensitive—3 | Tripeleennamine | 3.9                          | 6.4    |
|            | Hypersensitive—3 | None            | 4.9                          | 6.9    |
|            | Unsensitized     | None            | 2.1                          | 0.4    |

We used the drugs SA-97 and UML-491 (the antiserotonins) in the largest quantities compatible with the physical well-being of treated mice. By coincidence, the dose for each was determined, by preliminary experimentation, to be 1 mg. dissolved in 0.1 ml. buffer and injected intraperitoneally. Although

this dose was applied in Experiments I and II and, for SA-97, also in Experiment III, our original supply of UML-491 was exhausted by the first two experiments and a second batch was utilized for the third. The first injection of this batch proved it to be too toxic (inducing profound lethargy and extreme hypothermia in all mice injected) and so the second and third injections of UML-491 for this experiment consisted each of 0.5 mg. instead of 1 mg. Injections of these two drugs were given 30 minutes before skin testing, 1 hour before the 24-hour reading, and 1 hour before the 48-hour reading.

TABLE III. EFFECTS OF UML-491, OF SA-97, AND OF TRIPELENNAMINE ON THE CONTACT DERMATITIS REACTION IN MICE TO CDNB

| EXPERIMENT | GROUP          | DRUG TREATMENT  | MEAN REACTION DIAMETER (MM.) |        |
|------------|----------------|-----------------|------------------------------|--------|
|            |                |                 | 2 HR.                        | 24 HR. |
| I          | Hypersensitive | UML-491         | 1.3                          | 3.5    |
|            | Hypersensitive | SA-97           | 0.8                          | 0.8    |
|            | Hypersensitive | None            | 2.8                          | 4.7    |
|            | Unsensitized   | None            | 1.3                          | 0.9    |
| II         | Hypersensitive | UML-491         | 0                            | 5.1    |
|            | Hypersensitive | SA-97           | 0                            | 2.4    |
|            | Hypersensitive | None            | 5.2                          | 7.1    |
|            | Unsensitized   | None            | 1.6                          | 0      |
| III        | Hypersensitive | Tripeleennamine | 3.3                          | 6.0    |
|            | Hypersensitive | UML-491         | 0                            | 6.2    |
|            | Hypersensitive | SA-97           | 0                            | 3.2    |
|            | Hypersensitive | None            | 4.3                          | 6.5    |
|            | Unsensitized   | None            | 1.9                          | 1.3    |

The data in Tables II and III show that immediate and delayed hypersensitivity reactions of mouse skin to CDNB can be depressed by one or another of the drugs employed. However, the drug effects obtained with the contact dermatitis reactions have not all proved to be predictable on the basis of our previous work with protein allergens in mice. For example, the depressing effect of tripeleennamine upon protein allergen immediate hypersensitivity skin reactions is weak but definite.<sup>8</sup> By contrast, this drug appears to be impotent against the immediate phase of contact dermatitis. Although cortisone readily depresses both immediate and delayed skin reactions in mice to tuberculo-protein,<sup>8</sup> unexpectedly, but like tripeleennamine, it, also, appears to be helpless against immediate hypersensitivity reactions to CDNB. However, it is moderately effective against the delayed reactions.

Particularly interesting is the clear differentiation between immediate and delayed phases of contact dermatitis achieved by UML-491 treatment. Since the pharmacologic activity of this drug is mostly that of an antiserotonin and since its only other known activity (antihistamine potentiator) probably has little effect in these experiments, if the results with tripeleennamine are considered, this sharp differentiation suggests (1) that serotonin or some derivative of it is the prime agent in the development of immediate hypersensitivity skin reactions to a simple chemical in the mouse, and (2) that serotonin is not an essential factor in the genesis of reactions of delayed hypersensitivity dermatitis in the mouse to CDNB.

Because SA-97 also is a potent antiserotonin, one might expect from the effects just discussed of UML-491 that this drug, too, would inhibit the immediate phase of CDNB dermatitis; and, indeed, it does. Nevertheless, none of its known properties suggests that it should have a marked inhibiting effect on the delayed hypersensitivity phase of this dermatitis. Yet its effect on this is equal or superior to that of cortisone.

