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HYPERSENSITIVITY TO SIMPLE CHEMICALS*

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I. Introduction

THE topic for our discussion has long been an engrossing problem.† It remains so. Historically, a fresh emphasis and approach was provided when Karl Landsteiner—chemist, pathologist, and immunologist—commenced his systematic investigations in 1933 with Dr. John Jacobs. It was my great privilege to have begun work in this area with Dr. Landsteiner in 1936 and to have been able to continue this research subsequently. What I have to say represents, as it were, a projection of Landsteiner as a mentor.

The relationship between contact dermatitis and environmental agents, whether fur-dye, nickel-plated ornaments, plant "oils," iodine, turpentine, salvarsan or whatever, had stimulated European dermatologists to carry out studies on individuals of known sensitivity and to attempt experimental sensitizations of man. Deliberate sensitization was, of course, requisite for studying the sensitizing process itself.

Especially important breakthroughs, to use the current phrase, occurred when Bloch with Steiner-Wourlisch (1926) concentrated ethereal extracts of primrose leaves and used this concentrate successfully to sensitize human subjects, and when Rudolf Mayer (1928) was able to sensitize human volunteers with *p*-phenylenediamine. Both of these workers then applied their sensitizing material to guinea pigs and found this species to become sensitized also (Bloch and Steiner-Wourlisch, 1930; Mayer 1931). Moreover, in this period the Russian worker Wedroff (see Wedrow,

* Lecture delivered April 21, 1966.

† Previous Harvey Lectures have dealt with this subject (Pappenheimer, 1958; Eisen, 1964).

1927; Wedroff, 1932; Wedroff and Dolgoff, 1935) reported the sensitizing capacity of 2,4-dinitrochlorobenzene for man.

Landsteiner seized upon this and other simple chemicals which were reported to sensitize man. Relatively "simple" structures such as are provided by a benzene ring bearing chloro or nitro substituents, or both, were procured and used to sensitize guinea pigs by repeated intradermal injection of microgram amounts, a method suggested to Landsteiner by Kolle (1933). Seven out of 17 such compounds were found to sensitize: these were distinctly different from the others in that they possessed one substituent on the ring that could split off and do so at a sufficient speed to permit covalent bonding with the organic base aniline (Landsteiner and Jacobs, 1936). In proteins, negatively charged groups necessary for conjugation are provided chiefly by the protruding ϵ-amino groups of lysine residues. Further support for the interpretation of sensitization as a response to hapten-modified structures was lent by studies in man (Sulzberger and Baer, 1938). Selected compounds from those studied in guinea pigs were found to behave in man as in the guinea pig. Eisen and his colleagues (1952) later confirmed and extended the principle in the guinea pig.

At once Landsteiner commenced the preparation of hapten-protein conjugates *in vitro,* feeling that his prior studies with "artificial antigens" in rabbits presented the proper model for understanding contact sensitivity. From this well established work, it was known that certain simple structures, attached by diazotization to carrier proteins, would direct the synthesis of immunoglobulins, some of which would display so-called "hapten specificity." One could picture contact sensitivity as a consequence of formation of antigenic complexes by attachment of contactant to body protein *in vivo,* stimulation of the immunological apparatus, and subsequent capacity to react when the same hapten was encountered later. The expected proof was to be provided by demonstrating the presence of hapten-specific antibodies in sensitized animals. While contact-sensitivity was not transferable by serum, vagaries of the immunological apparatus and local fixation of antibody might be operative.

Although this endeavor, viewed in retrospect, was based on

a mistaken premise, it led to needed information and it fulfilled the criterion once stated to me by Leonor Michaelis: "You do not need to understand *first*. Just keep working, working hard, and the prepared mind will see." Indeed, by means of hapten-protein conjugates antibody could at times be found—for example, in Schultz-Dale tests with uterine horns from sensitized animals (Landsteiner and Chase, 1941)—by examining sera by means of passive cutaneous anaphylaxis (PCA), which was developed for the purpose independently (Chase, 1947, 1953). It became quite clear that applications of simple chemical allergens always provided, in variable degree, a stimulus to the antibody-synthesizing apparatus, but that contact sensitivity preceded the appearance of detectable antibody and persisted after antibody was no longer to be found.

A new finding brought encouragement to continue a search for antibody: the tuberculin-sensitizing capacity of killed mycobacteria was reported by Saenz (1935) and Freund *et al.* (1937) to be enhanced greatly by the presence of paraffin oil. Studies were begun on the directive effect of oil-potentiated mycobacteria in inducing sensitization with simple chemicals and hapten–protein complexes. Consequently, picrylated stromata of guinea pig red blood cells—a material that must be regarded solely as an "antigen"—and tubercle bacilli in paraffin oil were injected separately into the peritoneal cavity. The animals indeed became positive to contact tests with the simple hapten (Landsteiner and Chase, 1941; *cf.* Chase, 1954), reinforcing the idea that contact sensitivity arises by stimulation of the immunological apparatus.

The search for antibody turned to the lesions arising in the omentum from injection of picrylated stromata and mycobacteria in paraffin oil. Just as exhaustive (and fruitless) attempts had been made to extract antibody directly from skin, so the swollen roll of omentum was extracted, and extract, peritoneal fluid when present, and serum were all examined for antibody by ring tests; all were injected intradermally and overpainted with picryl chloride in triglyceride oil. Nothing positive was found. Next, use was made of "focal" tuberculin reactions. Because the donors were sensitive to tuberculin, mycobacteria injected intraperitoneally in saline sufficed to provoke viscid peritoneal exudates.

Some of these exudates were transferred to new animals without being fully clarified, whereupon the first clue as to cellular transfer was obtained in December, 1940. Sensitivity being feeble, exudates were transferred on three or four successive days.

One of the first experiments is given schematically in Fig. 1. Four injections, indicated by arrows, were made into one recipient, totaling 75 ml. entire exudate from 7.5 donors. Contact tests were applied at 48, 72, and 96 hours (T_2, T_3, T_4). The observed

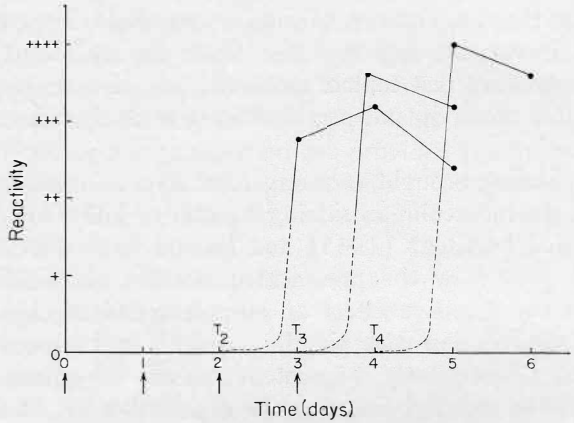

FIG. 1. Transfers of peritoneal exudates from guinea pigs sensitized to picryl chloride were made into a normal recipient on days 0, 1, 2, 3 (see text). Contact tests were made with 1 drop of 1 per cent solution of picryl chloride in olive oil on days 2, 3, and 4.

sensitivity is scored on a scale of 4. The response of this recipient to contact tests T_2 and T_3 is shown in Fig. 2. Of the two pairs of duplicate tests shown, those shown on the left were 2 days old, the right pair 24 hours old. Further tests of day 4 were strongly positive with picryl chloride, but practically negative with 2,4-dinitrochlorobenzene.

