IMMUNOCHEMICAL STUDIES OF ANTITOXIN PRODUCED IN NORMAL AND ALLERGIC INDIVIDUALS HYPERIMMUNIZED WITH DIPHTHERIA TOXOID

IV. Differences between Human Precipitating and Non-Precipitating Skin-Sensitizing Diphtheria Antitoxin as Shown by Electrophoresis

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It has been found that at least two varieties of diphtheria antitoxin are formed in subjects who are hyperimmunized with diphtheria toxoid (1, 2). In a large proportion of individuals the immune response is characterized by the simultaneous production of precipitating and non-precipitating antitoxins. A small proportion of subjects form only one or the other of these antitoxins.

Because of its close similarity to allergic reagin of the hay-fever variety, skin-sensitizing antitoxin has been selected as a model for further investigations of the qualities peculiar to reagin. The advantages of the diphtheria system over other less well characterized antigen-antibody systems have been enumerated in an earlier publication (1). In view of studies of the egg white system by Vaughan and Kabat (3) which showed that the immediate type of skin reaction is caused by antibodies to trace substances rather than to the major antigenic component, it was important in our studies to demonstrate that skin sensitization is specifically effected by non-precipitating antitoxin. Evidence has been presented (2, 4) which indicates that immediate wheal reactions are caused by non-precipitating skin-sensitizing antitoxin, and that reactions elicited following the intradermal injection of toxoid are due to specific interaction between toxoid and this variety of antitoxin.

In order to gain additional information concerning the nature and specificity of immediate wheal reactions, individual protein components of various sera were isolated with the object of analyzing their comparative skin-sensitizing abilities. It has been demonstrated (5) that the highly purified gamma globulin fraction obtained by cold ethanol precipitation (6) from a serum containing non-precipitating, skin-sensitizing antitoxin has lost most of its ability to cause immediate wheal reaction although a corresponding antitoxin loss does not occur. The reason for this behavior is as yet unknown. However, it

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is possible that exposure to ethanol under some conditions may alter a labile serum component necessary for this type of reaction. It was for this reason that the more gentle method of electrophoresis in a starch-supporting medium was selected as a means of serum fractionation.

The present report is based upon differences observed between precipitating and non-precipitating skin-sensitizing antitoxin utilizing the technique of zone electrophoresis.

Materials and Methods

The purified diphtheria toxin[1] and toxoid,[2] and the techniques employed in carrying out intracutaneous neutralization tests in rabbits, passive transfers to human skin, and precipitin reactions were the same as described previously (7, 1, 8). Human sera containing antitoxin were obtained from adult subjects 9 to 19 days after a single booster dose of diphtheria toxoid.[3] Specimens containing titers of at least 40 units per cc. were found most suitable for these studies. Nine sera were examined, including those from subjects Hu and O'D which were described in earlier investigations (2, 5).

Electrophoresis on a starch-supporting medium was carried out using the technique of Kunkel and Slater (9). Because the potato starch (Eimer and Amend) contained pyrogenic materials, the starch used for each experiment was thoroughly washed prior to use with at least 2.0 liters of physiological saline solution and 0.5 liter of pH 8.6 barbital buffer. The amount of serum used for each experiment was 2 cc., to which was added 0.5 cc. of barbital buffer. Electrophoresis was carried out at 4°C. using barbital buffer at pH 8.6 and ionic strength 0.1 (10). A current of 500 volts and 50 to 60 milliamperes was applied and maintained over a period of from 18 to 20 hours. Migration of certain electrophoretic components was estimated by observing the movement of serum pigments during the electrophoretic run (11). In many experiments a few grains of brom-phenol blue powder were added to a serum prior to electrophoresis. This made it possible to observe the subsequent movement of the dye-stained albumin (12). After the electric current had been discontinued, the starch block was cut into ½ inch segments and each segment was suspended in 2 cc. of physiological saline. The protein content of eluates from the starch segments was determined by use of Folin-Ciocalteu reagent (11, 13). Correction factors for the relative color values of the different fractions were not applied.

Gelatin was added to the eluates in a concentration of 200 μg. per cc. in order to stabilize the dilute proteins. In instances in which pooling of several eluates was desirable, the pool was lyophilized, reconstituted to approximately the original serum volume (generally 2 to 3 cc.), and dialyzed against normal saline. Following filtration through a Swinny type filter[4] and the addition of merthiolate to a concentration of 1:10,000, the antitoxic activity of individual eluates or appropriate pools of eluates was determined by intracutaneous rabbit tests, the capillary precipitin technique, and the method of passive transfer in human Schick-positive recipients. Immunological tests of eluates were performed immediately following electrophoretic separation of the serum proteins.