However these results may be interpreted after more is learned about the effects of these drugs on contact dermatitis, they at least suggest a convenient, practical method for differentiating immediate and delayed aspects of contact dermatitis in mice based on the apparently highly specific effect of UML-491.

*Passive Transfer Experiments.*—Despite the fact that immediate and delayed hypersensitivities to a given allergen usually can be distinguished from each other by several methods, such as by the use of appropriate inhibiting drugs as shown above, the most trustworthy criterion presently available for this purpose depends upon the idea that each is due to a different type of antibody, one commonly found circulating freely in the plasma and the other apparently attached to lymphoid cells, blood leukocytes, and other types of cells related to the reticuloendothelial system. Thus, if a hypersensitized animal simultaneously has both types of allergy, each can be separated from the other and identified by appropriate passive transfer experiments. Serum obtained from the allergic animal and injected into a normal one temporarily confers upon the latter mouse immediate but not delayed hypersensitivity; lymphoid cells transferred from the allergic to a normal animal give the recipient a temporary delayed hypersensitivity but not an immediate one. We have recently shown that this classic method of differentiation, so useful in man and in the larger laboratory animals, can also be employed in mice.<sup>7, 8</sup> Consequently, we now have used it to analyze mouse contact sensitivity to CDNB.

Donors, verified hypersensitive by skin tests usually performed a week preceding their use, were anesthetized lightly with ether vapor and bled out from severed armpit vessels. Their bloods were pooled and used as a source of serum for passive transfer. Immediately after each mouse was exsanguinated, its thoracic cavity was breached, and the thymus gland was removed and placed with others, similarly obtained, in Tyrode solution kept at 37° C. When enough donors had been sacrificed to provide the required quantity of thymus tissue, the solution in which these glands had been pooled was poured off and then replaced with fresh solution at the same temperature, and these were ground briefly and gently in a Ten Broeck tissue grinder, centrifuged at 500 g, resuspended in an appropriate volume of warm Tyrode solution, and injected intraperitoneally into recipient mice, as detailed in previous publications.<sup>7, 8</sup> For successful transfer in experiments of this type performed so far, we have found it essential to use a quantity of thymus cells such as is obtained from at least twice the number of donors as there are recipients. In the first two of the following three experiments, each recipient mouse received the equivalent of 0.006 ml. of packed thymus cell homogenate (approximately  $15 \times 10^6$  cells) within 1½ hr. after these cells were obtained from donors. Injected suspensions

consisted mostly of individually suspended thymocytes, 90 per cent of which were alive, according to an eosin viability stain<sup>14</sup> and a modification of this in which we used Evans blue dye. Recipients of the third experiment each received the equivalent of 0.009 ml. of packed cells.

In each experiment, the thymus cell suspension was injected 48 hours before recipients and appropriate controls were skin tested. On the other hand, donor serum was injected, also intraperitoneally, 24 hours before skin testing. In the two experiments with regard to passive transfer of delayed hypersensitivity

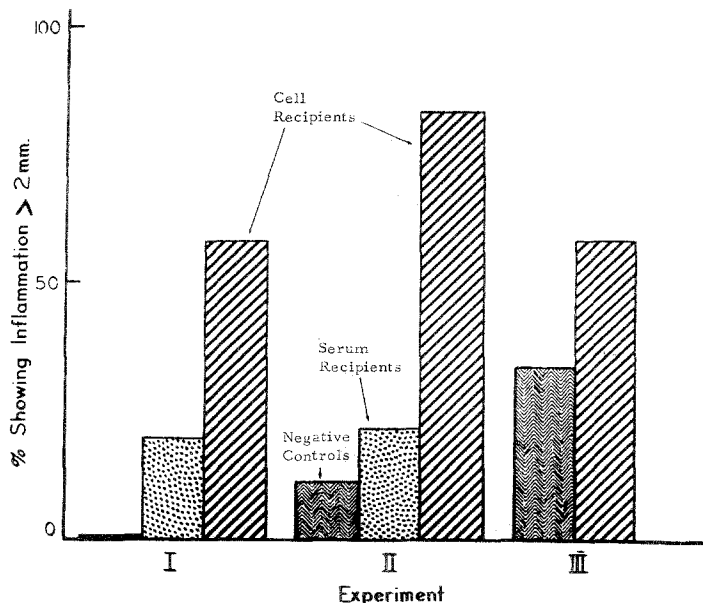


Fig. 4.—Bars showing proportion of mice in three different passive transfer experiments developing skin reactions exceeding 2 mm. in diameter 24 hours after skin test with CDNB. The first two experiments include groups of normal mice and of recipients of thymocyte cells or serum obtained from hypersensitive donors; the third experiment has only cell recipient and normal control groups.

in which it was used, 0.4 ml. of serum from hypersensitized donors was injected into each recipient; that is, its volume equaled nearly half the natural supply of serum in a mouse.

