STUDIES ON THE SENSITIZATION OF ANIMALS WITH SIMPLE CHEMICAL COMPOUNDS

XIII. SENSITIZATION OF GUINEA PIGS WITH PICRIC ACID*

BY HENRY C. MAGUIRE, JR. AND MERRILL W. CHASE

(From The Rockefeller University, New York 10021, and The Hahnemann Medical College and Hospital, Philadelphia, Pennsylvania 19102)

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A previous report has dealt with contact-type sensitization to picric acid (1). The clinical appearance of sensitivity to this weak allergen differs from the more usual laboratory sensitizations, such as with dinitrochlorobenzene, picryl chloride, substituted benzoyl chlorides, and the like, particularly in the first appearance of contact sensitivity to picric acid as a micropapular reaction at 24 hr, which slowly ascends in intensity over several days. In these early experiments, subsidiary agents were employed to effect sensitization, such as dermal irritation by cantharidin and often the use of Butesin picrate instead of picric acid itself (1).

In experiments reported herein, we have reinvestigated this type of sensitivity, extending to picric acid a special so-called “split-adjuvant” technique (2). By this means, guinea pigs can be sensitized to picric acid much more regularly and intensely. In its usual form, the technique consists of injecting into skin sites mycobacteria in paraffin oil and, later, allergen in saline. Subsequent contact tests made with allergen result in stepwise increases in the degree of hypersensitivity.

Our particular interest in this method of sensitization arose from experiments with dinitrochlorobenzene and picryl chloride, in which high contact-type sensitivity was established in the near-absence of circulating antibody. This situation contrasts sharply with the relatively high titers of antibody found when such chemical allergens are incorporated into Freund’s complete adjuvant and administered as a single injection. The split-adjuvant technique has also been compared with use of a type of Freund’s complete adjuvant for inducing sensitivity to picric acid.

In the course of the work with the split-adjuvant technique in the guinea pig, we successfully applied the method to a number of relatively weak sensitizers, in particular formaldehyde, quinine, and picric acid. We now report in detail experiments dealing with picric acid.

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Our results emphasize the variety encountered in the type of challenge reactions to simple chemical allergens, and add to the distinctly limited record of experience with weak allergens. In the following paper, several types of experiments are presented, designed to investigate the differences and the complex interrelationships between sensitivities to picric acid and to picryl chloride.

Materials and Methods

Animals.—Rockefeller albino strain guinea pigs (Moen-Chase) were progeny of a closed colony originating from animals especially bred for high susceptibility to sensitization with 2,4-dinitrochlorobenzene (DNCB) and so maintained (3, 4). Sewell Wright's Family XIII guinea pigs propagated by brother-sister matings were also used, as well as closed colony albinos of the Hartley strain originating in a hardy stock from Pine Bluff Arsenal, Pine Bluff, Ark.

Reagents.—Picric acid (PA) was purchased from Eastman (Eastman Organic Chemicals, Rochester, N.Y.) and was recrystallized from absolute alcohol. Picryl chloride (1 chloro-2,4,6-trinitrobenzene) designated PC1, was twice recrystallized as a nearly colorless product from a 2:1 mixture of absolute alcohol-benzene mixture with use of Norit-A (m.p. 83°C). These chemicals were stored at room temperature away from light. For some tests, twice recrystallized picryl chloride was freed of trace contamination with picric acid by passage in benzene through 100-mesh silicic acid (method of Roderick A. Barnes, personal communication). Redistilled, dry benzene (stored over Na2SO4) was used to form a slurry containing 80 g of silicic acid. A glass column (2.5 X 29 cm) with all-glass fittings was packed under air pressure (2.5 lbs./sq inch). 15 g of recrystallized picryl chloride in dry benzene was passed through the column at a rate of 4-5 drops/min. Colored picric acid was retarded on the column. The faster moving, essentially colorless fraction was taken and reduced by evaporation to one-fifth its volume. Crystallization was induced upon cooling by rubbing with a glass rod. The crystals, almost white, were dried on hardened filter paper. All work with benzene was carried out in a well-ventilated chemical hood well distant from direct light. Drying was carried out in relative darkness since picryl chloride in the wet state is light sensitive (5, 6).

Tubercle bacilli (Jamaica No. 22 strain obtained originally as a culture from Dr. Jules Freund) were killed by heat and dried (5). Medium weight paraffin oil was used (Saybolt Universal viscosity 175-180 sec at 100°F). The oil was autoclaved at 121°C for 45 min in 60-ml portions. The dry mycobacteria were ground under sterile conditions in a small mortar (45 mm diameter), and the paraffin oil was added dropwise during the grinding. Stock suspensions of 10 mg/ml were diluted appropriately by weight in the same sterile paraffin oil.

Sensitization Procedures.—The guinea pigs were usually sensitized in the manner described under Results, by the successive (and separate) administration of allergen and adjuvant to the same given skin sites, the so-called split-adjuvant technique (2). In different experiments of this type, allergen or adjuvant was given first.

In some experiments, 3.4 ml of an Aquaphor-paraffin oil-mycobacterial suspension containing 580 µg of Mycobacterium tuberculosi was blended as described in (7) with 2.4 ml of saline containing 24.2 or 4.8 mg of picric acid; the ganged syringes were loaded and thoroughly chilled before commencing the emulsification. The emulsion was stable for 36 hr at 37°C, when observation ended. Injection of 0.03 ml was made into each footpad (reference 7, p. 278) to deliver, in 0.12 ml, 12 µg of mycobacteria and 500 or 100 µg of picric acid.

