

REPOSITORY ANTIGEN INJECTIONS

Absorption Studies by Immunologic and Radioactive Methods

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THE INJECTION of a pollen extract emulsified in mineral oil has been used by Loveless¹ and Brown² as a repository form of treatment for desensitization. They have referred to the efficacy of this method as being due to a slow absorption of the antigen. Before any new or modified form of therapy is adopted for clinical use, it is the responsibility of physicians concerned to investigate the safety, efficacy, and mechanisms of its action. Several questions arise with respect to the repository treatment: (1) How much of the antigen is in the free aqueous phase ready to produce early local or constitutional reactions? (2) How long and to what extent does the antigen persist at the site? (3) Is the antigen available distant from the site or is it perhaps metabolized in situ? (4) Is there a possibility of inducing immediate or delayed sensitivity? (5) What effect might result from the presence of a foreign body locally? (6) Can objective methods be utilized to show the efficiency or superiority of such treatment over the conventional method?

The investigations to be described here do not purport to give the answers to all these questions, but it is hoped that the data obtained on some phases will help stimulate further investigations.

METHODS AND PROCEDURES

Emulsions were prepared as recommended by Brown, with slight modification. One part of a ragweed pollen extract which had been prepared by extraction with a Coca-Merthiolate solution was mixed with 2 parts of a 10 per cent solution of Arlacel-A in light paraffin oil and emulsified with the aid of two syringes connected with a joint needle. The details will be described in a subsequent article.

Reaction of Passively Sensitized Sites Following Deep Subcutaneous or Intramuscular Injection of Antigen.—Several years ago we had shown³ that the speed and degree of absorption of an antigen from a subcutaneous or in-

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tramuscular injection could be determined by the rate and end point of reactions of sites sensitized with serial dilutions of reagins. The skin of the forearms or back was injected intradermally with 0.1 ml. quantities of serum containing ragweed reagins. On the following day deep subcutaneous injections of 1.0 ml. of an aqueous extract of ragweed, containing 10,000 pollen units, were made in the arms of nonallergic subjects. In other subjects, 1.0 ml. of the emulsified preparation, containing the same number of pollen units, was injected. In some instances, after new sites were sensitized the emulsion was injected in the same subject who had had aqueous injections several weeks earlier.

Titration of Aqueous Phase by Direct Scratch Tests on Very Sensitive Allergic Subjects.—Several ragweed-sensitive subjects with varying degrees of skin sensitivity were titrated with serial dilutions of pollen extract. Ragweed pollen extract, containing 50,000 pollen units per milliliter, was prepared in ten numbered threefold serial dilutions so that the range varied from 50,000 units per milliliter in No. 1 to 2.55 units in No. 10. A drop of each solution was placed on a scratch on the forearm. Those subjects suspected of being highly sensitive were not tested with the first two or three strengths. Occasionally even more dilute solutions than the No. 10 were required to reach an end point. The emulsified extract was tested by scratch on the same subject. A positive reaction to the emulsion indicated that it had at least as much (and possibly more) free active antigen as the weakest aqueous extract giving a positive test. Failure of the emulsion to react indicated that the amount of free antigen was less than that contained in the weakest reacting aqueous preparation. The testing of several subjects with varying degrees of reactivity made possible an approximate assay of the emulsion. The following example (Table I) illustrates the method. An emulsion containing 5,000 pollen units

TABLE I. EXAMPLE OF TITRATION OF RAGWEED EMULSION BY COMPARISON WITH SCRATCH TEST REACTIONS TO DILUTIONS OF AQUEOUS EXTRACTS ON ALLERGIC SUBJECTS