The 24-hour readings from these three passive transfer experiments are presented in Fig. 4 in terms of the percentage of mice in each group showing a skin reaction diameter exceeding 2 mm. in diameter, that is, developing a reaction. Evidently, the hypersensitivity to CDNB responsible for contact dermatitis in mice measured 24 hours after performance of a skin test can be passively transferred with thymocytes and therefore is of delayed (cellular antibody) type. However, the results also suggest that although such transfer succeeded by the methods employed, it was not very efficient. Thus, the mean diameter of reactions in recipients was at best scarcely half that commonly seen in actively sensitized mice. Moreover, only slightly more than half the recipients were regularly rendered hypersensitive. The presumed identification of non-humoral antibodies as being responsible for the delayed phase of mouse

contact sensitivity permissible on the basis of these experiments is corroborated by our failure, as Fig. 4 shows, to achieve significant passive transfer with massive quantities of donor serum.

The drug studies, particularly those in which UML-491 was employed, have indicated that mouse contact dermatitis has two phases effected by different mechanisms and which might be equivalent to classic immediate and delayed types of hypersensitivity. The passive transfer experiments just described prove the existence of delayed type; the presence of immediate type due to humoral antibodies also has been actively detected by similar passive transfer experimentation. Thus, recipients of serum obtained from mice hypersensitive to CDNB show a rapidly developing and then receding hypersensitivity skin reaction to CDNB. For example, a group of mice, each of which received intravenously 0.25 ml. of donor serum and was then skin tested with this chemical 6 hours later, developed a mean reaction diameter of 4.3 mm. within 2 hours of the test, while the comparable measurement in normal control animals was 1.2 mm. Eight out of 10 serum recipients reacted to the skin test, while only 2 out of 10 controls did. While the skin reactions of passively transferred delayed hypersensitivity are strongly evident 12 hours after skin test, reactions due to the allergy passively transferred with hypersensitive donor serum had entirely disappeared by this time, both recipients and normal control animals showing no skin reactions.

*Course of Hypersensitization.*—The chemical CDNB is one of the most potent allergens used in immunologic research.<sup>4</sup> A single drop of its solution properly applied to the skin of a susceptible species of animal or man usually suffices to hypersensitize that individual. The following experiments show this to be true also of mice.

For hypersensitization in the following experiments we employed our 4 mm. diameter template rather than the larger one which we use currently. We utilized 0.5 per cent CDNB solution in acetone for the first treatment in both experiments but, because subsequent treatments doubled also as skin tests and because this original dose caused measurable skin reactions too frequently in normal mice, we began employing a 0.125 per cent solution thereafter.

Four test groups of 10 mice each were used for the first experiment. All 4 were exposed to 0.004 ml. of 0.5 per cent CDNB solution at the same time at the beginning of the experiment. The first group was skin tested 1 week later, the second at 2 weeks, the third at 3 weeks, and the fourth group 4 weeks later. All were tested once again 6 weeks after the initial sensitizing treatment. Along with each test group, a group of 10 unsensitized (normal) control mice was skin tested. Readings were performed after each test in the usual manner. Fig. 5 records only 24-hour induration diameter measurements since, as we have shown above, these reflect reliably the true delayed-type contact sensitivity reaction to CDNB in mice.