It was soon learned that tuberculin sensitivity, also, was transferable by means of white cells from sensitized donors (Chase, 1945); the older, occasional successes of Bail (1910), who found tuberculin sensitivity in guinea pigs after transfer of mortarized tuberculous lesions, became understandable. White cells can even

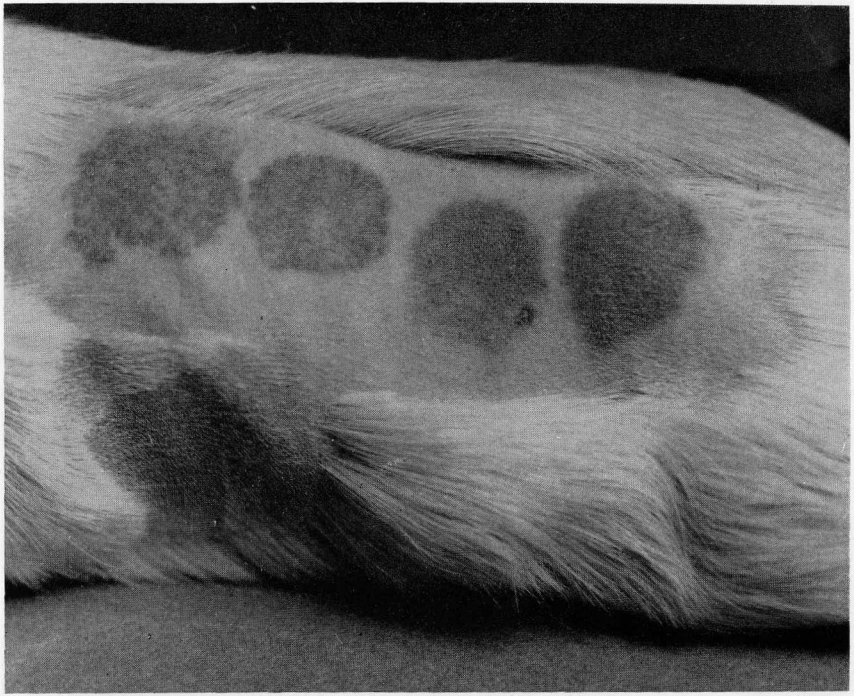

FIG. 2. Photograph on day 4 of tests made on 3281 (PCl 192), shown schematically in Fig. 1. The two contact tests on the left side were duplicate test sites of day 2, 48 hours prior to the photograph; the two at the right had been made 24 hours previously (test of day 3).

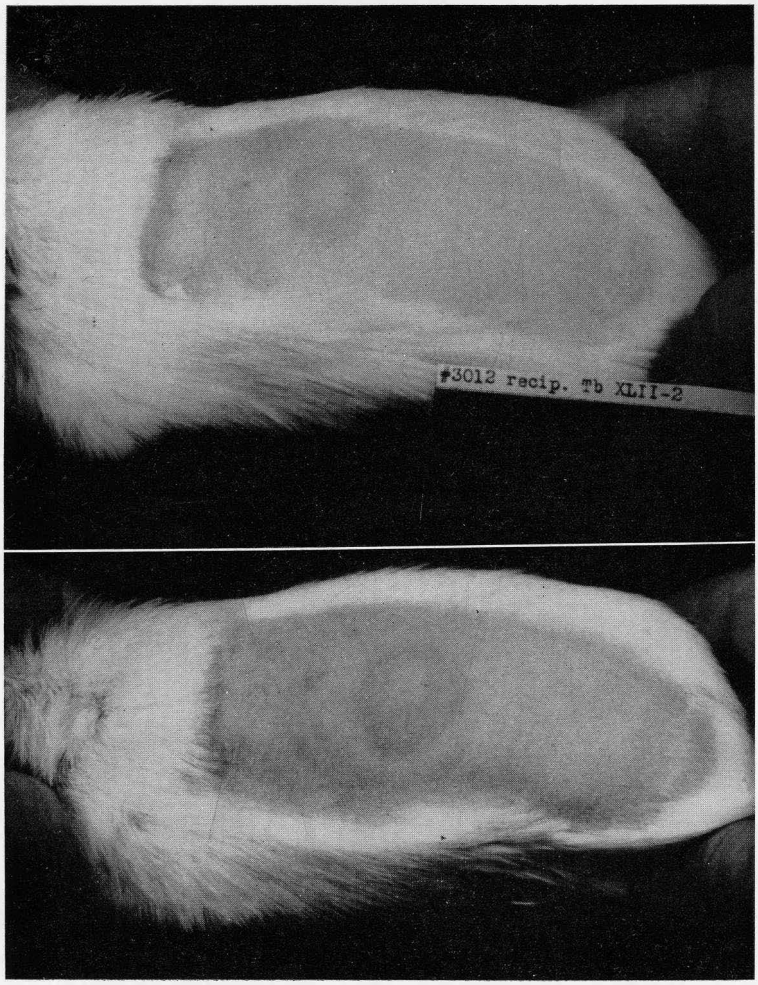

FIG. 3. Cellular transfer of tuberculin hypersensitivity from donors infected 8 weeks previously by subcutaneous injection of living Jamaica No. 22 strain of *M. tuberculosis* var. *hominis*. The recipient animal shown (3012, Tb 42-2) was injected intraperitoneally with 0.45 ml. of peritoneal exudate cells harvested from 6 donors 48 hours after injection of paraffin oil into the peritoneal cavity. The recipient was tested intradermally, once, with 1:5 dilutions of deglycerinated control broth (anterior) and deglycerinated Old Tuberculin. At the top is the reaction to tuberculin 19 hours later. One day later, an

transfer systemic reactivity to tuberculin, when the cells have especially high competency. Figure 3, for example, shows a transfer of cells taken from guinea pigs infected with virulent human tubercle bacilli; the recipient was tested with tuberculin before and again after a nonfatal systemic tuberculin shock. The large increase in the extent of the single tuberculin test because of the systemic shock is clearly seen. Overlooking the significance of deep allergization by infection, we turned to animals sensitized by killed mycobacteria in paraffin oil and could not repeat the systemic reactivity convincingly. The complete demonstration came a year later from Kirchheimer, Weiser, and Van Liew (1949), who succeeded by superinfecting their donors with living BCG before sacrifice.

II. Experimental Sensitization with Allergenic Chemicals

It will be useful, prior to further discussion of the role played by white cells, to review the various methods of establishing sensitization to allergenic chemicals (Table I). With many of the compounds most often used for experimentation, adequate sensitization is secured by the methods of intradermal injections or of percutaneous absorption. In the study of chemicals of low sensitizing capacity, ancillary procedures are helpful. For percutaneous absorption, for example, it is often useful to establish an irritated dermal site in which cells have accumulated. Among the devices listed to secure irritated dermal beds, the use of dilute cantharidin allowed the writer to sensitize guinea pigs to penicillin during the last war. Indeed, the common use of 2% dinitrochlorobenzene in alcohol for application to the skin is successful owing to the irritant effect of this concentration. The general principle applies to the experimental sensitization of human subjects also, the purpose for which Kligman and Epstein (1959) introduced use of Freon 12 as a spray. It may be added that absorption

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injection of 0.25 ml. of Old Tuberculin subcutaneously in the groin provoked a temperature rise to 40°C. in 4.5 hours, accompanied by pronounced livid streaks in the center of the dermal test site; at 9.5 hours, the animal reacted hyperacutely to touch. The view at the bottom was taken on the following day, after recovery had started.

through noncutaneous epithelia such as the colon can lead to sensitization of the skin (Macher and Dörner 1966).

The use of dead mycobacteria in paraffin oil for its adjuvant qualities has been noted already. Mycobacteria in oil and picrylated stromata, for example, can be included in one injection material by using an emulsifer as introduced by the late Jules Freund.

Most interestingly, the fourth method listed—a primary preparation of animals as in method 3 followed by percutaneous absorption of the simple chemical—exaggerates markedly the degree

TABLE I
METHODS OF SENSITIZING GUINEA PIGS

1. Intradermal injections of microgram quantities
   Banal solvents, e.g., 4 per cent alcohol in saline; corn oil
2. Percutaneous absorption
   Local irritation often helpful, e.g.,
   Dinitrochlorobenzene, 2 per cent in alcohol
   Applications of dilute cantharidin
   Freon 12 (Kligman)
   Incorporating irritants, as organic peroxides
   Excitation of a previously acquired sensitivity
3. Use of mycobacterium-liquid hydrocarbon adjuvant[a]
   a. With simple chemical
   b. With hapten–carrier complexes
4. "Combination"—(3) followed by (2)[a]
5. Intradermal injections of hapten–carrier complexes (0.1–3 μg.)

[a] Tuberculin hypersensitivity established concomitantly.

of hypersensitivity. The full increase in sensitivity occurs in about three steps, as Dr. Battisto and I found, using successive contact tests, so that the animals finally respond to testing with between 0.007 down to 0.002 per cent of the simple chemical in an appropriate vehicle.