DILUTION	POLLEN UNITS/ML.	SUBJECT A	SUBJECT B	SUBJECT C
1. 1:20	50,000	+	+	...
2. 1:60	16,666	+	+	...
3. 1:180	5,555	+	+	...
4. 1:540	1,852	+	+	+
5. 1:1,620	617	+	+	+
6. 1:4,860	206	+	+	+
7. 1:14,580	69	+	+	+
8. 1:43,740	23	0	+	+
9. 1:131,220	7.66	0	0	+
10. 1:393,660	2.55	0	0	+
11. 1:1,180,980	0.85	...	...	+
12. 1:3,542,940	0.28	...	...	0
13. 1:10,628,820	0.09	...	...	0
Emulsion reactions		0	+	+

per milliliter tested on the subjects, whose sensitivities are noted, reacted in subjects B and C but not in subject A. A reaction in subject C indicates that the preparation contained more than 38 units per milliliter. A reaction in subject B indicates that it contained more than 7.66 units. A negative reaction

in subject A indicates that there were less than 69 free units. Thus, it would be interpreted that this emulsion contained between 38 (0.76 per cent) and 69 (1.38 per cent) units in the aqueous phase.

The amount of free antigen could be judged also from the local subcutaneous reaction in allergic subjects. This will be discussed further in the article to follow. By suspending 0.1 ml. of radioiodinated emulsion in 2 ml. of Coca's solution and determining the radioactivity in the surrounding Coca's solution within a very short time, we made an additional estimation of the free aqueous phase.

Neutralization of Passively Sensitized Sites.—The problem here was to note whether the presence in the circulation of antigen, presumed to be released gradually from the emulsion, could be ascertained by its neutralizing effect on passively sensitized sites. The appropriate dilution of reagin was determined in the following manner. Several nonallergic subjects were sensitized with twofold serial dilutions of reagin. A subcutaneous injection of 10,000 pollen units in aqueous extract was made at a remote site. A dilution of reagin which was weaker than that reacting by this method was selected for intradermal injection in a number of sites in the same subject. One row of sites was injected intradermally with 0.02 ml. of threefold serial ragweed dilutions, ranging from 1:1,000 to 1:729,000 and the lowest concentration of pollen giving a reaction was noted. This titration established the base-line reactivity. A subcutaneous injection of 10,000 units of pollen, in either aqueous extract or emulsion, was injected immediately thereafter. Twenty-four hours later another row of sensitized sites was tested with various pollen concentrations in order to note any change in reactivity. In the case of the emulsions, we repeated this procedure at intervals of days, then weeks, always using fresh sites. In order to account for normal inactivation of reagin in the site, control sites were simultaneously sensitized with an unrelated reagin (*Alternaria*) and subsequently tested with its antigen.

Radioiodinated Ragweed Pollen Extracts.—Preparations of I^{131} -labeled ragweed extract were made by iodination of protein, according to the method of Pressman.⁴ As determined by scratch-test titrations on allergic subjects, this procedure did not alter the allergenic activity of the extract. These preparations were injected in emulsion form into three nonatopic and two ragweed-sensitive persons. In aqueous form, a deep subcutaneous injection of the I^{131} -labeled preparation was given to one nonatopic and one ragweed-sensitive person. Two separate batches of this labeled extract were made up. The first, which was given to the three nonatopic subjects who received emulsion and the two subjects who received aqueous antigen, contained approximately 1 microcurie of radioactivity per injection. The second batch, containing approximately 10 microcuries per 1 ml. of emulsion and 3,000 pollen units of antigen, was administered to the two allergic subjects. The fate of the antigen was studied by two methods. In all subjects, radioactivity at the site of injection was counted with a sodium iodide crystal and large 2 or 3

inch aperture lead shield. Counts were made immediately after injection and at intervals thereafter until the activity became impossible to measure, due to either biologic elimination or radioactive decay (half-life of $I^{131} = 8.07$ days). In the two atopic persons who received labeled emulsion, the radioactivity in the urine and blood was counted in a well scintillation counter. It was not possible to obtain radioactivity counts in the other subjects because of the relatively low radiologic activity of the injected material. The radioactivity eliminated in the urine was determined in one subject for the first six days after injection. Unfortunately, because of the unavailability of the other subject for a period of time after the injection, it was possible to collect the urine specimens only in the first six hours.

In one nonallergic subject, an attempt was made to detect radiation at the site of passive transfer of reaginic serum after injection of I^{131} -labeled emulsion at a distant site. Activity over the regional lymph nodes and thyroid gland was investigated in all subjects who received I^{131} -labeled material.