[1] Purified diphtheria toxin was kindly supplied by Dr. A. M. Pappenheimer, Jr., Department of Microbiology, New York University, College of Medicine.

[2] Purogenated diphtheria toxoid used in certain portions of this study was provided through the courtesy of Dr. Henry Piersma, Lederle Laboratories Division, Pearl River, New York.

[3] The author wishes to thank Dr. James McComb, Massachusetts Antitoxin Laboratory, Jamaica Plain, Boston, Massachusetts, for providing Schick testing materials and diphtheria toxoid for purposes of immunization.

[4] Becton, Dickinson and Company, Rutherford, New Jersey.

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RESULTS

Comparison of patterns obtained by starch electrophoresis demonstrated a difference in the migration of the precipitating and skin-sensitizing antitoxins. Practically all of the antibody was included within the gamma globulin fraction, but the skin-sensitizing antitoxin was concentrated in the faster moving portion (γ_1) as shown in Fig. 1, while most of the precipitating antitoxin migrated in a slower moving (γ_2) component (Fig. 2).

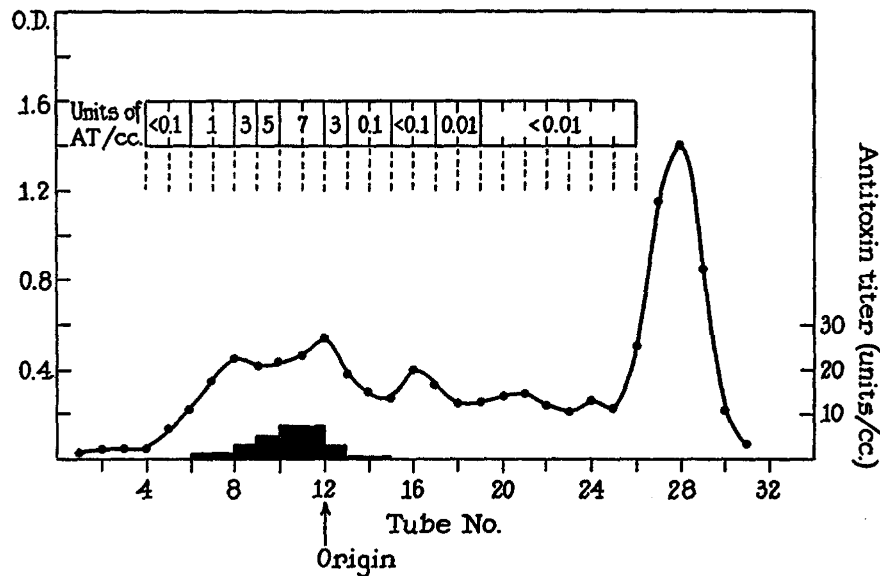

FIG. 1. Electrophoretic pattern of non-precipitating skin-sensitizing antitoxic serum Hu showing distribution of antitoxic activity in serum fractions. The curve represents the relative protein concentration of individual eluates as determined spectrophotometrically. Antitoxin content is depicted by the underlying solid black portion.

Non-Precipitating Skin-Sensitizing Antitoxic Serum.—Two specimens designated Hu (40 units of antitoxin per cc.) and Chr (50 units of antitoxin per cc.) were analyzed. Under the conditions of these experiments, separation of serum into five components (gamma, beta, $alpha_1$, and $alpha_2$ globulins, and albumin), spread out over 31 to 33½ inch wide starch segments, occurred in a period of from 18 to 20 hours. The shift of diphtheria antitoxin toward the cathode (solid black, Figs. 1, 3, and 4) is less than the electroosmotic flow and thus represents migration in the direction of the anode. Migration of skin-sensitizing antitoxin was such that demonstrable antitoxic activity occurred over a considerable range of the gamma globulin and portions of the beta globulin (shown in Fig. 1) with a preponderance of activity localized near the origin or faster moving portion (γ_1) of the gamma globulin (segment 12).