The last item listed, intradermal injections of tiny amounts of hapten-carrier complexes, establishes a short-term delayed-type sensitivity that is revealed best by reinjection of like hapten-carrier complexes into the skin. Occasionally by use of this method contact sensitivity is expressed against the simple chemical as well,

TABLE II

"COMBINATION" *vs.* ADJUVANT METHODS OF SENSITIZING[a]

Treatment	Contact test with PCl in olive oil			
	1/5%	1/15%	1/50%	1/150%
PGPSt and Tb	(+++++±)	(++++)	(f. tr.)	(f. tr.)
	(++++±)	±	prac 0	prac 0
	(+++++)	(+++)	tr.	tr.
	+++++±	(+++)	0	tr.
	(++++)	0	0	0
	(+++±)	(++±)	(prac 0)	(prac 0)
	(+++±)	(tr.)	0	prac 0
	(tr.)	0	0	0
PGPSt and Tb followed by contact tests	+++++±	+++++±	++++±	++++
	+++++±	+++++±	(++)	++±
	+++++±	+++++±	(+++±)	+±
	+++++±	+++++	(+±)	+±
	+++++±	+++++±	(+±)	±
	++++±	+++++	(++)	++
	++++±	++++±	(+++)	+±
	++++±	++++±	+	(+)
	++++±	+++	(f. tr.)	+±
	+++±	+++	(+++)	(+±)
	++++	+++++±	±	(±)

[a] All guinea pigs were sensitized by intramuscular injection in 5 sites of 0.2 ml. each, to deliver 10.0 mg. picrylated guinea pig erythrocyte stromara (PGPSt) and 0.5 mg. killed *M. tuberculosis* (Jamaica No. 22) as described by Chase (1954). Data in the lower part of the table relate to animals treated, in addition, with simple contact tests of 1% picryl chloride in almond oil on days 22, 28, and 35, the two successive types of sensitization being termed the "combination" method. Contact tests are shown on day 40, made with single drops of olive oil containing stated concentrations of picryl chloride. Readings were made at 24 and 48 hours, and the highest reaction is recorded (48-hour readings are shown within parentheses). Legend in this and the following tables: f. tr., faint trace; tr., strong trace; ±, scattered, very faint pink spots; ±, faint pink spots and patches, not confluent; +, confluent faint pink reaction, well-delineated margin; ++, pale pink, slightly indurated reaction; ++++, pink, indurated reaction; +++++, excoriated lesion with intense skin injury. Intergrade readings between + and +++++ are entered as appropriate for description.

a phase of sensitization about which we shall have more to say later. Such experiments are usually limited to a 9-day period and permit only one or two tests, since reinjection stimulates production of antibody, the presence of which shortly dominates the type of reaction observed at an intradermal test site—an antibody-induced reaction of Arthus type.

The so-called "combination" method of sensitization is an especially effective tool. An example is given in Table II. The eight animals shown in the upper portion of the table demonstrate that contact-type sensitivity to picryl chloride has been established quite well. Yet animals of the same group, listed in the lower half of the table, exhibit a greatly increased degree of sensitivity owing to the application of a few spaced contact tests of picryl chloride. The advantage of this method is indubitable. Severe eye reactions are given by such animals, quite unlike those possessing

TABLE III

SUSCEPTIBILITY OF DIFFERENT GUINEA PIG STOCKS TO SENSITIZATION

Stock	Contact tests to picryl chloride			Sensitivity to tuberculin[c]
	Intradermal method	"Adjuvant" method[a]	"Combination" method[b]	
Rockefeller	+++++	++++	++++	+++++
Hartley	++++	++++ (except ♀♀)	± (some animals are excellent)	++++
XIII (Wright)	++	++++	++	+++
II (Wright)	+++	±	+++	±

[a] Sensitization by hapten–carrier conjugate + mycobacteria.
[b] Sensitization as in the lower part of Table II.
[c] By dead mycobacteria in paraffin oil. Test dose 0.5 μg. PPD i.d.

the usual range of sensitivity. And with use of donors so sensitized, cellular transfer can be accomplished with only 0.1 ml. of moist packed cells or less.

Not all the methods of sensitizing guinea pigs act equally in all stocks of guinea pigs. Thus in Table III the differences in behavior of two albino stocks and two broken-color, highly inbred stocks are shown to four modes of sensitization vis-à-vis picryl chloride and tuberculin. The latter two stocks, Sewell Wright's Family XIII and Family II, were established before 1915; each is approaching the status of isologous stocks. The two outbred albino stocks have gene pools from which, by selective breeding, it is possible to establish various sublines having special properties.

The Rockefeller stock shown here was bred from animals selected for high susceptibility to sensitization with dinitrochlorobenzene.

The ability to manipulate responses by proper gene selection is illustrated in Table IV, selection of grandparents and parents

TABLE IV

GENETIC CONTROL OF SUSCEPTIBILITY TO UNDERGO SENSITIZATION BY MEANS OF MYCOBACTERIAL ADJUVANT[a]

Subline	Parents	Animal No.	Contact tests with picryl chloride 1%	0.2%	0.06%
Selection for	243 × 66	4013 ♀	+++++±	++++±	+
efficient	204 × 50	4018 ♂	+++++±	+++±	0
utilization	204 × 50	4017 ♀	++++	tr	0
of adjuvant	65 × 66	4014 ♀	+++++	+++	±
property of	65 × 66	4015 ♀	++++±	±	±
mycobacteria	47 × 50	4011 ♂	++++++	+++++±	++++
	47 × 50	4012 ♂	+++++	+++++	tr
	47 × 50	4009 ♀	+++++	+++±	±
	47 × 50	4010 ♀	++++±	++	±
Selection for	637 × 626	4008 ♂	±	0	0
inefficient	265 × 99	4007 ♂	++±	tr	0
utilization	265 × 99	4006 ♀	+	tr	0
of adjuvant	627 × 626	4005 ♂	+++±	±	tr
property of	627 × 626	4004 ♀	+++	±	0
mycobacteria	85 × 1265	4002 ♂	+	f.tr.	0
	85 × 1265	4016 ♂	prac 0	0	0
	85 × 1265	4001 ♀	±	0	0

[a] All animals were sensitized at one time by the simple adjuvant method shown in the upper half of Table II and were rested on day 34 with single drops of olive oil containing stared concenttations of picryl chloride; readings at 48 hours are listed. Legend as in Table II. Parental selection, made within The Rockefeller Institute albino colony, determines the susceptibility to sensitization; the F_2 generations are shown.

having been made on the basis of the type of response to mycobacterial adjuvant with picrylated guinea pig stromata. It may be added that other offspring of the two sublines displayed no differences in sensitizations effected by intradermal injections of simple picryl chloride.

Still another type of response to simple chemical allergens

TABLE V

UNRESPONSIVENESS TO SENSITIZATION SECURED BY PRIOR FEEDING OF 2,4-DINITROCHLOROBENZENE[a]

Treatment	Contact tests		
	1%	0.33%	0.1%
Feeding of DNCB	+±	tr	0
	(+±)	(tr)	(tr)
	(±)	0	0
	(±)	0	0
	±	tr	f.tr.
	(tr)	tr	0
	tr	f.tr.	0
	(f.tr.)	0	0
	(f.tr.)	0	0
	f.tr.	0?	0
	0?	0	0
None	(+++++)	(+++++)	(+)
	+++++	++++	(±)
	+++++	+++±	(±)
	+++++	+++	±
	+++++	(+++±)	tr
	++++	+++	±
	+++++	++	±
	++++	(++)	±
	+++±	+±	tr
	+++	±	0

[a] Male guinea pigs (375 to 450 g.) were fed 3 mg. of 2,4-dinitrochlorobenzene (DNCB) in 0.3 ml. of olive oil on days 1 to 6; 5 to 20; and 29 to 34. Intradermal sensitizing injections of 2.5 μg. of DNCB were made on days 50, 51, 53, 55, 57, 59, and 61. Contact tests of single drops of differing concentrations DNCB in olive oil, placed on day 78, were read at 24 and 48 hours. The highest reaction is recorded (within parentheses for 48-hour readings). Animals were tested also with the oil vehicle, without reaction. Four normal animals were tested in the same way, also without reaction. Entries are as in Table II.

should be noted, viz., depression of the sensitization process by special pretreatments with specific allergen before a sensitizing procedure is applied.* Only a few conclusions need concern us from the several studies devoted to this subject (Chase, 1963;

* The phenomenon described by Sulzberger (1929) with neosalvarsan and the unresponsiveness secured by feeding these chemical allergens are alike in principle (Chase, 1963, 1966).