The results of this experiment demonstrate that a single exposure of a mouse to 20 mcg. of CDNB is sufficient to hypersensitize it to this chemical. Allergy after such exposure has not appeared (Fig. 5) as early as 1 week after treatment, but it begins to be detectable in an occasional mouse by the end of

the second week, after which it rises rapidly to reach a peak 3 weeks after the primary application of allergen. Of the group of mice tested 2 weeks after the first exposure, only 3 clearly showed hypersensitivity, suggesting that this was a critical time of transition for the experimental animals from the nonallergic state. The hypersensitivity induced by primary exposure to only 20 meg. of CDNB apparently is short lived for, as Fig. 5 indicates, there was a rapid decline in reactivity between the third and fourth weeks after sensitization.

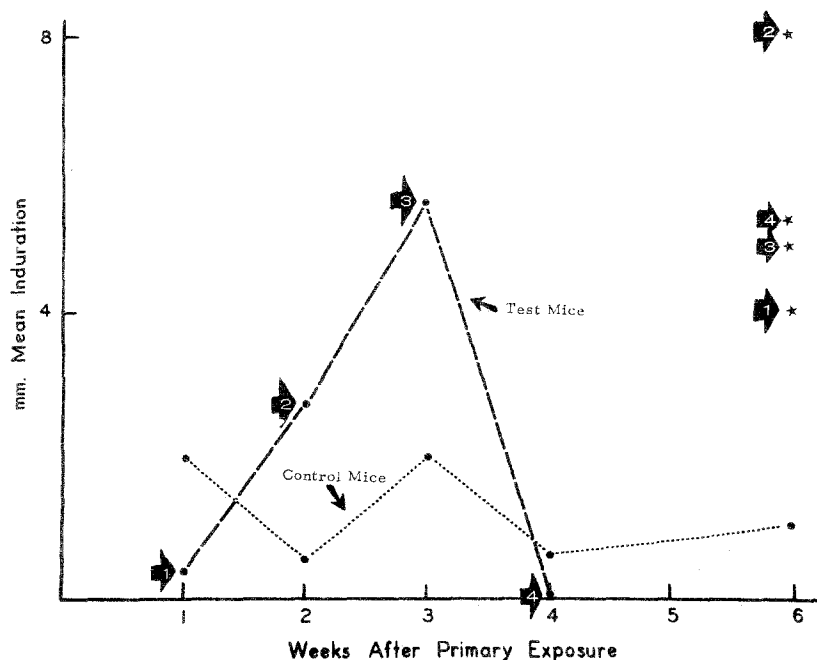


Fig. 5.—Development of contact sensitivity in four groups of mice tested individually at different intervals after single exposure to CDNB; skin tests read at 24 hours. Each test group is numbered so that it can be matched with a corresponding skin test reading obtained for it 6 weeks after primary exposure, and which indicates the level of hypersensitivity in this group which has developed as a result not only of the primary exposure but also of the boosting effect of its one previous skin test. Each time test mice were skin tested, a group of 10 normal control mice also was tested for comparison.

Skin tests performed 6 weeks after the beginning of the experiment, of course, measured hypersensitivity in each group after secondary stimulation, which was furnished by the preceding skin test. Since these tests were performed with only 5 meg. of CDNB, they suggest not only that an anamnestic response can be elicited readily in mice primarily sensitized to CDNB but also that this response is strongest when the secondary exposure is applied 2 weeks after the first, that is, when allergy is first becoming evident. The anamnestic response is most strikingly demonstrated, however, among the mice skin tested (i.e., given a second exposure) 4 weeks after the primary exposure. Their reactivity rises in only 2 weeks from a negative reaction to one as high as that of any other group, except the one tested 2 weeks after primary exposure, and to a value and incidence considerably higher than could be expected merely 2 weeks after primary exposure, particularly with a 5 meg. quantity of allergen.

This first experiment on the course of hypersensitization of mice to CDNB has established that a single exposure to this chemical, used as described, suffices to induce allergy and that double exposure can boost this hypersensitization. From these observations arise such questions as to whether multiple exposures can elicit more hypersensitivity than double exposure, if so what the maximum reactivity would be in mice to CDNB and when, during the course of an experiment probing these two questions, maximum hypersensitivity would be achieved. To obtain answers to these questions, we set up the following experiment.