1 Chase, M. W., and H. C. Maguire, Jr. Studies on the sensitization of animals with simple chemical compounds: cross-reactivities of picric acid and picryl chloride on sensitive and tolerant guinea pigs. Unpublished manuscript.

2 Abbreviations used in this paper: CFA, complete Freund's adjuvant; DNCB, 2,4-dinitrochlorobenzene; PA, picric acid.
For epicutaneous administration picric acid was made up at a 10% concentration in dibutyl phthalate, with subsequent dilutions in irritant-free olive oil or corn oil to the concentrations required by the particular experiment. Standard dilutions in the mixed solvents were stored in the dark at room temperature.

Contact Testing.—Challenge tests were made on sites on the flank prepared by close-clipping with an electric hair clipper bearing a readjusted No. 0000 blade (John Oster Mfg. Co., Milwaukee, Wis.). The rear feet of the guinea pigs were booted before challenge by a wrapping of commercial water-proof adhesive tape so as to prevent self-scratching of the test sites and scratches from clambering by cage mates. One drop of the test solution was let fall from a Pasteur pipette hand-held at an angle of about 45°. This drop represented approximately 0.02 ml and was atraumatically spread with the heat-polished tip of a glass rod so as to cover an area 2 cm in diameter. The reactions were read at 24, 48, 72 hr and at 1 wk. Sites were recorded as word descriptions (see legend of Table I), later translated into a score (e.g. 0, tr, ±, +, ++, ...). The scoring system, developed to describe contact challenge responses, deals basically with confluent reactions of differing intensities (5): +, faint pink and not elevated; ++, pale pink, usually slightly elevated; ++++, pale pink to pink, usually moderately elevated; +++++, pink and definitely thickened. Reactions of even greater intensity will be encountered in highly sensitized animals: ++++, bright pink, well thickened; ++++++, bright pink with central tissue destruction ("plaques" covering creamy cellular accumulations). Several degrees of reactivity are seen between "negative" and "one-plus" (+): tr (trace) or tr (strong trace), traces of dotted erythema; ± ("one-half"), either faint pink but patchy or uneven, or a confluent very faint pink; ± ("three-quarters"), faint pink but slightly mottled. With picric acid, the scoring system was modified slightly to accommodate to the special types of nonconfluent reactions seen with this compound. These modifications apply only to the following grades: +, a few faintly pink small papules; ±, a few coarse spots or streaks; +, many coarse pale pink patches; +str (one-plus, but strong), the same + but with patches distinctly elevated.

Biopsies.—Full-thickness skin biopsies were made on different days. The specimens were fixed in buffered formalin; sections were cut at approximately 7 μm and different sections stained with hematoxylin and eosin, Mallory's trichrome stain, and Alcian blue.

RESULTS

Sensitizing to Picric Acid by the Split-Adjuvant Technique

The split-adjuvant technique for securing high-contact sensitivity to chemical allergens is described in reference 2. In applying the method to picric acid, Rockefeller albino guinea pigs were injected intradermally with 0.05 ml of paraffin oil containing 2.5 μg of heat-killed tubercle bacilli in each of five sites along one flank. (One control group received saline intradermally.) All sites were indicated by straddling marks made with a skin marking pencil. Next day, each of these same sites were reinjected with 0.1 ml of saline containing 100 μg of picric acid. Contact tests were made with different concentrations of picric acid, usually on days 12, 19, 26, and 33, beginning usually with 5 or 1% picric acid in equal parts of dibutyl phthalate and olive oil or, more informatively, with two tests (5 and 1%). The reactions tabulated in Table I are illustrative. The daily readings are listed for tests T12 and T18, but only maximal readings are shown here for tests T26 and T33. It should be added that in a control group in which saline was injected instead of picric acid into sites of adjuvant preparation and the same series of picric acid contact tests was given, sensitization to picric acid did not result.

Hypersensitivity reactions to picric acid are those of a dermatitis. However, they are rather curiously different from reactions of guinea pigs similarly sensi-
tized to DNCB or PCI. With these compounds, reactions tend to develop rather uniformly over the site of contact, usually within 24 hr, and show well-circumscribed margins. In contrast, picric acid reactions develop very slowly, particularly at the initial testings. Thus by 24 hr there usually appear several isolated, mildly erythematous papules; during the next 2 days these papules expand, develop heightened color, and often become confluent (Fig. 1). During this time, the test area thickens, often in irregular fashion. Reactions of great intensity can develop; some show an erythematous zone extending well outside (6–12 mm) of the area of application, while others present angry purplish red reactions. In the strong reactors, a thick adherent micaceous scale forms over the test site, starting as early as at 3 days and not uniformly associated with prior erythema. Often this scaling persists for more than a week (Fig. 2), the site frequently healing with some degree of scarring (a permanently hairless area). In secondary testing, the reaction usually evolves more abruptly, leading to coarse scaling as early as at 2 days. It is indeed surprising that reactions which appear relatively mild at 24 hr may progress to severe inflammation and ultimately to scar. A scale of this adherent type is only rarely seen in comparable experiments with guinea pigs sensitized to and tested with DNCB or PCI.