Chase *et al.,* 1963), particularly with Dr. J. R. Battisto (Battisto and Chase, 1963, 1965) and in the work of Drs. Coe and Salvin (1963) and Salvin (1966). Several methods of treatment exist for securing this type of unresponsiveness. The one used chiefly in my laboratory has been feeding of the chemical allergen intermittently over the course of 3 to 5 weeks. This procedure alters the responsiveness of almost every guinea pig, as illustrated in Table V.

The control of the immunological apparatus that is seen to be exerted here in sensitization of contact type extends also, partially, to synthesis of immunoglobulins of the same haptenic specificity. Such an animal will not respond to injection of picrylated guinea pig proteins to form either anaphylactic or precipitating antibodies, but will synthesize hapten-specific antibody if picryl bovine gamma globulin is used instead (Battisto and Chase, 1965). The resultant defect in responsiveness seems to pertain to synthesis only, for cellular transfer establishes adoptive sensitization in specifically unresponsive animals fully as readily as in normal recipients.

One feature worth noting is the fact that animals can apparently become stabilized at a fixed intermediate level of responsiveness. Of particular interest, however, has been the recent observation of Drs. Battisto and Bloom (1966) that unresponsiveness can follow injection of picrylated particulates—cells or stromata, a procedure that can hardly influence every lymph node in the body.

The relation of the factors developed so far—cellular transfer, the participation of immunoglobulins, unresponsiveness, and the role played by the skin—will now be described in more detail.

III. Transfer of Cells Synthesizing Immunoglobulins

While treatment with simple chemical allergens stimulates the antibody-producing apparatus, there is no present reason to view ordinary varieties of hapten-specific antibodies as mediating delayed contact sensitivity: in the course of sensitization, such immunoglobulins cannot be detected as early as contact sensitivity, they do not appear regularly, and the time of appearance can be varied by choice of dose and frequency of the sensitizing injections. For example, Dr. Charles W. Johnson in my laboratory made

a careful examination of the relative times at which contact sensitivity, Arthus-type reactivity, and antibody capable of giving reactions of passive cutaneous anaphylaxis (PCA) appeared in the course of sensitization. Three allergenic chemicals were used in varying concentrations, for daily injection or as a single treatment of 5 simultaneous injections; and the response to topical applications of 2 per cent alcoholic solution of DNCB was studied in addition. Contact tests with the simple chemicals, intradermal injection of hapten coupled to guinea pig serum, and serum samples were studied. With all three sensitizing compounds and all doses, contact sensitivity invariably arose first, between the fifth and the seventh days according to the chosen concentration of hapten. The interspace between time of positive, delayed-type contact and intradermal tests and the times at which Arthus reactions and PCA antibody appeared could be varied at will, from much longer than 6 days down to 2 days. The time required for synthesis and accumulation of PCA antibody, accordingly, was found to bear no fixed relation to the onset of contact sensitivity; one can conclude that delayed-type sensitivity is a different process. It must be added, however, that positive "contact" reactions can be obtained following serum transfer provided sufficiently high concentrations of antibody are present, as well as especially prominent Arthus-type reactions to intradermal injection of hapten-protein complexes. Such contact reactions are well developed and essentially maximal at 7 hours, persist for about 17 hours, and then fade rapidly. It is probable that both of the immunoglobulins of the guinea pig, termed $7S\gamma_1$ and $7S\gamma_2$, can contribute to the effect.

Concern about a possible relation between immunoglobulin and contact sensitivity following cell transfer led us to special experiments. It became clear, first, that antibody was not discernible at any time after successful transfer of delayed sensitivity when usual methods were employed for sensitizing the cell donors. Second, when donors sensitized by the "combination method" were employed, having both extraordinary reactivity to contact testing and high titers of antibody (PCA variety, as well as some hapten-specific precipitins), antibody could be detected in cell recipients. The upper part of Fig. 4 shows, as a dashed line,

accumulation of antibody in the serum following transfer of lymph node or splenic cells (0.4 to 0.6 ml.); in contrast, peritoneal exudate cells induced by paraffin oil have proved inadequate to lead to antibody synthesis. After about 7 days, the amount of antibody declines, presumably owing to homograft-type rejection of the transferred cells, and it then decays at a rather constant rate. In contrast, contact sensitivity (shown as solid lines) can

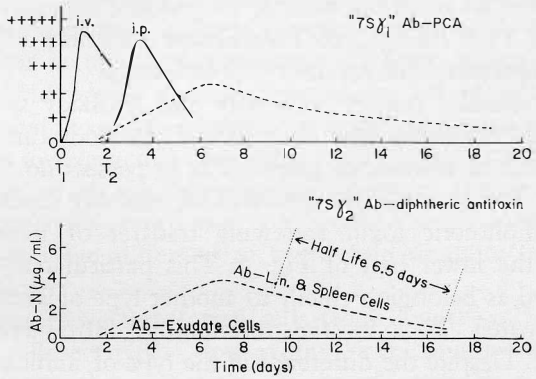

FIG. 4. Cell transfer of contact sensitivity and antibody synthesis. The upper part of the figure is a schematic representation of the temporal differences, following cellular transfer, between the appearance of contactant reactivity (solid lines) and the appearance of antibody (dashed line) having the specificity of the hapten employed for sensitizing the donors, as determined in randomly bred guinea pigs. The antibody was evaluated by the method of passive cutaneous anaphylaxis (PCA).

The lower part of the figure shows a similar temporal course of appearance and decay of another type of immunoglobulin, diphtheric antitoxin, following similar cellular transfer, in the absence of delayed-type sensitivity.

be secured by peritoneal exudate cells even more readily than by white cells of the spleen or lymph nodes, and can be established by much smaller numbers of spleen and node cells than are required for antibody-synthesis. By intravenous administration of cells, contact sensitivity is fully established within 19 hours or less; cells given by the intraperitoneal route establish sensitivity in about 3 days. By testing at suitable intervals it is found that the first phase of contact sensitivity wanes rather rapidly.

The antibody was measured by passive anaphylaxis in a reasonably quantitative way. Such antibody we now recognize—owing to newer studies by White, Jenkins, and Wilkinson (1963) and, in especially excellent detail, by Benacerraf *et al.* (1963)—as belonging to the "$7S\gamma_1$" variety of guinea pig antibodies. Cells killed by freezing and thawing, or suspension in chloroform-saturated saline solutions, failed to induce antibody formation; the absence of antigenic material within the cells was confirmed by failure either to induce sensitivity or produce antibody when incorporated, even living, into Freund-type complete adjuvant and injected as potential antigen into new recipients.

Unquestionably, contact sensitivity and antibody synthesis are separable events in the new host. Indeed, antibody can be synthesized by cells of animals so prepared as to possess no contact-type sensitivity. For example, the synthesis of antibody capable of neutralizing diphtheric toxin following transfer of living cells is shown in the lower half of Fig. 4. This particular antibody can be regarded as belonging chiefly to another type of immunoglobulin, designated $7S\gamma_2$ in the terminology introduced by Dr. Benacerraf. Despite the difference in the type of immunoglobulins under study, the appearance of neutralizing antibody shown here is markedly like that of PCA antibody. In the latter part of the curve a decay rate of 50 percent in 6.5 days could be established, showing cessation of synthesis.* (One advantage of choosing the toxin–antitoxin system is that measurement of antitoxin is accurate to within 5 per cent and quantitation is possible with only small samples of serum.)