Induced Sensitivity.—Several nonallergic subjects who had received aqueous or emulsified pollen extract were tested for immediate and delayed skin reactivity with intradermal injections of pollen, varying in strength from 1:100,000 to 1:100. Several allergic subjects who had received the emulsions were also tested for delayed sensitivity.

Assay of Skin, Eye, and Nose Sensitivity.—This will be described in the article to follow.

RESULTS

Amount of Active Antigen in Aqueous Phase Ready for Release.—When deep subcutaneous injections of 10,000 units of aqueous pollen extract were given to a passively sensitized person, the sites sensitized with the reaginic serum, diluted 1:32, were the weakest in which reactions were usually obtained. An emulsified antigen administered later in such subjects or in other subjects sensitized with the same serum failed to show reactions at the sites sensitized, even with the undiluted serum. The direct scratch test with the emulsion indicated that approximately 1 per cent of the preparation was in the active aqueous phase. This estimation was further corroborated by the 1 per cent radioactivity found in the Coca's solution in which a small amount of emulsion was placed. Preparations made by us with very strong pollen extracts and with reduction of the proportion of Arlacel A-oil combination showed a considerably greater percentage of free antigen. The reactivity of the emulsion, as noted by the reaction after the subcutaneous injection in the allergic person, varied. For example, subject A, who had received treatment with 200 units of aqueous extract with moderate reaction, showed no reaction when injected with 2,500 units of the emulsion. Obviously, all that could be ascertained from that trial was that the emulsion was less than 8 per cent active; how much less is impossible to say. Another preparation containing 3,000 units in emulsion evoked a slight reaction in one person

whose reaction to 50 aqueous units was moderate, while in another subject with a moderate reaction to 25 units a marked swelling resulted from the same emulsion. This would indicate perhaps that between 1 and 2 per cent of that emulsion was in the free aqueous phase.

It was found that the radioactivity readings over the site of injection of the emulsion for the first few hours were unreliable.

Persistence of Antigen at Site.—The one allergic person who received emulsion and showed a significant drop (37 per cent) in the first two and one-half hours was the most sensitive of the subjects studied. Both of the ragweed-sensitive persons who received emulsion injections showed a more rapid rate of disappearance of I^{131} from the injection site than did the non-

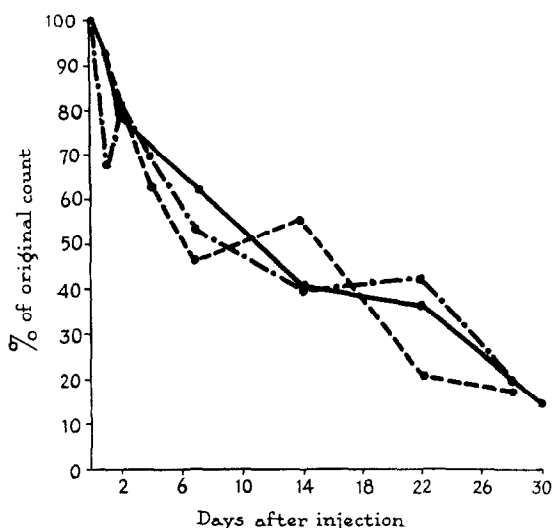


Fig. 1.—Disappearance of radioactivity from sites of injection of 1 ml. of I^{131} ragweed emulsion in three nonallergic subjects. Readings expressed as per cent of original count after correction for radiologic decay of I^{131} .

atopic subjects who received emulsion (Figs. 1 and 2). There was a gradual disappearance of radioactivity from the site until only about 20 per cent (corrected for loss due to radioactive decay) remained after four weeks in the nonatopic group (Fig. 1). Because of the relatively short half-life of I^{131} (8.07 days), this was as long as readings could be obtained in this group of those who had received approximately 1 microcurie of radioactivity in the original injection. In the two allergic subjects who received emulsified pollen extract containing 10 microcuries, it was still possible to show significant radioactivity (corrected to about 5 per cent) at the injection site after eight weeks (Fig. 2).