Peculiarly, reactions to epicutaneous application of 1% concentration of picric acid are sometimes greater than to 5% solutions. Further, among simultaneous tests made on normal control animals with various concentrations (e.g., 5, 1, 0.2, and 0.06% solutions), paradoxically the weaker concentrations are more likely than the stronger to give minor irritation reactions, usually not discernible before 48 or 72 hr. Such irritative responses impose no barrier to the

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**TABLE I**

*Illustrative Picric Acid Contact Tests*

| Animal | Test No. | 1% | 0.06% | 0.00% | Maximal readings* | Maximal readings* |
|--------|----------|----|-------|-------|-----------------|-----------------|
| No.    | 24 hr | 48 hr | 72 hr | 24 hr | 48 hr | 72 hr | 0.0% | 0.06% | 0.00% |
| 45     | +     | ++++ | ++++ | ++str | ++++ | ++++ | (++++±) | (++++±) | +++ | + |
| 44     | +     | ++++ | ++str | ++     | ++++ | ++++ | (++++±) | (++++±) | +++ | + |
| 47     | +     | +±± | ++str | ++     | ++±± | ++±± | (++++±) | (++++±) | ++ | ++str |

Representative animals displaying differing degrees of initial sensitivity are shown. The actual readings, represented by plus symbols, were as follows for test T12 at 24 hr intervals. (a) No. 45: large diffuse faint pink patches; pale pink to pink and thickened; bright pink, very well thickened, crust; (b) No. 44: strong trace; faint pink to pale pink and lightly mottled; pale pink to pink and moderately thickened; (c) No. 47: negative; many coarse, faint to pale pink spots; pale pink and patchy, thickened.

* * Tests made on days 23 and 32 are listed only at times of maximal readings, either at 24 or at 48 hr (shown within parentheses) or at 72 hr (within square brackets).

† Zone of color outside and surrounding the site of application.
study of sensitivity but must be taken into account in evaluating readings at 72 hr.

As a rule, the peak of the reactions is attained more rapidly in the late chal-

Fig. 1. Four representative guinea pigs sensitized by the split-adjuvant technique are shown, 72 hr after the initial test T₁₂ made with 1 drop of 5% picric acid in dibutyl phthalate:olive oil (1:1). The reactions consisted of small dots on papules at 24 hr, increasing sharply by 48 hr. Discrete papules and discrete groups of confluent papules produce a mottling and an irregular margination of the reaction. The reaction shown in the upper left exhibits some erosion and crusting. Reproduced from Kodachrome photographs.

lenges. Thus at the fourth test the maximal reactions tend to occur by 24 or 48 hr, whereas the initial tests generally require at least 3 days to develop fully.

As with prior studies using DNCB in the split-adjuvant technique, one of the constant findings with picric acid was the stepwise increase in sensitivity incurred by making several successive contact tests (2). The increase in sensitivity represents an actual boosting effect of successive testings (Tables II, III); sensitivity does not simply ascend with time (Table II). In order to most clearly document the boosting effect, it is well to use, in the initial tests, concentrations
of allergen which do not result in maximal reactions. For instance, in Table III, group A, five of six animals clearly demonstrate increase of sensitivity with successive testing, while the higher sensitivity of animal No. 22 hinders post facto analysis.

It will be noted in Table III that animals sensitized to picric acid give positive contact tests with picryl chloride dissolved in olive oil. The cross reactions to picryl chloride at 24 hr were nearly fully developed, confluent, and not papular as in PA testing. The cross-reactivity between picryl chloride and picric acid is examined in the following paper. 1

**Sequence for Picric Acid and Adjuvant.**—Experience with dinitrochlorobenzene and with picryl chloride suggested that allergen could be given before as well as after adjuvant (2). This was tested for with picric acid (Table III).

Rockefeller guinea pigs were divided into two groups. One group was injected with mineral oil containing 2.5 μg of tubercle bacilli 1 day before these same skin sites were injected with picric acid. In the other group, skin sites were prepared with picric acid and 1 hr later the sites were injected with the adjuvant. (A lessened time interval has been indicated when allergen is injected first [2].) For undetermined reasons, the degree of sensitivity observed in the animals of Table III was considerably more intense than was our usual experience.

It is clear that picric acid hypersensitivity could be induced with either the allergen or the adjuvant delivered first into the skin sites. In this particular ex-
### TABLE II