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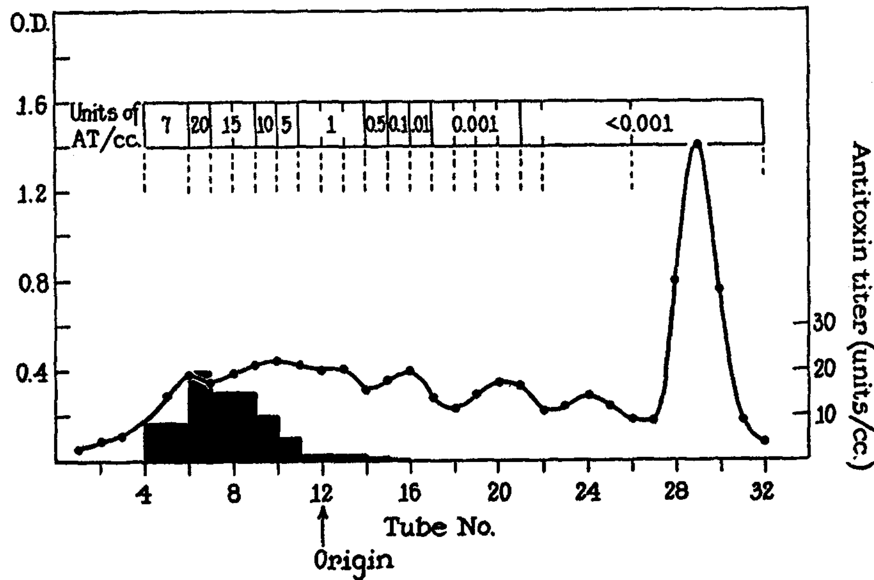

FIG. 2. Electrophoretic pattern of precipitating antitoxic serum O'D showing distribution of antitoxic activity in serum fractions. The protein concentration and antitoxin content are represented as in Fig. 1.

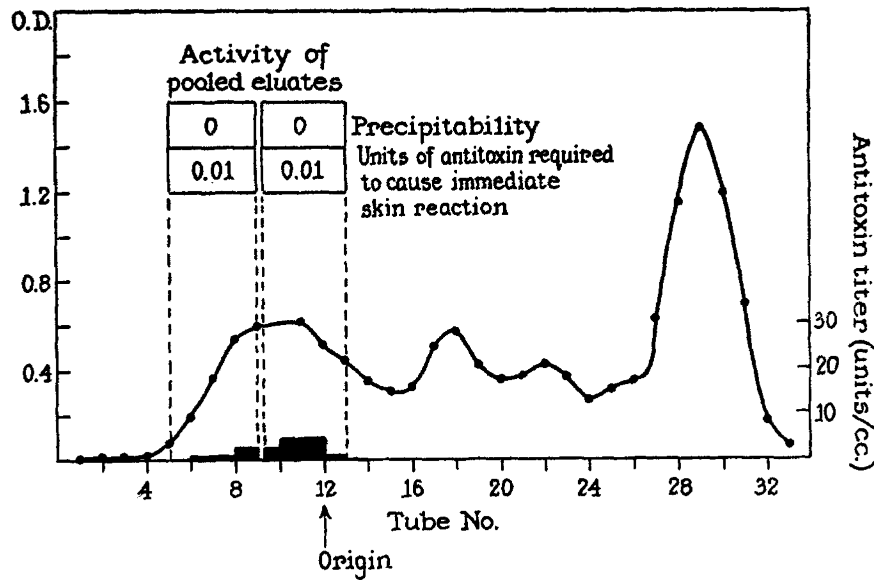

FIG. 3. Electrophoretic pattern of non-precipitating antitoxic serum Chr showing distribution of skin-sensitizing activity in gamma globulin. The precipitability and skin-sensitizing activity of pooled and concentrated eluates is indicated in boxed portions above the electrophoretic curve.

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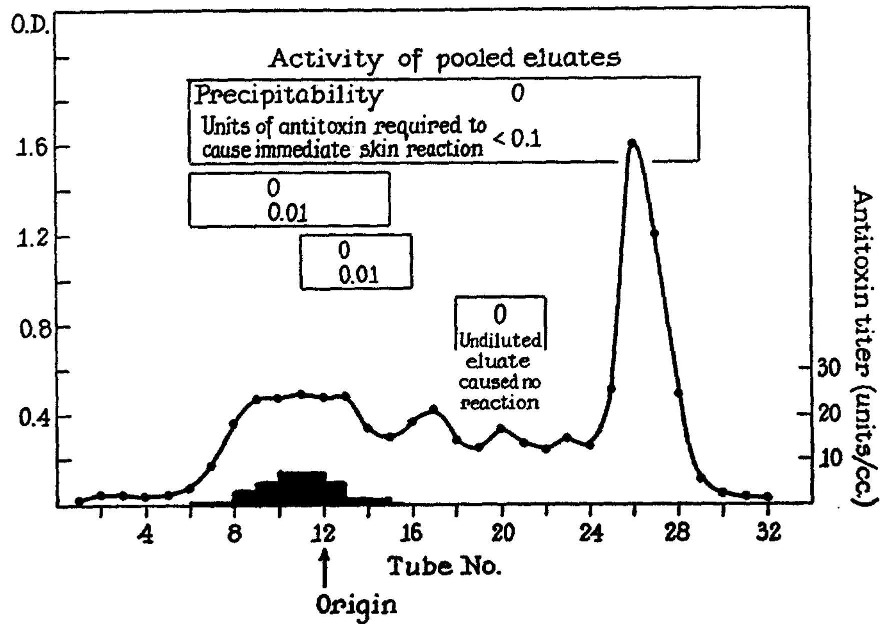