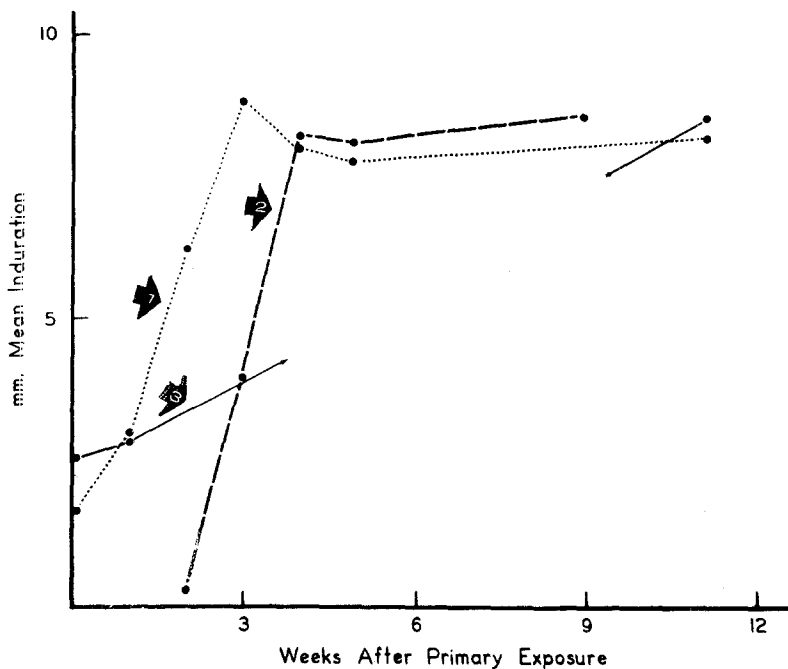


Fig. 6.—Development of contact sensitivity in three groups of mice exposed more than once to CDNB. Group 1, exposed repeatedly at weekly intervals, began to develop hypersensitivity (mean induration exceeding 3 mm., in this experiment) between the first and second weeks after first exposure, reached a maximum by the third week, and thereafter apparently was unaffected by additional skin tests with allergen. The results for Group 2 were similar except that hyperreactivity developed somewhat earlier, being detectable 1 week after first exposure. Group 3 mice were exposed only twice and then were not skin tested between the first and the eleventh weeks; therefore, progress in the development of hypersensitivity in this group between these two times is not known, as indicated by an incomplete line. This group is interesting principally because it developed hypersensitivity equivalent both in degree and longevity, within the confines of this experiment, to that in the other two groups with multiple exposure.

It consisted of seven groups of 10 mice each. Mice of the first group were skin tested on alternate sides of the abdomen weekly for 5 weeks, animals of the second group weekly for 4 weeks starting 1 week later than the first group so that its first skin test served as a negative control for the first group, those of the third group weekly for 3 weeks beginning 2 weeks after the start of the experiment, and so on through the sixth group, animals of which were tested only the last week. The seventh group was used primarily for negative

control skin tests for the first- and second-week groups at the beginning of the experiment. Only selected representative results from this experiment are presented in Fig. 6, since to present all of the results would be confusing and not provide any more essential information. Each plotted point for a curve in Fig. 6 represents a skin test and, also, an exposure to CDNB.

The principal findings of this experiment, which are illustrated in curves for three of its seven groups in Fig. 6, are as follows. Delayed hypersensitivity is not usually developed as early as 1 week after primary exposure (Curves 1 and 3) but occasionally can be perceived this early (Curve 2). While, as Fig. 5 of the preceding experiment indicates, it still is only marginal 2 weeks after single exposure to chemical, it has developed nearly to a maximum 2 weeks after primary exposure when this is followed within a week by secondary exposure. A boosting effect induced by secondary exposure is, then, clearly evident. Maximum hypersensitivity apparently is attained between 2 and 3 weeks after primary exposure, even when this is succeeded by the boosting exposure 1 week later. No higher reactivity is attained, within the period encompassed by this experiment, by exposing a mouse more than twice to CDNB (Curves 1 and 2 compared with Curve 3). In contrast to its rapid disappearance when induced by only a single exposure to allergen (Fig. 5), mouse contact sensitivity provoked by double successive exposure, separated by a 1-week interval, apparently is long lived (Curve 3). Thus, as readings from the last skin tests performed in this experiment show, the maximum reactivity attained 2 to 3 weeks after primary exposure has shown no tendency to decrease in nearly 3 months.