**Stepwise Increase in Contact Sensitivity to Picric Acid (Anamnestic Effect)**

| Animals | $T_{0}$ | 9% | 1% | 0.2% | $T_{1/4}$ | $T_{42}$ | $T_{2}$ | $T_{34}$ |
|---------|---------|----|----|------|----------|--------|--------|--------|
| Group A₁ |
| 92      | ++      | ++++ | ++  | ++  | N.D.     | ++      | ++      | ++      |
| 77      | +str    | +w  | +m | +m  | N.D.     | +       | +       | +       |
| 68      | (±)     | ±w  | ⌂  | ⌂   | N.D.     | (±)     | (±)    | (±)   |
| 97      | ++      | +   | +  | +   |          |        |        |        |
| Group A₂ |
| 95      | [++]    | +   | +  | +   | N.D.     | ++      | (±str) | +      |
| 63      | ++      | ±w  | ⌂  | ⌂   | "        | +       | +       | +       |
| 82      | [++]    | ⌂  | ⌂  | ⌂   | "        | +       | +       | +       |
| 62      | [++]    | +str| +  | +   | "        | +       | +       | +       |
| Group B |
| 67      | No test |     |    |     |          |        |        |        |
| 54      | [++]    | +   | +  | +   |          |        |        |        |
| 53      | [++]    | +str| +  | +str|          |        |        |        |
| 55      | [++]    | ⌂  | ⌂  | ⌂   |          |        |        |        |
| 56      | [++]    | ⌂  | ⌂  | ⌂   |          |        |        |        |
| Toxicity controls |
| 38 TC   |     |     |    |     |          |        |        |        |
| 39 TC   |     |     |    |     |          |        |        |        |
| 88 TC   |     |     |    |     |          |        |        |        |

Guinea pigs were sensitized with the split-adjuvant technique (100 µg of PA into each of five sites prepared the previous day with 2.5 µg of heat-killed mycobacteria in paraffin oil). Animals of group A₁ and group A₂ were topically tested with picric acid as shown, whereas group B received only the final testing ($T_{34}$). Control animals (TC) were tested in parallel with groups A₁, A₂, and B.
TABLE III

Split-Adjuvant Techniques Used With Picric Acid

| Guinea Pig | Sensitization | Contact tests with picric acid | PCI contact |
|------------|---------------|-------------------------------|-------------|
|            |              | Day -1 | Day 0 | Day 0 + 2 hr | T\(_w\) 1% | T\(_w\) 0.2% | T\(_w\) 0.02% | T\(_w\) 0.00% |
|            |               |        |       |              | T\(_e\)% | T\(_e\)% | T\(_e\)% | T\(_e\)% |
|            |               |        |       |              | 0.8%   | 0.02% | 0.02% | 0.00% |
|            |               |        |       |              |        |        |        |        |
| Group A    |               |        |       |              |        |        |        |        |
| 22         | P.O. + Tbc*   | Nil    | PA    | (+++++±)     | (++++++) | (++++++) | (+++++)  | (+++++) |
| 12         | " + "        | "      | "     | (+++++)      | (+++++)  | (+++++)  | (+++++)  | (+++++)  |
| 13         | " + "        | "      | "     | (+++++)      | (+++++)  | (+++++)  | (+++++)  | (+++++)  |
| 10         | " + "        | "      | "     | (+±)         | (+++++)  | (+++++)  | (+++++)  | (+++++)  |
| 11         | " + "        | "      | "     | (+±)         | (+++++)  | (+++++)  | (+++++)  | (+++++)  |
| 25         | " + "        | "      | "     | (+±)         | (+++++)  | (+++++)  | (+++++)  | (+++++)  |

Group B

|            |               |        |       |              | P.O. + Tbc | (++++)   | (++++)   | (++++)   |
| 144        | Nil           | PA     | P.O.   | (++++)       | (+±±)      | (+±±)    | (+±±)    | (+±±)    |
| 46         | "            | "      | " + "  | (++++)       | (+±±)      | (+±±)    | (+±±)    | (+±±)    |
| 43         | "            | "      | " + "  | (++++)       | (+±±)      | (+±±)    | (+±±)    | (+±±)    |
| 147        | "            | "      | " + "  | (++++)       | (+±±)      | (+±±)    | (+±±)    | (+±±)    |
| 48         | "            | "      | " + "  | (++++)       | (+±±)      | (+±±)    | (+±±)    | (+±±)    |
| 49         | "            | "      | " + "  | (++++)       | (+±±)      | (+±±)    | (+±±)    | (+±±)    |

Animals of group A received five intradermal injections of 2.5 µg of mycobacteria in 0.05 ml of paraffin oil; 1 day later, 100 µg of picric acid in 0.1 ml of saline was injected into each mycobacterial site. Animals of group B received the same treatment but reversed in order and with the period between injections reduced to 1 hr. A single contact test was made on day 23 with picryl chloride in olive oil to determine cross-reactivity.

* P.O. + Tbc, paraffin oil containing killed tubercle bacilli.
periment, adjuvant preparation of the skin (group A) appears to have been more efficient.

Variation in Amounts of Tubercle Bacilli and Picric Acid.—

The routine first adopted successfully (2.5 μg of mycobacteria and 100 μg of picric acid into each of five sites) was varied. Rockefeller guinea pigs were divided into six groups of six guinea pigs each. The sensitization schedule used consisted of injecting allergen (100, 25, or 2.5 μg) first, in each of five sites on the flank, followed by adjuvant (2.5 or 1.0 μg) into these same sites 1½ hr later. All guinea pigs were challenged with picric acid on days 12 (1%), 18 (1%, 0.3% and 0.06%), and 32 (0.06% and 0.02%).

Typical reactivity to picric acid developed in all groups. It was found that the amount of picric acid could be varied widely, with about equal results, provided the dosage of tubercle bacilli was 2.5 μg. When the mycobacteria were reduced to 1.0 μg, both 100 and 25 μg of picric acid were effective in sensitizing, while distinctly poorer sensitivities were encountered in the group given 2.5 μg of picric acid. Striking was the relative insusceptibility of the sensitization technique to large differences in picric acid when an adequate amount of mycobacteria was employed. In our usual experiments, however, picric acid was used at 100 μg per site by arbitrary choice.