FIG. 4. Electrophoretic pattern of non-precipitating antitoxic serum Hu showing skin-sensitizing activity of eluates obtained from various serum fractions. The specific activity of pooled and concentrated eluates is indicated in boxed portions above the curve.

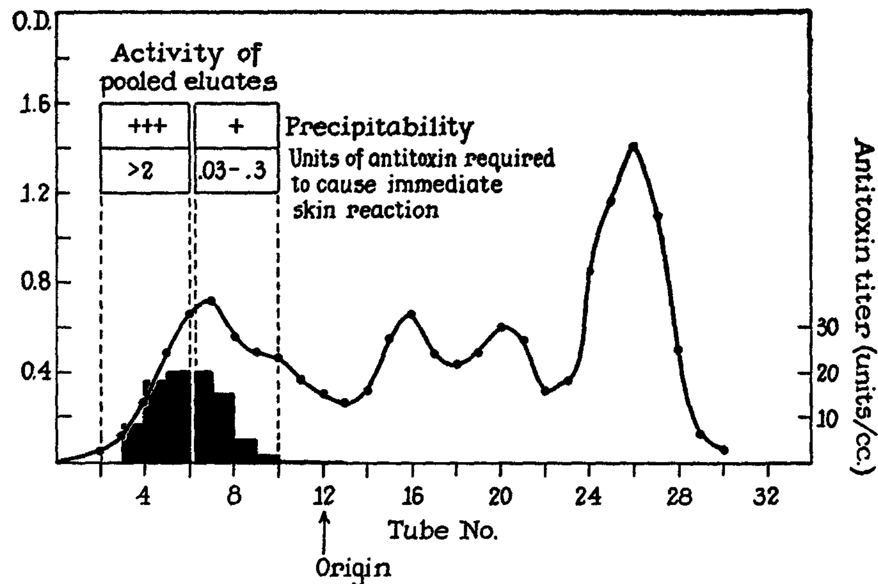

FIG. 5. Electrophoretic pattern of precipitating antitoxic serum Bas showing distribution of antitoxic activity in gamma globulin. Characteristics of pooled and concentrated eluates are shown as described for Figs. 3 and 4.

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Recovery of antitoxin appeared to be of the order of 50 to 75 per cent, although no attempts were made to obtain maximum antibody recovery. Examination of the appropriate eluates containing antitoxin, using the capillary precipitation technique, showed no precipitation in fractions obtained from sera Hu and Chr. The skin-sensitizing activity of fractions was determined by passive transfer experiments using appropriately diluted

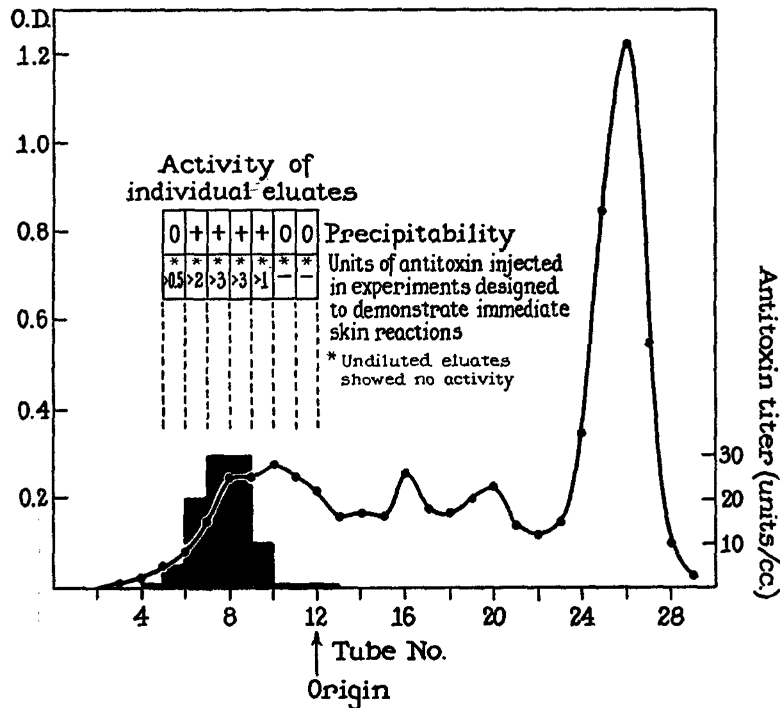

FIG. 6. Electrophoretic pattern of precipitating antitoxic serum Ow showing distribution of antitoxic activity in gamma globulin. Characteristics of individual eluates are represented as described above.