A lurking feeling persisted that antigen, degraded or converted to some special intermediate, might be contained within the cells and be responsible for a rapid response by the immunological apparatus of the recipient animal. Others have since turned to X-irradiation to suppress the capacity of the recipient to respond to a new immunological stimulus. We chose to study the avidity (affinity) of the antibody that appeared in wholly unaltered re-

* Cells transferred only a few days after first injection of diphtheric toxoid contained antigenic toxoid, as Dr. Odd Wager found, for even when cells were killed there occurred a primary response of antitoxin production after 12 days. (If living, such cells would, in addition, synthesize antitoxin in the pattern shown in the lower part of Fig. 4.)

cipients. It happens that diphtheric antitoxin differs in its avidity for toxin according to the way chosen for stimulating the animal. Man, monkey, and guinea pig all respond to initial injection of fluid toxoid by producing antibody of low avidity. By subsequent injections, average avidity will increase in 3 or 4 steps, until finally strongly avid sera are produced, like the serum of horses from which standard horse diphtheric antitoxin is obtained following many months or even years of injections. Jerne (1951) had worked out a most excellent method for measuring avidity. In this, a comparison is made between the amounts of antibody required for complete neutralization of toxin under *highly unfavorable* and *highly favorable* conditions for forming antigen-antibody complexes. The degree of avidity is shown both by the differences in requirement for antitoxin in the two methods and by the way in which the test mixtures approach the neutral point. The two methods accord within a fourfold range for highly avid antitoxin and can differ up to twentyfold or more for antibody of low avidity.

Accordingly, we transferred cells from donors synthesizing antibody of different types—low avidity, or medium avidity, or high avidity. It turned out that the quality of antitoxin appearing in the recipients was always that of the donors. This finding provided assurance that the antibody synthesized did not arise because of antigen transferred with the cells, for a primary antigenic stimulus would have caused only low-affinity antibody to appear. [Others have approached this problem, particularly Taliaferro and Talmage (1955), by studying the incorporation of radiotracer amino acid into newly synthesized antibody.]

We are left, then, to conclude that common types of immunoglobulins will be synthesized by transferred living cells of lymph nodes and spleens whenever the donor of the cells is actively synthesizing antibody, but that the appearance of antibody occurs *after* cell-transferred contact-type sensitivity. Also, it will be recalled that contact-type sensitivity can be transferred without detection of any antibody.

IV. Transfer of Contact-Type Sensitivity by Cells

Cellular transfer has joined together both microbial hypersensitivities and contact dermatitis, two types that were once held to

be quite different in mechanism. It must be stated that cellular transfer of contact dermatitis in man, although not without positive reports, must be regarded at present as entirely unsatisfactory. One cannot evaluate whether technical difficulties in handling cells or in selecting proper donors have affected the outcome of such studies. There are some species differences. In the guinea pig, there is extreme vascular dilatation in the bed beneath the contact site, and one can test for delayed sensitivity with hapten-coupled serum proteins. Human beings sensitive to dinitrochlorobenzene, on the contrary, do not react to dinitrophenylated human serum proteins (author's laboratory experience; *cf.* Eisen, 1959), but they can be tested by contact with the chemical directly. Eventually, a satisfactory method of effecting transfer of contactant sensitivity in man with cells should be worked out.

In the guinea pigs, many technical simplifications were introduced in transferring contactant sensitivity over the initial experiments described above. Only a single transfer of washed cells was subsequently used. Mycobacteria are not needed for sensitizing the donors.

Living cells are necessary, and with these we early ran into the problem—and failed to grasp its significance—known as the graft-*vs.*-host reaction: the recipients of pooled cells, particularly of lymph nodes, at times would show cutaneous rashes within 3 to 5 days and would often die within a further 3 or 4 days. Dr. J. R. Battisto (1960) took up this subject; he has shown clearly that a second principle may be involved, for a high proportion of animals in the random-bred Rockefeller albino stock possess cells (and serum factors) that prove directly injurious to the tissues of certain other individuals within the colony, a condition that represents a spontaneous iso-hypersensitivity that may well accelerate graft-*vs.*-host responses. With use of pools of smaller size and transfer of small amounts of cells, this finding fortunately is now rare and constitutes no major obstacle.

Many attempts were made with Bloom to transfer with disrupted instead of living cells (Bloom, 1963; Bloom and Chase, 1966). Living cells from three sources, as in Table VI, were entirely competent to transfer contact reactivity. Yet with freezing and thawing or controlled sonication all competency disappeared, none

of the 16 recipients displaying sensitivity when an equal or much increased number of disrupted cells were transferred. In the course of these studies, certain other facts turned up, from which it seems wholly improbable that any of the scattered reports of transfer with nonliving material among guinea pigs yet deserves serious consideration.

TABLE VI
FAILURES IN TRANSFER OF DISRUPTED CELLS[a]

Cell source	Transfer of living cells	Frozen and thawed (+ DNase)	Sonicated
Peritoneal exudates	2.5×10^8 +++++	3×[b]	0.68× 2× 3.3×
Lymph nodes			
Pool 1	9.5×10^8 ++++	3.4×	1× 2× 2.5×
Pool 2	7.6×10^8 ++++	1.3× 3.3×	
Spleens			
Pool 1	5.6×10^8 +++	3.2×	1× 2× 2.8×
Pool 2	7.1×10^8 ++±	1.3× 2.7×	
Results of transfer of disrupted cells:		0/7	0/9

[a] Cells were taken from guinea pigs sensitized chiefly to picryl chloride by the "combination method" of Table II. Freezings and thawings, in quite varied menstrua, were done in succession five to eight times. Sonication was conducted in polypropylene tubes within cooled chamber of a 9 kv. Raytheon oscillator until cells were disrupted as seen by phase contrast microscopy.

[b] Multiples of living control cells.

In all transfers effected within random-bred stocks, the transferred sensitivity persists for only a few days, so that it is necessary to apply a test within the first few days after transfer (Table VII). In the upper two lines, the loss experienced by withholding the first contact test until day 5 (T_5) or until day 7 or day 10 can be seen. The lower half of Table VII shows 23 recipients

studied by Dr. Bloom with initial testing postponed to day 12 or later. A persisting sensitivity was seen in only one animal out of the 23, probably a fortuitous clonalization within outbred stock. (Transfers made within well-nigh isologous stocks such

TABLE VII

TRANSIENT NATURE OF CELL-TRANSFERRED CONTACT SENSITIVITY[a]

Pool	Reactions in recipients to first contact (cells transferred on day 0)				
	$T_1 - T_2$	T_5	T_7	T_{10}	$\geq T_{12}$
No. 1 Exudate cells of 10 donors, divided among 3 recipients i.p.	++++±	+	+±		
No. 2 Exudate cells of 12 donors, divided among 4 recipients, i.v.	++± +++±		0	0	
Various pools Exudate or node cells or splenic cells divided among 16 recipients, i.p.	(4) +++++ (1) +++ (1) ++				(2) ± (8) 0
As above, divided among 18 recipients, i.v.	(4) +++++ (1) ++++				(1) ++++ (1) + (1) ± (10) 0

[a] The transient nature of contactant sensitivity, as effected by cellular transfer in randomly bred guinea pigs, is shown. Animals not brought to test early, as by day 4 following intravenous injection of cells, have minimal or wanting sensitivity as compared with tests made within the first few days on animals receiving equal portions of cells of the same pool. Numbers in parentheses indicate total individuals possessing the same class of reactivity.

as Wright's Family XIII, in which homograft-type rejection of skin does not occur, show persisting sensitivity.)

Despite the transient nature of cell-transferred hypersensitivity, the early testing leads to a curiously efficient active sensitization

(Fig. 5). The aftermath of early testing is shown on the upper schema as a "bimodal" response. No such degree of active sensitization is acquired by normal animals as a consequence of one to three successive contact tests made in parallel with tests on

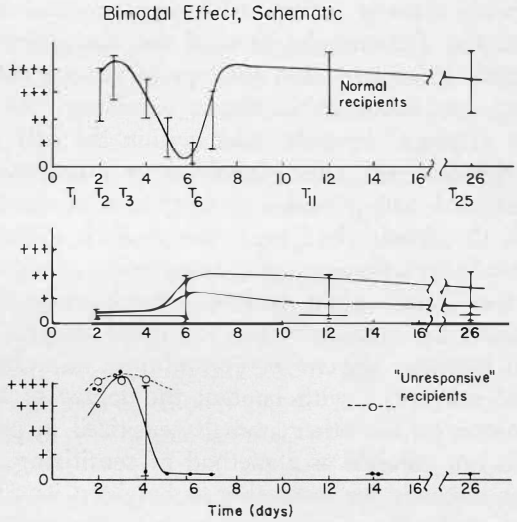

FIG. 5. Various features of sensitivity induced by cellular transfer at day 0 are shown, contact tests having been made with 1% picryl chloride in olive oil at various times (1 to 25 days) after transfer. The upper schema, in which variations in degree of response are indicated by vertical bars, indicates an initial and waning sensitivity followed by an upsurge of active sensitization that is induced by the joint procedure of transferring living cells and making contact tests. The degree of low-grade sensitivities that are encountered in normal animals, tested in parallel with animals that receive "competent" cells, is indicated in the middle schema. The lower schema indicates that, in animals rendered unresponsive to the hapten by prior feeding, as reported in Table V, and used as recipients of "competent" cells, the second response of active sensitization owing to contact testing is nearly obliterated.

recipients of cells (middle schema). Yet the active sensitization represented in the second burst of sensitivity seen on the upper schema is hardly encountered in recipients that have been rendered specifically unresponsive to picryl chloride (lower schema) by feeding this chemical. In these "fed" animals, the first phase

(left-hand) was entirely normal; in three such recipients the second phase was entirely wanting (closed circles shown at the baseline) and was positive in only one, an animal which had received a large volume (over 0.4 ml.) of packed white cells (open circle). (It must be remembered that such "hapten-fed" animals are accepted as being actually unresponsive; experimental sensitization, necessary to test for completeness of the unresponsive state, is not permissible beforehand for this type of experiment.)