Sensitization to Picric Acid Effected in Tuberculin-Positive Guinea Pigs.—It seemed important to inquire whether guinea pigs already sensitized by mycobacteria would be able to attain high levels of sensitivity to picric acid by means of the split-adjuvant technique.

In order to keep the local dermal reactions to mycobacteria within tolerable limits, the content of tubercle bacilli was decreased from 2.5 to 0.1 μg per site. Rockefeller guinea pigs were sensitized to tuberculin by intradermal injections of mycobacterial adjuvant on one flank. 2 months later, when they were highly sensitive to tuberculin, five sites on the opposite flank were injected with paraffin oil containing 0.1 μg tubercle bacilli. On the next day, picric acid (100 μg) was injected into each fresh adjuvant site in one-half of the guinea pigs and saline was injected similarly in the remainder of the animals.

Challenge tests with picric acid on day 11 resulted in moderate to intense reactions in all those animals that had received picric acid 11 days previously, whereas their saline controls were nonreactors. The positive picric acid reactions were fully comparable in intensity and evolution with those of guinea pigs sensitized by the standard split-adjuvant technique (cf. Table III, group A).

In another experiment we again used guinea pigs that had been sensitized to tuberculin by means of adjuvant. Sensitizing injections with mycobacteria followed by picric acid were made as above. In addition, one-half of these guinea pigs received contact applications of picric acid as testings on days 12 and 18; the remainder of the animals were rested. At a final common challenge with 0.2% PA on day 27, 7/8 of the guinea pigs in the group that had received the intermediate tests showed definite reactions (one-plus or more), while certain sensitization was observed in only 1/8 of the group not given the prospectively boosting contact applications of picric acid on days 12 and 18.
Topical Sensitization with Picric Acid.—In a preceding paper (2) it proved possible to induce sensitivity by treating skin sites epicutaneously with chemical allergens (DNCB, PCI, quinine) either before or after depositing mycobacterial adjuvant intradermally. A similar experiment was undertaken with picric acid.

12 inbred guinea pigs of Sewell Wright’s Family XIII were each injected intradermally in five clipped sites on the flank with 0.05 ml of paraffin oil containing 5 μg of tubercle bacilli.

### TABLE IV
The Percutaneous Route for Sensitizing with the Split-Adjuvant

| Guinea pigs | Mycobacterial injections | Allergen applied | Contact tests |
|-------------|--------------------------|------------------|--------------|
|             | Day 0                    | on days 0-5      | T0           | T00          |
|             |                          |                  | PA 1% | Form. 37% | PA 1% | Form. 37% |

#### Group A

|     |     | Picnic acid |     |     |
|-----|-----|-------------|-----|-----|
| 72  | P.O. + Tbc | (+++) (+±) | 0   | (++++ (+) |
| 06  | " + " | (+)     | 0   | (+++) |
| 73  | " + " | (+±)     | 0   | (+) |
| 05  | " + " | (+)     | 0   | (+) |

#### Group B

|     |     | Formaldehyde |     |     |
|-----|-----|--------------|-----|-----|
| 153 | P.O. + Tbc | 0 | (+) | N.D. | (+str) |
| 111 | " + " | 0 | (+±) |
| 08  | " + " | 0 | tr   |
| 112 | " + " | ±? | tr   |
| 20  | " + " | 0 | (+)  |
| 110 | " + " | (0) | (+)  |
| 1763 TC | + (eroded | 0 | tr   |
| 1764 TC | center | 0 | tr   |

Family XIII guinea pigs were injected intradermally in each of five sites with 0.05 ml of paraffin oil containing 5 μg of tubercle bacilli. Commencing immediately after the injection, and once daily for 5 days, contact applications, as for skin testing, were made to the sites either with 1% picric acid in oil (group A) or with droplets of undiluted formalin (group B). Testing was done on days 17 and 25. Symbols defined in text. Significance of entries in parentheses as in Table I. Note Str as indicating an extremely faint trace.

Immediately thereafter, and also on the following 5 days (days 1, 2, 3, 4, and 5) six of the guinea pigs received droplets of 1% picric acid in dibutyl phthalate:olive oil (1:9) spread gently across the adjuvant sites, totalling about 0.05 ml each application. These applications did not grossly irritate the adjuvant sites. The other six guinea pigs had aqueous formaldehyde (37%) spread similarly and served as specificity controls.

Tests on days 17 and 27 showed that the picric acid treated guinea pigs had indeed acquired a hypersensitivity to picric acid (Table IV), but of quite variable intensity. The formaldehyde-treated guinea pigs had also developed a definite, although not strong, hypersensitivity to formaldehyde.

The question arose whether old inflamed sites, existing as a consequence of
injections of mycobacterial adjuvant some weeks earlier, would serve as well as freshly prepared sites for sensitizing with chemical allergens. A limited exploration was made in Family XIII guinea pigs; sites prepared 1 month before with 5 μg of mycobacteria in paraffin oil, showing considerable inflammation and even sometimes superficial ulceration, were painted 15 times over a period of 20 days with either picric acid (1% as described above), quinine monohydrochloride (10% in butyl Cellosolve), or 37% formaldehyde. The animals failed to acquire any sensitivity. Evidently freshly prepared adjuvant sites are required.