Our experience from numerous other experiments on CDNB sensitivity in mice has been that the maximum skin reaction diameter readings in this experiment which we have observed to range between 8 and 10 mm. are not exceeded. We believe that this maximum in diameter may be somewhat artificial, although none the less important, because we use a template to contain the spread of the test solution until it has been air dried onto the skin, and because lateral spread of the allergen after this is governed by the properties of mouse skin which are relatively constant from animal to animal. In contrast to this consistent maximum induration diameter which we have observed in mice sensitized by various procedures, we find that reaction depth, measured by thickness of the pinched-up skin, can be increased over what is seen in the present experiment by a more aggressive method of hypersensitization. For example, the greatest mean skin thickness attained at a 24-hour skin test reading by any group of mice in the experiment above was only 2.0 mm., significantly but not much above that of normal skin. On the other hand, in later experiments in which we have employed our large Plexiglas sensitizing template instead of the smaller one, and consequently have exposed mice to seven times the volume of CDNB solution over seven times more skin area than we are able to with the small template, the skin reaction has been obviously deeper, thickness averaging 3 mm., with necrosis more severe and persistent.

## DISCUSSION

Delayed hypersensitivity is presumed to be primarily responsible in mice, as well as in other species of animals, for rejection of homografts, that is, for the homograft reaction. Until recently no direct experiments on delayed hypersensitivity in mice have been performed and, indeed, by some workers they have been thought impractical. Assuming that the homograft reaction is primarily a delayed hypersensitivity phenomenon and using it to study this type of allergy indirectly in mice, principally because they are so readily available in the highly inbred strains required for such experiments, has resulted in accumulation of much valuable theoretical and practical information. Nevertheless, the homograft reaction is so complex that many basic aspects of research on delayed hypersensitivity are impractical to study with it. One, for example, is the antigenic specificity of the allergy. The present experiments not only support our previous reports that delayed hypersensitivity can be studied directly in mice,<sup>5-8</sup> but they also suggest that problems such as specificity can be investigated by employing allergens as simple as 1-chloro-2,4-dinitro-benzene.

Contact sensitivity, of course, can be and has been studied in guinea pigs and man. What, then, is the value of studying it in still another species? We already have mentioned the outstanding experiments on tissue transplantation and on the homograft reaction which have been performed in mice. There is little prospect that any other species will replace the mouse in such investigation. Yet interpretation of all these experiments regarding the probable role of delayed hypersensitivity in tissue graft rejection or acceptance has had no direct information on the characteristics of delayed hypersensitivity in mice upon which to rest. The use of mice in delayed hypersensitivity research is justified, then, as an aid to this work. Conversely, the large store of knowledge which has been accumulated with mice on tissue transplantation and related problems is unmatched for any other species and presents a rich background upon which to depend for information needed to set up experiments on delayed hypersensitivity or to help interpret their results.

The "antibody" of delayed hypersensitivity has defied definition. Only in experiments employing human volunteers has moderate success been achieved in this endeavor.<sup>2, 3</sup> Merely to demonstrate its existence in laboratory animals usually requires heroic passive transfer experiments<sup>15</sup> with the sacrifice of many animals but acquisition of few results. Mice are cheap and can be kept and used in large numbers. Not only do they open the field of delayed hypersensitivity investigation to the modestly equipped laboratory, but they also permit an experimenter to readily use enough animals to be able to obtain results having statistical validity.

We have alluded to the known striking differences in delayed hypersensitivity phenomena which occasionally are seen between species. Although these differences may at first be confusing, their elucidation contributes to more complete understanding of delayed hypersensitivity and consequently has considerable theoretical appeal. Undoubtedly the mouse will have its own peculiarities in this vein as it has been found to have in connection with immediate

hypersensitivity phenomena. For example, our experiments already have demonstrated that its skin reactions to either protein or chemical allergens do not show the erythema characteristic of such reactions in the larger species and man.