Regarding the prompt initial rise in sensitivity following cellular transfer displayed by tests made within the first 4 days, one could ask whether the effect involved an anamnestic response of the transferred cells provided by carry-over of chemical hapten with which the donors had been tested. Such a concept would be applicable to sensitizations effected either by use of simple hapten or by the "combination method." Accordingly, donors were sensitized solely by injection of the "complete antigen," picrylated erythrocyte stromata, given in Freund-type adjuvant, and the donors were not tested with hapten; the degree of sensitization was determined on the other animals sensitized in parallel. This procedure is not efficient as a method of sensitizing, but by improving our methods we were able to heighten its efficiency. Indeed, transferred cells of such donors gave the same initial rise in sensitivity that is displayed in Fig. 5 and showed the same bimodal type of response: the secondary rise is not anamnestic. Other recipients tested only at 16 days failed to respond.

It seems, from studies of Bloom in my laboratory with Dr. Leonard Hamilton, then at the Sloan-Kettering Institute (Bloom *et al.,* 1964), that transferred cells must continue their synthetic functions in the new host in order to secure the appearance of contact sensitivity. Competent cells were treated with critical concentrations of mitomycin C, which fail to kill but block synthesis of DNA-dependent RNA. Cells so treated gave rise to contact sensitivity after transfer, provided injection was made intravenously and recipients received their contact test at once. This finding apparently depends upon synthetic functions of cells temporarily sustained by existing stores of DNA and RNA, for if aliquots of mitomycin C-treated cells were given by the intraperitoneal route and testing was practiced on the third day (T_3),

the recipients hardly responded to contact testing. This finding, which deserves much further elaboration, casts strong doubt on the oft-voiced idea of "cell-bound" antibodies in the sense of cells carrying with them significant amounts of preformed "anti-substance." Rather, continuing synthesis seems required after the transfer of competent cells.

Quite recently, a new look has been taken at the well-established inhibition of monocytes in tissue culture by adding allergen to which the cell donor is sensitive. Improvements in technique were introduced by George and Vaughan (1962), living cells being packed in a 2- to 3-mm. stub of capillary tubing, the tubing placed in fluid medium, and the cells allowed to migrate outward for 24 hours as a "brush." Dr. John David (David *et al.*, 1964) used this technique with cells of animals sensitized by hapten–protein complexes. In these highly significant studies, the complete sensitizing structure is found to be necessary as antigen for securing inhibition of cellular migration. Just as with the mitomycin–C experiments of Bloom *et al.* (1964) in controlling the sensitiveness of the whole animal, so David (1965) finds that macrophage migration from capillary tubes proceeds normally even in the presence of specific allergen when puromycin is added. If such experiments parallel, or perhaps even serve as an *in vitro* model of delayed sensitivity, two other findings become of paramount importance: first, the migration of macrophages is largely or fully controlled *by the admixed population* of lymphocytes which react to the allergen. It has been found by both Dr. David and Dr. Bloom—the latter having studied tuberculin hypersensitivity—that macrophages from *normal* animals are sufficient provided that lymphocytes from sensitized animals are present. Secondly, Dr. Bloom has purified lymphocytes from peritoneal exudates, and it was recently reported (Bloom and Bennett, 1966a, b) that incubation of tuberculin with competent lymphocytes for 24 hours releases a soluble substance which, by itself, controls migration of macrophages. The role which could be attributed to such a factor in eliciting reactions at cutaneous test sites, where monocytes are not always a prominent part of the invading cellular population, clearly calls for investigation. One could guess that high local concentrations of such a factor would be necessary,

requiring close association of epithelial cells and competent lymphocytes at the test site.

V. The Manner of Sensitization

Our goal in these studies, fundamentally, is to know how the individual who meets an allergenic chemical becomes sensitized to it. Whatever the tissue that makes a complex with the hapten—or perchance with several haptens—the individual acquires a specific recognition of the sensitizing material, and the dermatologist can hope to discover the particular contactant to which a patient is sensitive.

Recent studies have pointed to the importance of the carrier configuration and have provided excellent evidence that the specificity of delayed sensitivity is ordinarily directed not only against the haptenic structure but against some adjacent part of the surface of the carrier as well. These studies have been due in large part to Drs. Gell and Benacerraf (1961), and Drs. Salvin and Smith (1960). When delayed sensitivity is initiated by, say, picrylated protein A and tests are then made not only to this, but to picrylated proteins B, C, D, and so on, delayed sensitivity arises both to the original complex and also to carrier A tested alone. Whether the other picrylated proteins will cross-react depends upon biological kinship of the alternate carrier to that used in forming complex A, and so on. For inducing delayed sensitivity to hapten-protein complexes, several methods can be employed: a single intradermal injection of 1 to 3 μg. of conjugate can be made, or conjugate can be injected in Freund's "incomplete" adjuvant (mycobacteria omitted), or antigen can be coated with specific antibody before injection. Testing for delayed sensitivity to the hapten-proteins is carried out by one or a few intradermal injections, made before circulating antibody appears and masks delayed sensitivity; the first skin test usually serves as an anamnestic stimulus for synthesis of immunoglobulins.

Very useful information has been gained by studying sensitization induced by hapten–protein complexes. Yet an accompanying contact sensitivity to the simple chemical itself is encountered only seldom. We have speculated that the clinical problem of contact dermatitis should be approached in other ways. Put in

simplest form, one can render a guinea pig sensitive to contact test within 5 or 6 days by making several simultaneous injections into the dermis or several successive daily injections of a chemical allergen in 0.25 to 2 μg. doses, for example of picryl chloride (1-chloro-2,4,6-trinitrobenzene). When successive injections are made, a lighting-up becomes evident in these sites at about the fifth day, and coincidentally, a contact test with picryl chloride will be positive. The desideratum would be to substitute, for injections of the simple hapten, a preformed picrylated complex that would result in inducing sensitivity to the simple chemical. We realized even in 1939 that complexes of hapten coupled to serum proteins could not substitute for the simple hapten itself. In 1961, Drs. Salvin and Smith produced evidence that soluble proteins of the skin were more efficient as carriers of haptens than those of the serum in rendering animals sensitive. They injected dinitrophenylated skin solutes, incorporated in incomplete adjuvant, into the footpad of guinea pigs and found some positive contact reactions to dinitrochlorobenzene. This was putative evidence that special proteins within the cells of the skin played a prominent sensitizing role. Our own experience, begun with Drs. Kawata and Macher, have led us to somewhat different conclusions.