Histology of the Picric Acid Challenge Reactions

Histologic study was made of biopsy material from typical picric acid contact reactions.

In one experiment Hartley guinea pigs were sensitized to picric acid by the injection of 100 μg of picric acid into each of five sites that had been injected intradermally the day previously with 0.05 ml of paraffin oil containing 5 μg of heat-killed tubercle bacilli. The animals were boosted by the topical application of picric acid on days 17, 23, and 30. On day 55, tests were made with 1% picric acid (in dibutyl phthalate:olive oil, 1:9) on three sites on the flank. Biopsy specimens were secured at 24, 48, and 72 hr.

The reactions of the test sites, immediately before biopsy, were as follows:

| Guinea Pig No. | 24 hr | 48 hr | 72 hr |
|---------------|-------|-------|-------|
| 7             | ±     | +±    | +++   |
| 21            | +±    | +++   | +++++ |
| 10            | ±     | +     | +++++ |

The histological changes of the epidermis are characterized by hyperkeratosis, acanthosis, exocytosis, and both intra- and extracellular edema. Intra-epidermal microvesicles are seen, being particularly prominent in later specimens. In some areas there are focal collections of lymphocytes and degenerating polymorphonuclear leukocytes that form intra-epidermal abscesses (Figs. 3, c, d). In the dermis, there is edema and an infiltrate consisting of round cells and polymorphonuclear leukocytes (including eosinophils). The infiltrate concentrates about hair follicles and frequently can be seen invading the external root sheaths (Figs. 3, a, b). Although microscopically the inflammation tended to be somewhat more intense in the older reactions, the histologic changes in clinically mild 24-hr reactions were often quite severe. The perifollicular distribution of the infiltrate suggests that the early papules of the picric acid reaction are distributed in relation to hair follicles.

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\(^{3}\) The variable percentage of dibutyl phthalate used with olive oil or corn oil as mixed solvent for picric acid should not be a major factor in the unique appearance. One of us (M.W.C.) uses only triglyceride oil for PA concentrations of 1% and below, and the manner of development is no different. In PCI sensitivity, straight dibutyl phthalate as solvent or straight triglyceride oils as solvents produce exactly equivalent reactions in titrations.
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Figs. 3 a and b.  Histology of 24-hr reactions to test T_{55} made with 1% picric acid dissolved in dibutyl phthalate:olive oil (1:9). (a) 24 hour picric acid reaction demonstrating cellular infiltrate concentrating about the upper portion of the hair follicles. × 41. (b) 24 hr picric acid reaction showing follicular and perifollicular edema and cellular infiltrate. × 165.
FIGS. 3 c and d. Histology of 72-hr reactions to test T55 made with 1% picric acid dissolved in dibutyl phthalate:olive oil (1:9). (c) 72 hr picric acid reaction. There is extensive spongiosis and exocytosis with a collection of inflammatory cells (round cells and degenerating polymorphonuclear leukocytes) forming a pustule just below the fully developed keratin layer. × 200. (d) Picric acid reaction at 72 hr. There is an abscess, consisting mainly of lymphocytes, enclosed in the keratin layer. Spongiosis and a dermal infiltrate can also be seen. × 250.
Sensitization with Complete Freund’s Adjuvant (CFA)

To examine whether the split-adjuvant technique possessed especial advantages, picric acid was injected into the footpads in a type of complete Freund’s adjuvant emulsion, consisting of 12 µg of M. tuberculosis per animal (strain Jamaica No. 22), paraffin oil, Aquaphor, and picric acid in saline. When 100 µg of picric acid was so injected, only trivial cutaneous sensitivity arose in all of 15 animals and retesting failed to boost their sensitivity. In another experiment, the amount of picric acid was increased to 500 µg.

Under these conditions, 9 out of 16 guinea pigs sensitized quite satisfactorily, and typical anamnestic boosting by the first epicutaneous test with picric acid was observed in 8 of the 11 animals given a secondary test; five of these became high reactors. Yet the remaining seven animals showed the same trivial sensitivities encountered in all of the 100-µg group. In contrast, sensitization to picric acid by means of the split-adjuvant technique, even when only 2.5 µg of picric acid was injected into each of five prepared sites, occurred much more uniformly. However, in those guinea pigs in which sensitization was secured either by the split-adjuvant technique or the footpad method with CFA, responses to either intradermal or contact tests were indistinguishable.

DISCUSSION

Sensitization with picric acid has been reported in man (8–10). Such sensitivity was particularly common in the preantibiotic era when antibacterial ointments made with picric acid and picric acid derivatives were widely used. Experimental sensitization of guinea pigs was first attained by Landsteiner and di Somma in 1940 (1). These authors chiefly applied dilute cantharidin to the clipped skin to obtain a mildly inflamed area; on this site Butesin picrate in olive oil was painted daily. By means of this treatment, up to one-half of the animals developed sensitivity. These authors pointed to special features of the reactions, and they took note of the marked variation in susceptibility both individually and between different guinea pig stocks.

The Split-Adjuvant Method.—Recently, a split-adjuvant technique was described (2) which consists in the separate administration to skin sites of adjuvant and allergen (to be followed, importantly, by a succession of several skin tests during which the sensitivity ascends). In a typical experiment, five sites on the flank were injected with paraffin oil containing heat-killed tubercle bacilli and about 24 hr later these same sites were injected with a saline solution of an allergen such as DNCB or PCl. Testings with allergen at weekly intervals, beginning at about 2 wk, resulted in an increasing sensitivity for three or four testings.