In the two subjects sensitive to ragweed pollen, radioactivity was removed from the site at a much more rapid rate (Fig. 2). Both of these persons had eliminated approximately 1 per cent of the calculated injected radioactivity in the urine within two hours and a total of 4 per cent in the first eight hours. A total of 31 per cent of the activity appeared in the urine of the one person

TABLE II. NEUTRALIZATION OF SENSITIZED SITES BY 10,000 UNITS OF RAGWEED EXTRACT IN AQUEOUS AND EMULSIFIED FORMS

SUBJECT	PREIN- JECTION TITER	1 DAY AFTER AQUEOUS INJECTION	PREIN- JECTION TITER	TITERS AFTER EMULSION INJECTION									
				1 DAY	1 WEEK	2 WEEKS	3 WEEKS	4 WEEKS	5 WEEKS	6 WEEKS	10 WEEKS		
A	1:81,000	1:27,000	1: 81,000	1:27,000		1:27,000	1:27,000						
B Alternaria	1:81,000	1:27,000	1:243,000	1:81,000	1:27,000	1:27,000		1:27,000					
			1: 9,000	1: 9,000	1: 9,000			1: 9,000					
C Alternaria	1:81,000	1:27,000	1:243,000	1: 9,000	1: 9,000	1: 9,000			1:9,000				
			1: 27,000	1:27,000	1:27,000	1:27,000							
D Alternaria	1:81,000	1:27,000	1: 81,000	1: 9,000	1: 9,000		1: 3,000			1:3,000			
			1: 27,000	1:27,000	1:27,000		1: 3,000					1:1,000	1:1,000

in whom the excretion of I^{131} in the urine was followed for six days. The chemical combination of I^{131} as it appeared in the urine or blood is not known. It was no longer bound to protein, as it would not precipitate with ammonium sulfate at 50 or 100 per cent saturation, trichloroacetic acid, or heat coagulation. By these methods it was readily precipitable in the original iodinated extract.

The studies of the absorption of I^{131} ragweed extract in aqueous solution showed no difference in the rate between the ragweed-sensitive and the non-atopic persons. As expected, the absorption rate was much more rapid than in those persons who received emulsified preparations. However, over 20 per cent remained after twenty-four hours and between 10 and 15 per cent

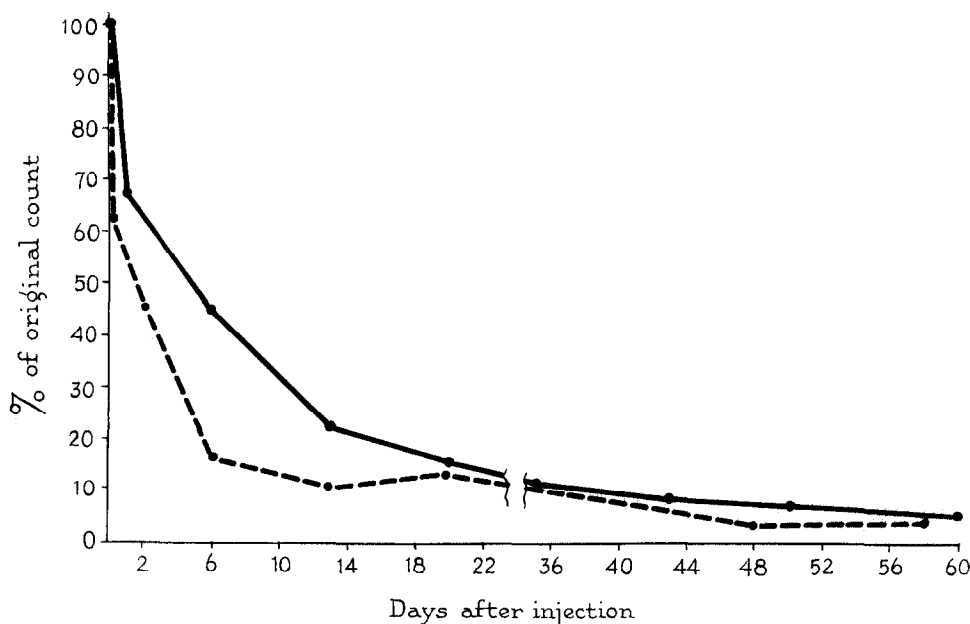


Fig. 2.—Disappearance of radioactivity (corrected) from sites of injection of 1 ml. of I^{131} ragweed emulsion in two allergic subjects.

after forty-eight hours. Between 2 and 4 per cent of the radioactivity originally injected was present at the site as long as three weeks after injection in one subject and two weeks after injection in the other (Fig. 3).