Let us consider first how self-coupling probably proceeds in the skin. Dr. Nicholas T. Macris in my laboratory studied the reaction of picryl chloride with a soluble protein under physiological pH and temperature (Fig. 6). At a molar hapten:protein ratio of 50:1, reaction with BSA proceeds well and 18 haptenic groups become attached per protein molecule in 1 hour, 65 per cent of the hapten remaining free. Similar observations have been made with several chemical allergens (Table VIII) with which BGG and BSA were used as model carriers. Under these same conditions, light coupling—3 to 12 groups depending upon the hapten and the particular protein—occurs after 8 to 16 minutes. Yet the molar ratios used here are more than 300 times higher than could exist in the dermis after injection of 2.5 μg., if the hapten is distributed over a sphere 5 mm. in diameter which contains approximately 85 per cent water and 15 per cent tissue and soluble proteins. Consequently, with ordinary sensitizing injections of chemical allergens, in which the dose injected is not

frankly toxic to the tissues, we may expect that rather few haptenic groups will couple per molecule of carrier, that several hours will be required to complete coupling, and that uncoupled hapten in solution or dissolved in lipid membranes of cells may be carried elsewhere. The rate of coupling will depend upon the properties

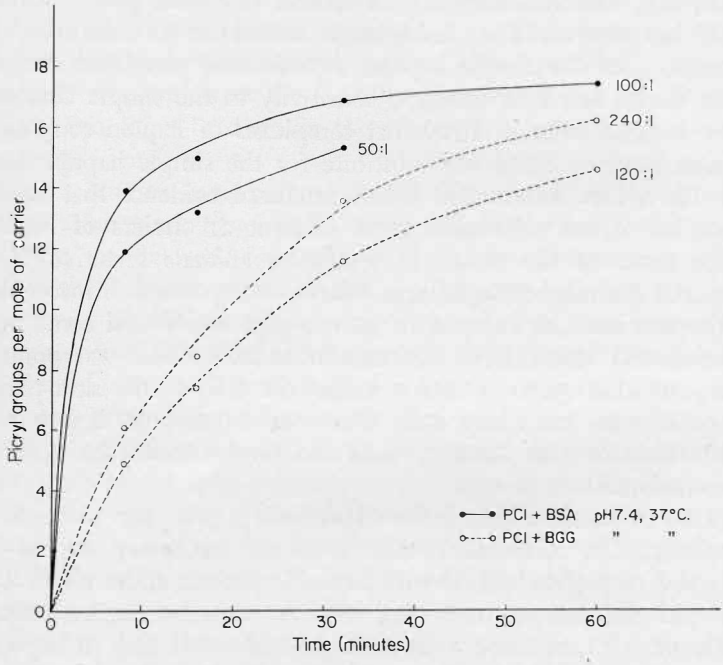

FIG. 6. Coupling rates of picryl chloride (PCl) with 0.3% bovine serum albumin (BSA) or bovine serum globulin (BGG) at pH 7.4 and 37°C. (N. T. Macris).

of the allergenic chemical, exemplified by the reaction constants of the compounds shown in Table VIII. Here, the labile substituents are those listed in position 1 of the benzene ring. They split off, with alkaline hydrolysis, at widely differing rates. The speed of coupling to glycylglycine under a given highly favorable, nonphysiological condition demonstrates rate differences more clearly—picryl sulfonate couples completely in 2 minutes, picryl

chloride in 12 minutes, and dinitrofluorobenzene in 30 minutes. We have not attempted to study DNCB, that excellent allergen which exhibits only 0.4 per cent of the reaction rate of DNFB. Within tissues, coupling naturally will be much slower owing to pH, dilution, and availability of lysine groups.

TABLE VIII

COUPLING UNDER PHYSIOLOGICAL CONDITIONS (pH 7.4, 37°C.)[a]

	DNFB, 140 mg./g.			PCl, 185 mg./g.			PSO_3H, 260 mg./g.		
Carrier	Molar ratio	Time (min.)	Groups added	Molar ratio	Time (min.)	Groups added	Molar ratio	Time (min.)	Groups added
BGG	120:1	16	3.2	120:1	8	4.9	120:1	8	8.0
		60	8.3						
BSA	50:1	8	8.3	50:1	8	11.9	—	—	—
		60	14.0						

	Reaction constants	Conjugation with glycylglycine
DNCB: DNP-1-Chloro	3.25 at 15° (Na-Eth)	—
DNFB: DNP-1-Fluoro	686. at 15° (Na-Eth)	30 min., pH 8.0, 25°, 100:1
PCl: TNP-1-Chloro	Very great (Na-Meth)	12 min., pH 8.0, 25°, 100:1
PSO_3H: TNP-1-Sulfonic	Very great (NaOH)	2 min., pH 8.0, 25°, 100:1

[a] The number of haptenic groups added per mole of bovine gamma globulin (BGG) or bovine serum albumin (BSA) at pH 7.4, 37°C., is shown for three allergenic chemicals, 2,4-dinitrofluorobenzene (DNFB, or "DNP-1-Fluoro"), picryl chloride (PCl, e.g., 1-chloro-2,4,6-trinitrobenzene or "TNP-1-chloro"), and picryl sulfonic acid (PSO_3H, or "TNP-1-Sulfonic"). These couplings are compared with the time for full reaction with glycylglycine at a molar excess of 100:1 at pH 8.0 and with the respective reaction constants for cleavage of the substituent at position 1 with sodium ethylate (Na-Eth) or sodium methylate (Na-Meth) or sodium hydroxide.

In contrast to the conditions suggested as being applicable to *in vivo* coupling, most laboratory attempts to induce delayed sensitivity with conjugates have been carried out with highly coupled proteins. We attempted to duplicate the result of intradermal injection of picryl chloride by picrylating various materials, but not highly. Skin proteins were prepared by Dr. H. Kawata from full-thickness skin and from epithelial scrapings. In the superficial layers of the skin, besides serum proteins in low concentration, there were at least 5 soluble skin antigens and, in addition, special

materials that appeared not to be antigenic. Residual tissue, after full extraction of soluble material, was sonicated and coupled as insoluble particulates. The "ghosts" of guinea pig erythrocytes were coupled also; some stromata lots were laked by very mild methods. Techniques were used to ensure the absence of any uncoupled hapten in these preparations.

The results of our current studies are shown in Table IX. Various picrylated materials were injected, either intradermally

TABLE IX
CONTACT SENSITIVITY INDUCED BY PICRYLATED PROTEINS[a]

Picrylated conjugate		Nos.	I.D. ++/++++ (%)	Footpad, incomplete adjuvant ++/++++ (%)	Each injection (μg)
I. TNP-serum proteins	3–5×	46	19 (2)	—	30–250
	1×	27[b]	—	15 (0)	75
TNP-skin solutes	4–5×	18	35 (5)	—	30–75
	1×	30	—	40 (10)	30–75
II. TNP-serum proteins (with alumina)	3–5×	42	64 (28)	—	10–250
TNP-skin solutes (with alumina)	4–5×	22	76 (**63**)	—	30–75
	1×			33 (0)	75
III. TNP-skin residue	**4**×	18	61 (**50**)	—	75–125
	1×	12	50 (8)	—	375
	1×	7		58 (**42**)	75
TNP-skin residue (with alumina)	4×	12	92 (**75**)	—	75
	1×	3	All (***all***)	—	75
TNP-rbc-Stromata (Low coupling heated or not)	3–5×	15	26 (13)	—	125–750
(Higher coupling)	2×	8	49 (37)	—	25–100
	3–5×	25	90 (**64**)	—	150–750

[a] Contactant sensitivity to picryl chloride induced by injecting picrylated materials (trinitrophenylated, or TNP) intradermally in the dorsum (I.D.) or, incorporated in incomplete adjuvant (lacking mycobacteria), into the footpads of guinea pigs. Injections were completed in 4 to 7 days; the number of injections and amount of material are listed. The reaction to a first test with 1 per cent picryl chloride in olive oil is given, usually following testing on the ninth day (T_9), although tests between T_8 and T_{25} are included. Figures given in parentheses indicate the percentage of reactors in the +++/++++ category; the other values list the percentage of reactors rated ++ or greater (indubitable sensitization). Boldface figures show highly significant values.

[b] Six of these received TNP-serum + alumina in incomplete adjuvant.

on the back of the animal or, incorporated in "incomplete" Freund's adjuvant as used by Dr. Salvin, into the footpad. Each injection of 30 to 100 μg. of conjugate represented 0.6 to 1.5 μg. of picryl residues. The results of contact testing with 1 per cent picryl chloride in olive oil are shown in the third column, the first figure being the percentage of animals responding indubitably (a rating of "two-plus" or greater), and the figure within parentheses indicating the percentage of animals that exhibited reactions of +++ or ++++ intensity, equivalent to the reactivity found in animals sensitized with the simple chemical.