Applied to picric acid, the method was highly successful; moreover, as with allergens previously studied, successive contact tests resulted in significant increases in the degree of hypersensitivity. For example, Tables I–III present animals of typical experiments, sensitivity being detected at 0.02% concentration; higher sensitivity has been encountered on occasion (Table III).
It should be noted that, in the first step of this split-adjuvant method, prior experiences with DNCB indicated that allergen could precede adjuvant with about equal results provided allergen was administered within 12 hr or less. A similar result was had with picric acid (Table III, group B), five sites of picric acid being laid down 1 hr before injecting the mycobacterial adjuvant.

In exploring to establish optimal conditions, we found that wide variations in the quantity of picric acid delivered to the sites (100, 25, or 2.5 μg) gave apparently equal results as long as the mycobacterial adjuvant was maintained at 2.5 μg/site. None of the variations was clearly superior, hence in routine work 2.5 μg of tubercle bacilli and 100 μg of picric acid were employed, the interval between these being 24 hr when the adjuvant was given first, and 1.5 hr when the allergen preceded the adjuvant.

When picric acid is used successfully as a sensitizer, contact reactions with picryl chloride are observed (Table III), although the reciprocal experiment is hardly positive; this cross-reactivity displays special features, which are discussed in the following paper.¹

Most of our experiments to date have employed as adjuvant the virulent Jamaica strain 22 of *M. tuberculosis* var. *hominis* as the heat-killed mycobacterium in paraffin oil. In a few experiments with a commercially prepared adjuvant (Freund’s complete adjuvant H37Ra, Difco Laboratories, Inc., Detroit, Mich.) qualitatively similar results were obtained. Direct comparisons between strains of tubercle bacilli have not been made.

**Anamnestic Responses.**—The induction of sensitivity by means of mycobacterial adjuvant has the especial consequence that later skin tests increase the degree of sensitization markedly. The phenomenon was first encountered in effecting contact sensitization to picryl chloride by means of picrylated guinea pig stromata and mycobacteria (5); the later exacerbation of sensitivity by means of contact tests made with the simple chemical, picryl chloride, proved to be notable and consistent in the Rockefeller albino strain, although less pronouncedly in some guinea pigs of the similarly outbred Hartley strain (11; J. Turk, personal communication). In the present experiments with the split-adjuvant technique, “self-coupling” occurs each time after administration of allergen; Hartley strain guinea pigs and also Wright’s Family XIII guinea pigs have sensitized and boosted much like the Rockefeller University albino colony. Evidence for anamnestic responses in delayed sensitivity has recently been reported (12), effected directly with 2,4-dinitrochlorobenzene, without use of adjuvant. However, the extent of the increment in sensitivity, and its frequency, is indeed limited, in contrast with instances in which mycobacteria and paraffin oil are used. If the principle of anamnestic response pertains both to sensitization effected with and without use of adjuvant, the adjuvant effect is to be distinguished in that it prepares the animal to respond to contact tests by more than one increment in the level of sensitivity.

Injections in a Freund-type complete adjuvant emulsion were made in com-
parison with the split-adjuvant routine described here. When a total of 100 μg of picric acid in saline emulsified in Aquaphor, paraffin oil, and tubercle bacilli was injected into the footpads, sensitivity to picric acid was feeble or absent, and anamnestic boosting by sequential contact tests was not secured; increasing the total amount of picric acid to 500 μg did, however, lead to satisfactory sensitivity, and to boosting in 50% of the animals, whereas the other half did not respond. (With the split-adjuvant technique, five sites of 25 μg or of 2.5 μg picric acid would ensure rather uniform sensitization.)

Several further variations of the split-adjuvant technique were explored. It was determined that guinea pigs could be primed by topical application of picric acid to freshly prepared adjuvant sites (rather than by being injected intradermally with allergen). If such a variation should prove appropriate to other weak sensitizers, as other experiments with quinine and formaldehyde suggest, the split-adjuvant technique may well find application in rendering the guinea pig more readily sensitizable to a wide range of topical materials, many of which would not be suitable for testing by injection. Thereby the method might, for instance, facilitate the screening for allergenicity of prospective topical medicaments in the guinea pig. In later experience by one of us (H.C.M., Jr.), daily application to adjuvant-prepared sites of weak allergens under closed dressings is found to be superior to application of “open patches” as used in this paper.

The finding that guinea pigs already sensitive to tuberculin can be used in the split-adjuvant technique (the content of tubercle bacilli in newly administered adjuvant being then much reduced) implies that the steps leading to the acquisition of tuberculin hypersensitivity are not essential for the adjuvant priming of the guinea pig with chemical allergens. Further, animals so brought to reactivity towards picric acid undergo further anamnestic increases in sensitivity by successive contact testings.