Prolonged Release of Antigen From Emulsion.—The rate of release of antigen from the emulsion and the aqueous extract can be followed from the curves of diminished radioactivity at the site of injection. Whether the radioactivity released actually represents antigen capable of stimulating protection is not known. The experiments on reagin neutralization might offer a clue. Table II shows the effect of both aqueous and emulsified antigens on the reagin. It will be seen that, within the limitations of the experiment, neutralization cannot be shown to occur consistently after the first twenty-four hours. No radioactivity of remote passively sensitized sites was noted after the I^{131} emulsion injections.

No significant radioactivity could be found over the general body surface or in the regional lymph glands at any time after the injection. Radioactivity over the thyroid gland was considerable in the subjects who had received no iodides prior to the injections and moderate in those who had been given iodides.

Production of Delayed Hypersensitivity.—Of six nonallergic subjects who had received 10,000 units of emulsified ragweed pollen extract and who were tested subsequently, five showed positive delayed reactions to intradermal tests with pollen. These reactions varied from moderate to very strong. In one subject, a 1:1,000 solution injected intradermally produced marked inflammation

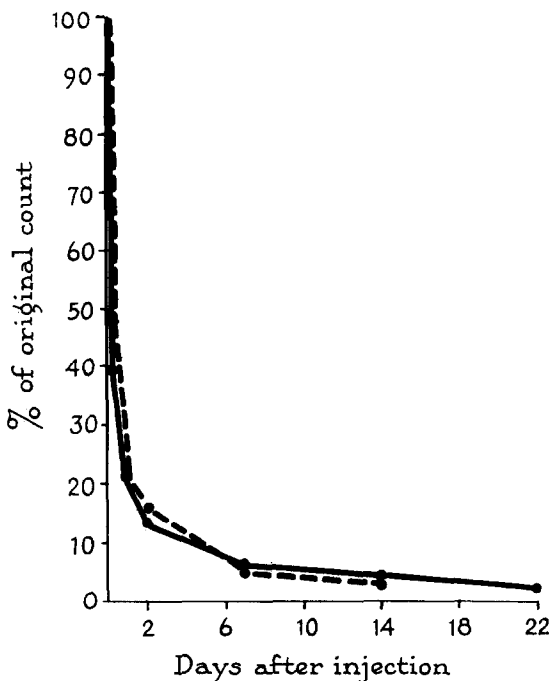


Fig. 3.—Disappearance of radioactivity (corrected) from sites of injection of 1 ml. of aqueous I^{131} ragweed extract in one allergic and one nonallergic subject.

with necrosis. It is not known when this delayed hypersensitivity began, but the first test was made five weeks after injection of the emulsion. In some of the subjects retested as long as three months after the emulsion injections, a positive reaction was still obtainable. Gel diffusion tests for precipitating antibodies to ragweed pollen antigens were negative.

The subject who had the most marked delayed skin reaction began to develop soreness, limitation of movement, and axillary gland enlargement in the arm which had received the emulsion two weeks after the injection. This reaction progressed to marked redness, swelling to about one and one-half to two times its size, then to softening and fluctuations, and finally to spontaneous drainage of sterile pus about three months after the injection. During the height of this inflammation the subject also developed typical lesions of

erythema nodosum. At the time of the development of the erythema nodosum, he informed us that he had had a sore throat a few days before and a cold at the time of the injection of emulsion.

Objective Evidence of Protection From Emulsified Antigens.—This will be discussed in a subsequent article.