When soluble skin proteins were compared with serum proteins as carriers of picryl residues, intradermal injections led to typical contact sensitivity in no more than 2 to 4 per cent of the animals, although the skin solutes were unquestionably more successful in inciting weak reactions. Animals sensitized by the special footpad method that Dr. Salvin (Salvin and Smith, 1961) had practiced are shown in the fourth column. Picrylated serum proteins were highly inefficient and picrylated skin proteins were again superior, but satisfactory in only 10 per cent of the animals. We may conclude that sensitization following intradermal injection of hapten ordinarily does not depend upon coupling with soluble constituents.

Yet it might be argued that such proteins, if not free to diffuse, could represent effective sensitizing structures. Therefore, these same products were adsorbed to alumina before injection. The result was indeed significantly improved, since 28 per cent of animals treated with insolubilized picrylated serum proteins and 63 per cent with alumina-fixed picrylated skin solutes were satisfactorily positive. Here, the marked superiority of coupled soluble skin proteins fixed *in situ* (at least temporarily) indicates a significantly superior structure for inducing sensitization from that represented by the proteins of serum. When such insolubilized conjugated skin solutes were injected into the footpad, however, only lowgrade sensitivity appeared.

The fully insoluble residue of extracted skin was then picrylated. After only four daily injections, high sensitivity was seen in 50 per cent of the animals in contact testing on days 7 or 9. A single intradermal injection was not sufficient, but by the

footpad route 42 per cent became highly positive by a single injection. Evidently we were closer to the proper carrier structure.

We injected these insoluble conjugates also with alumina cream, in part to study the superiority seen with alumina and soluble proteins. Here again, sensitivity was established more regularly, 75 per cent responding well to test T_5 when one injection per day was employed for 4 days, and even a single injection sensitized well the 3 animals so used, tested on the fifth day. The reason why alumina increases the percentage of high reactors is not known.

With use of another insoluble carrier, picrylated ghosts of guinea pig erythrocytes, low coupled products failed to sensitize significantly. In contrast, highly conjugated stromata given in a sufficient number of injections will lead to contact sensitivity providing the injections are made over the time of 2 weeks. Nevertheless we reject stromata as representing a sensitizing conjugate that can initiate sensitization such as is secured with the simple hapten, in view of the artificially high coupling and the need to inject over a long period of time.

Our attention is, consequently, centered at the moment on the insoluble constituents of skin, but even soluble constituents can approach these, in a degree, as carrier structure. It is interesting to note that, with use of insoluble skin residue as carrier, all-or-none responses have resulted—animals are either highly sensitive or are plainly not sensitive. These results do not stand alone, for dinitrophenylated conjugates, so far as we have carried out the work, have given entirely consistent data with contact tests made with dinitrofluorobenzene. The role that is played by the site in which simple hapten is injected is currently under study by my colleague Dr. E. Macher. One interesting finding suggests that the allergenic chemical can be used competitively, inducing sensitivity on the one hand and unresponsiveness on the other hand. The balance between these two effects may well determine the degree of sensitivity attained.

That the process of sensitization is complex is illustrated in Table X, which shows the reactions of selected individuals. These were sensitized in part with skin particulates, in part with skin solutes, and it happens that alumina was present in each of these

particular injection mixtures. At contact test T_5 (column 2), six were determined to be high reactors and three were found to be low reactors. As soon as the result of the contact test was known, intradermal injection tests were made with several picrylated soluble proteins. The first contact-positive animal listed was found to react to all the picrylated carriers—proteins of

TABLE X
DELAYED SENSITIVITIES INDUCED BY TNP-SKIN MATERIALS[a]

Material	Number of injections (daily)	Contact T_5 PCl O.O.	Intradermal tests, 75 μg. T_6 PGPS	T_6 PBSA	T_6 PChSA	Ab found (day)
TNP-skin residue + alumina	4	(+++±)	+++++	+++	+++	9
		(++)	0	0	0	≠7
		(+)	+++	+++	++	8(?)
TNP-skin residue + alumina	1	++++				8
		++++				8
		+++++				8
TNP-skin solutes + alumina	4	(+++±)	+++±	+	±	≪8
		+str	++++±	++	++	6
		(+w)	+++±	±	±	?

[a] Types of delayed sensitivities induced by trinitrophenylated (picrylated) skin particulates and soluble proteins. Complexes and sensitization as in Table IX. Contact testing with 1% picryl chloride in olive oil (O.O.). Intradermal testing with picrylated total proteins of guinea pig serum (PGPS) or bovine serum albumin (PBSA) or chicken serum albumin (PChSA). Antibody sought by method of PCA 17 hours after injection of serum in intradermal sites on recipients.

guinea pig serum, bovine serum albumin, or chicken serum albumin. The third animal, which reacted only feebly to contact, nevertheless possessed the same delayed-type reactivity to all three hapten-carrier complexes. The second animal, although well sensitive to contact, failed to recognize any of the three complexes as being related to its sensitivity. Such differences appear to be independent of the presence of PCA antibody (right-hand column), and the reaction to intradermal injection of conjugates, whenever positive, was judged to be solely of delayed type.

When picrylated soluble skin proteins were used to sensitize (Table X), all animals gave typical delayed reactions to picrylated serum proteins, and they showed varying degrees of recognition of equally picrylated but antigenically more foreign carriers. These responses were quite independent of the variable capacity of these same animals to react upon contact with the simple hapten. If contact reactivity is to be studied, obviously not all answers will be found by sensitizing with picrylated *foreign* proteins.

Delayed sensitivity has many facets. Studies are necessarily directed to individual phases with the expectation that the puzzle will eventually be fitted together. At the beginning I remarked that hypersensitivity to simple chemicals remains an engrossing problem. The presentation has been remiss, perhaps, in its emphasis on the guinea pig as a tool and in omitting several important phases of study and theories. For the reasons given, sensitization effected with conjugates where the carriers are far removed from guinea pig, have not been stressed, nor have studies with delayed sensitivity to foreign proteins, nor the engaging studies made by Drs. Uhr and Pappenheimer (1958) on desensitization, nor the use of synthetic polypeptides as carriers. We may touch on two items. Drs. Karush and Eisen (1962) have propounded a bold theory which in essence states that delayed sensitivity may be a manifestation of antibody that possesses extraordinarily high affinity for the antigen, an antibody not discernible in serum because it would be synthesized in quantity, i.e., sufficient for reaction at the test site, only after the making of a skin test. Immunocompetent cells would be able to synthesize it as well as splenic and nodal tissue. It would bind at the test site during the developing reaction and be the cause of it. Further synthesis would then be curtailed abruptly and it would be lost to view. For such a hypothetical antibody to be the mediator of delayed sensitivity, it must necessarily be synthesized effectively before other types of immunoglobulins arise and (as regards sensitization with simple chemicals) only when the skin route is chosen, or mycobacterial adjuvant is employed. Further, one animal should synthesize a spectrum of highly avid antibodies in order to account for the differences among individuals seen in Table X with regard to contact sensitivity and sensitivities to homologous conjugates and

to heterologous conjugates. (An explanation for such differences as these must be sought, of course, whatever the mechanism of delayed-type sensitivities may prove to be.)

Such complexities in the expressions of delayed sensitivity as are shown in Table X should pertain also to factors of the type met by Dr. Bloom when specifically competent lymphocytes were incubated with tuberculin for some hours. The question might be phrased, *what* competencies do we look for? The clinical implications of delayed sensitivity suggest that competency to secure contact sensitivity with simple chemicals must not be overlooked.

The second topic that has not been discussed is the relative probability that, in man, something analogous to Dr. Lawrence's "transfer factor" would play a role in contact dermatitis. The subject of transfer factor, studied thus far only in microbial hypersensitivities of man (reviewed in Lawrence, 1959; Lawrence *et al.,* 1963), has not been explored in regard to human contact dermatitis because of the difficulties experienced thus far in obtaining such a type of transfer. Persons with experience in transfer of microbial hypersensitivity should make the attempt to transfer sensitivity to chemical allergens in man. In 1963, I suggested that far-sighted dermatologists should commence banking cells of sensitive human beings so that extracts from large volumes of cells could be tested for a transfer factor. The area requires exploration, using both living cells and extracts.

Scientific pursuit cannot be undertaken without a vision. We recall, in words attributed to DeMott, that the scientific pursuit is one "where inquiry is undertaken with full expectation of rapid obsolescence in the results." It can only be hoped that current results will help to frame the next set of questions.

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