On the whole, contact sensitivity can be studied in mice with the same techniques as are used in larger animals. Differences in technique usually arise because of differences in animal size. Since erythema does not develop in mice, reactions must be measured primarily in terms of induration diameter. Mouse skin is thin and delicate and reactions usually are much smaller than are seen in guinea pigs, for example. Consequently, care must be taken that the skin is closely shaved but nevertheless that it is not unduly injured during this procedure. Useful skin area is small. To employ this to best advantage templates should be utilized to apply both sensitizing and test doses of allergen solution. Lymph node tissue in the normal mouse is discouragingly scanty as a supply of cells for passive transfer experiments, but the thymus gland, which is quite large in mice 3 or 4 months old, is a convenient and plentiful source of immunologically competent cells.

Contact dermatitis reactions to CDNB in mice, according to our present experiments, seem to have both immediate and delayed hypersensitivity phases, just as we have noted to be true in mouse protein allergies.<sup>7, 8</sup> The delayed phase clearly is proved by its passive transfer to normal mice with thymocytes but not with serum, used in large volumes, secured from allergic mice. On the other hand, the immediate phase is equally certainly identified by passive transfer with serum but not thymocytes from allergic animals.

While the two phases of mouse contact sensitivity merge into each other too much, during development of the dermatitis following challenge of an allergic animal, to permit their certain differentiation physically as can be accomplished for protein allergies, they can be distinguished sharply by the use of the highly specific antiserotonin drug UML-491. This completely suppresses the immediate phase but does not affect the delayed phase. We have reported previously that both tripeleminamine (an antihistamine) and cortisone can suppress immediate hypersensitivity skin reactions in mice to tuberculo-protein, and that cortisone also can suppress the delayed hypersensitivity (tuberculin) reaction.<sup>8</sup> In contrast to these findings, neither of these two drugs was able to suppress the immediate phase of contact dermatitis to CDNB, although cortisone did inhibit the delayed phase. Another drug (SA-97) which has antiserotonin activity, but also has several other pharmacologic effects, inhibited both immediate and delayed aspects of the contact sensitivity reactions. These results indicate that although histamine is involved to some extent in the events of immediate hypersensitivity skin reaction to tuberculo-protein, it is not in this aspect of contact dermatitis. Indeed, since the highly specific antiserotonin UML-491 can completely depress the immediate humoral antibody-mediated portion of contact dermatitis, histamine-like substances probably are not involved in this phase; in all probability it owes its appearance

to the effects of serotonin released locally by the allergen-antibody reaction. Since UML-491 has no depressing effect on the delayed phase of contact dermatitis in mice, serotonin probably is not significantly involved in this reaction.

Regarding comparison of some of the more salient characteristics of contact sensitivity in the mouse with those in the guinea pig,<sup>4</sup> the laboratory animal most commonly employed at the present time for studying this phenomenon, we can make some generalizations. The delayed-type skin reaction appears and peaks somewhat more rapidly in the allergic mouse than it does in the comparable guinea pig. Hypersensitivity itself takes about the same time after exposure to allergen to develop in both species, and experiments still in progress indicate that once present in the mouse, after adequate sensitization, it lasts at least as long as it does in the guinea pig. Finally, the mouse reaction conspicuously lacks erythema, but otherwise it closely resembles grossly the reaction in guinea pigs. As more is learned about contact sensitivity in mice, our range of comparison can be enlarged.

#### SUMMARY

Experiments reported in this paper demonstrate that mice can be induced to develop simultaneously both immediate and delayed types of hypersensitivity to the low molecular weight chemical 1-chloro-2,4-dinitro-benzene simply by applying to them percutaneously a minute quantity of this chemical dissolved in acetone. Both types of allergy can be detected by similar application of a test quantity of the chemical in acetone, occasionally as early as 1 week after primary exposure to it. They have been distinguished from each other by passive transfer experiments and by using appropriate drugs to suppress their respective phases of the contact dermatitis reaction. Thus, the delayed type is passively transferred by thymocytes, obtained from hypersensitive mice, but not with serum; the immediate type can be transferred with serum. The anti-serotonin drug 1-methyl-D-lysergic acid butanolamide tartrate (UML-491) specifically inhibits the immediate phase but does not affect the delayed. Neither an antihistamine (tripelennamine hydrochloride) nor cortisone acetate inhibited the immediate phase, but cortisone suppressed the delayed phase.

The characteristics of contact dermatitis in mice, the course of development of contact sensitivity in these animals, and special methods needed to study both of these phenomena have been examined and are discussed.

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