DISCUSSION

A more direct way to study the reactivity of passively sensitized sites undoubtedly would have been to determine first the smallest amount of antigen in aqueous medium and then the smallest amount of emulsified antigen required for production of a reaction. We had planned to do this later, since we realized that a large number of subjects would be required. However, the occurrence of delayed reactivity following injection of the emulsified antigen in nonallergic subjects dissuaded us from proceeding along these lines.

The scratch test technique for assaying the reactivity of the emulsion could be made more accurate if there were always available a number of subjects whose differences in reactivity varied by only one dilution of the aqueous extract. This is not easily attainable and, even if it were, only threefold differences in reactivity of the emulsion could be determined. However, the method is satisfactory for rough screening to ascertain whether an emulsion is poor, good, or excellent. The local reaction occurring in the intervening twenty-four hours or so following the administration of the emulsified antigen in a subject whose tolerance is known gives an approximate indication of the amount of readily releasable antigen. There is a marked difference in local reactivity, however, dependent on unrecognized variations in the administration of the antigen and perhaps fortuitous spread of the material close to the skin surface. An example will illustrate the point. One of us injected 0.5 ml. of an emulsion of 5,000 units per milliliter into one arm and an equal volume from the same syringe into the other arm. One arm had no reaction whatsoever. The other arm began to itch shortly after the injection, and by the end of twenty-four hours an erythema and swelling had extended over practically the entire arm.

The diminution of radioactivity at the site of injection of emulsion does not necessarily prove that the difference is a measure of release of active pollen antigen. The radioactivity loss during the first twenty-four hours varied markedly in different persons. Perhaps this may be partly explained by the spread of the material to different depths in the tissues. Horizontal spread of material was measured by radiographic scanning as is employed in thyroid studies (Fig. 4). Here it will be seen that there are variations in subjects or perhaps slight differences in the technique of injection. It is also possible that the pollen antigen may be metabolized to a considerable extent at the site of injection and thus not be available as an active antigen in blood and distant tissues. The possibility of such an occurrence is supported to some extent by the failure of neutralization of sensitized sites to proceed beyond a twenty-four-hour period. It may be that this is due to the fact that either the antigen is metabolized in situ or that small amounts released in active form are still

capable of being inactivated enzymatically before combining with antibody. The finding that a preponderance of radioactivity in the blood is in uncombined form also suggests that the antigen is rapidly metabolized.

It will also be noted that the rate of loss of radioactivity appears to be in the following order of increasing rapidity: (1) from emulsions in nonallergic subjects, (2) from emulsions in allergic subjects, and (3) from aqueous extracts. This, too, would suggest a more rapid metabolism of the antigen in situ by the allergic person. The apparent, more rapid diminution of radioactivity at the injection sites in allergic subjects who had received the emulsified antigen may be accounted for by the reaction of the tissues to the free aqueous antigen.

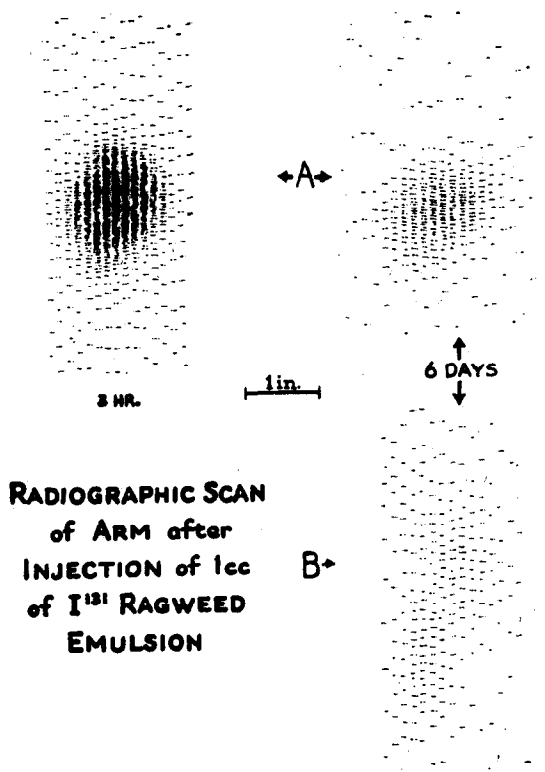


Fig. 4.—Pattern of spread of radioactivity in arms of two allergic subjects after injection of 1 ml. of I^{131} ragweed emulsion. Scannings obtained with a Model 1700 isotope scanner made by Nuclear Chicago. Each small bar represents thirty-two detected disintegrations.