**Genetic Differences in Susceptibility.**—Variation in susceptibility among individual guinea pigs can be noted in Tables I and IV, and irregularity in individual response was encountered even more markedly in the former attempts to sensitize with the cantharidin-butesin picrate method. The Rockefeller University albino colony (Moen-Chase) is maintained genetically highly susceptible to sensitization with dinitrochlorobenzene and has proven to be highly susceptible also towards picryl chloride (3, 4). That variable responses noted to picric acid have a genetic basis finds support in an exploratory breeding program done some years ago by one of us (M.W.C.) with the late Mr. Jerry Simunek. A modest program of selective breeding was undertaken over a 27 month period with the aim of establishing a subcolony uniformly susceptible to sensitization with picric acid, by means of selective breeding within the Rockefeller University albino stock, with use of a modified Landsteiner-di Somma sensitizing regimen. In the time allotted to the project, a measure of success was attained by means of parental selection, mating, and progeny testing (4) in attempts to breed respectively for high and for low susceptibility. Results on the F₂ generations were as
follows: breeding for susceptibility: five high reactors, six intergrade, one low; breeding for low susceptibility: no high reactors, four intergrade, and eight low reactors. The breeding program was not pursued further. For the bulk of the present work, unselected animals were drawn from the Rockefeller University albino colony.

While genetic susceptibility appears to play by far the major role in the degree of sensitivity attained, experiences with the split-adjuvant technique with picryl chloride in well-inbred sublines of Wright’s Family XIII guinea pigs suggest that nongenetic variations play a lesser role also. Such effects could be due to imprecision in injecting the chemical allergen into the prepared mycobacterial sites, or perchance to establishing varying degrees of partial tolerance as the bulk of the injected allergen escapes from the skin depots (13).

**Gross and Microscopic Appearance of Picric Acid Reactions.**—In the guinea pig, both clinically and histopathologically the picric acid reactions diverge from the reactions elicited with the well-studied strong sensitizers, DNCB and PCI. Further, our experience with other weak allergens, such as quinine and formaldehyde, suggest that, in general, there is a considerable variety of reactions in the guinea pig to allergic contactants. The picric acid reactions develop slowly, commonly starting as discrete papules and sometimes taking 3 or more days to reach a maximum, particularly in first testings; considerable inflammation (scaling, erythema, thickening) is often seen when the sites are examined at 1 week. The reaction may extend beyond the site of application of picric acid, a finding also recorded in clinical reports of picric acid sensitivity in man (9). Quite characteristic is the formation of a thick tenaceous whitish scale, usually covering most of the test site. This scale, often seen even in mild picric acid reactions, is encountered but seldom in reactions to DNCB and PCI, and then only as part of a maximal reaction to an inappropriately high concentration of test allergen. Also, the early micropapular response of the picric acid tests are quite unlike the response seen with DNCB or PCI tests of comparable intensity. Finally, the picric acid reactions often are found scarred after scaling has ceased, whereas scarring is relatively uncommon in DNCB and PCI animals excepting, again, in instances of exquisite hypersensitivity and inappropriately high concentrations of test allergen. This tendency to scar may be magnified owing to the chronicity of the picric acid challenge reactions. With one other weak sensitizer, quinine, a similar clinical reaction ensues after testing well-sensitized animals with 10% quinine hydrochloride in butyl Cellosolve.

Histopathologically, the challenge reactions of DNCB and PCI are characterized by a mononuclear cellular infiltrate in the dermis, concentrated, particularly in milder reactions, about small blood vessels. A few polymorphonuclear leukocytes may be seen in the dermis, constituting a distinct minority of the cellular infiltrate; in the dermis, there is acanthosis, intracellular and extracellular edema, and exocytosis of mononuclear cells (14, 15). Picric acid reactions, on the other hand, have an infiltrate that consists, in large part, of neutrophilic...
polymorphonuclear leukocytes. This infiltrate frequently extends upward, forming large or small intra-epidermal pustules (Figs. 3 c, d).

The pronounced concentration of infiltrating cells about hair follicles and root sheaths seen in histological sections suggests that the characteristic papules of early PA sensitivity are distributed in relation to the hair follicles. The prominence of polymorphonuclear leukocytes in picric acid reactions could allow a role for these cells in the developing reaction even though, as shown in the following paper¹, we hardly ever found precipitating antibody in animals sensitized to picric acid.

SUMMARY

A method of establishing regular and intense sensitivity to picric acid is described, based upon an initial sensitization by a “split-adjuvant” technique in which the intradermal injection of mycobacteria in paraffin oil precedes or follows the administration of allergen to the same sites. When subsequent contact applications of picric acid are later made, the degree of sensitivity rises in steps such that reactivity occurs in tests made with low concentrations of picric acid, in the range of 0.06–0.006 % but varying somewhat from one experiment to another. This heightening of picric acid reactivity represents an anamnestic response in the area of delayed hypersensitivity.

The characteristics of contact reactions to the weak allergen, picric acid, differ from those encountered with covalently binding haptens, PC1 and DNCB. A slow evolution from an initial micropapular reaction to full reaction requires about 3 days, leading often to a micaceous scale, with histological evidence of vesiculation even while the reaction is still feeble, and to an infiltrate containing a significant number of polymorphonuclear leukocytes.

Substitution of an emulsion of picric acid in complete Freund’s adjuvant as a priming experience proved to be much less efficient.

The split-adjuvant technique offers a general plan for sensitizing with weak allergens. Indeed, technically, sensitization can be acquired even when, for priming, the allergen is applied topically over intradermal depots of mycobacteria in paraffin oil. Compatibility between sensitizer and adjuvant is not required.

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