The resulting increased vascular permeability, possibly increased phagocyte infiltration, enzymatic mechanism dependent on the antigen-antibody reaction, or other inflammatory changes may be responsible. The finding of measurable radioactivity in situ from injections of aqueous extracts for as long as three weeks is rather surprising in view of previous concepts about absorption of aqueous extracts. In a similar study in rabbits with radioiodinated bovine gamma globulin and egg albumin, Talmage and Dixon⁵ in 1953, reported the disappearance rate from the emulsion to be similar to that which we found in

man. With the aqueous preparation, however, only 1 per cent of the bovine gamma globulin persisted at the site after two days, and less than 1 per cent of the egg albumin remained after one day. Is our finding true for ragweed protein and not for other proteins? Is it likely to be shown with injected uncombined I^{131} ? All these points require further investigation. Nevertheless, this finding does cast some doubt on the validity of the concept that it is only the persistence of antigen which accounts for the superiority of the repository treatment, since some antigen does persist even with the aqueous extracts. It should be realized also that the natural decay of I^{131} places practical limitations on the ability to locate remnants of the antigen for a long time with either the aqueous or the repository preparations. In eight weeks an initial count of 10,000 will be reduced to about 40, a count difficult to detect.

It should be emphasized here that because of the very nature of the difficulty of conducting all experiments with the same batch of emulsion the emulsions may have varied from time to time. This may account for the difference in quantities estimated to be released as determined by different methods. It should be noted at this point also that we may be far from correct in discussing radioactivity as if it were synonymous with antigen. It may be that only part of the radioiodinated pollen extract is actually the antigenic material. The separation of antigenic from nonantigenic components is of course a problem of issue in this and other fields of investigation. We are proceeding with attempts at such separation.

The production of delayed hypersensitivity poses both basic and practical questions. If the emulsion stimulates the immune response, it may produce several effects. It might induce delayed hypersensitivity to the injected antigen, as it has done in several of these subjects. It might produce sensitivity to another antigen, as to microbes present in the body at the time of injection. There is a strong suggestion of this possibility in the subject who developed erythema nodosum. This delayed reactivity appears to have occurred only in nonallergic subjects. However, more extensive investigations are planned to corroborate this phenomenon and to attempt to clarify it.

SUMMARY

1. Methods employed to study the degree of immediate release of ragweed pollen antigen from emulsions have consisted of the ability of emulsified and injected antigens to produce reaction in passively sensitized sites, the reaction on scratch test in allergic subjects after titration with aqueous extracts, the radioactivity of the blood and urine shortly after the injection of radioiodinated emulsion, the local reaction of the emulsion in allergic persons, and the radioactivity release from the emulsion placed in an aqueous solution.

2. With the emulsions studied, such immediate release varies from about 1 to 3 per cent, depending on the method used and the batch of the emulsion.

3. Persistence of the antigen at the site of injection has been studied in relation to the persistence of radioactivity of radioiodinated pollen extract. Traces of the radioiodination can be shown to exist for as long as four weeks

with the emulsion and three weeks with the aqueous preparation. It is possible that longer persistence might be shown with our use of more potent radioactivity.

4. By the method of reagin neutralization, it could not be proved that antigen is released from the emulsion in active form beyond the twenty-four-hour period. It is admitted that this is not final evidence that the antigen is unavailable.

5. The production of delayed hypersensitivity with the emulsified antigen in nonallergic subjects is of basic and clinical significance.

6. Further amplification of these and other studies is needed to perfect the antigenic preparation and to understand the immune mechanisms involved.

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ADDENDUM

In five additional nonallergic persons, we have been able to confirm the persistence of some radioactivity at the site of injection of aqueous ragweed extracts for periods up to three weeks.

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