eluates in human Schick-positive recipients. Skin-sensitizing activity is expressed in terms of the amount of antitoxin which upon challenge with intradermal toxoid (0.1 Lf in 0.02 cc.) yields reactions greater than a toxoid control (0.1 Lf at an unprepared skin site). Thus, extracts from appropriate portions of the gamma globulin of serum Chr were able to cause significant wheal reactions upon challenge in skin sites containing 0.01 unit of antitoxin (Fig. 3). Pooled eluates in these experiments were prepared from starch blocks 6 to 9 and 10 to 13. Fractions from serum Hu behaved similarly as shown in Fig. 4. The results summarized in this figure were obtained with

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pools prepared from aliquots of the individual eluates and the samples tested represented pools from the following starch blocks: (*a*) 6 to 30, (*b*) 6 to 15, (*c*) 12 to 16, (*d*) 18 to 22. Restoration of the combined total eluates (eluates 6 to 30) to original serum volumes as previously described yielded a preparation which appeared slightly less potent than whole serum when 0.1 unit of each specimen was challenged intradermally with toxoid. Preparations of the alpha globulin (eluates 18 to 22) were found to be completely unreactive.

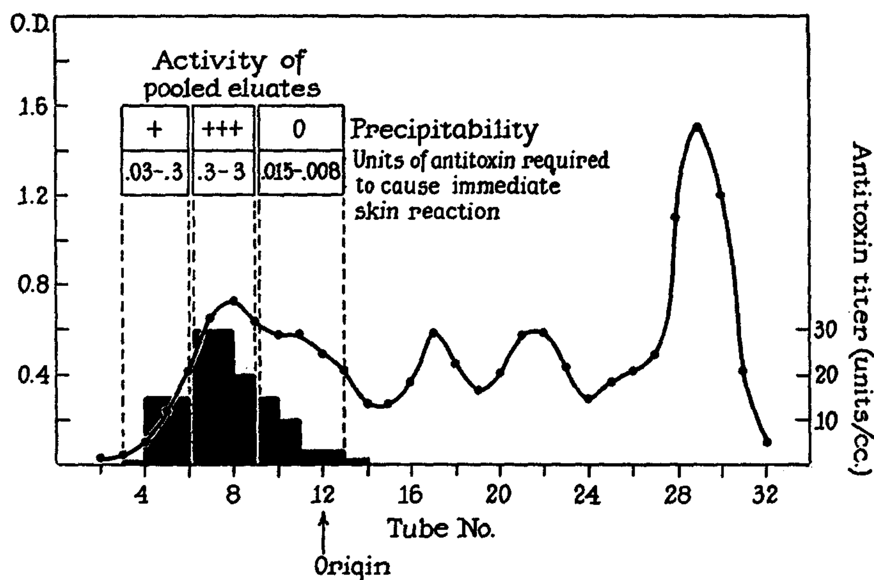

FIG. 7. Electrophoretic pattern of whole serum Pag containing mixture of precipitating and non-precipitating skin-sensitizing antitoxins showing distribution of activity in gamma globulin. Characteristics of pooled and concentrated eluates are represented as described above.

Gamma globulin eluates (6 to 15 and 12 to 16) were reactive in human skin at a level of 0.01 unit.

Hu serum obtained 20 months subsequent to hyperimmunization with toxoid and 18 months following withdrawal of the specimens described above still showed a high titer of antitoxin, displayed the same electrophoretic pattern, and also exhibited the property of passive transfer.

Precipitating Antitoxic Serum.—Four serum specimens were examined which are termed Har (100 units per cc.), O'D (110 units per cc.), Bas (160 units per cc.), and Ow (200 units per cc.). Qualitative precipitin tests showed rapid precipitation with toxin occurring maximally over a range of toxin concentrations below and close to the zone of antitoxin equivalency. A quantitative study of serum O'D showed that it contained 240 μg. per cc. of specifically

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precipitable antitoxic N. In the case of serum Bas, the antitoxic N specifically precipitable by equivalent toxin was 248 μg. per cc. Electrophoretic separation carried out on these specimens showed that migration of diphtheria-precipitating antitoxin was for the most part considerably slower than that of skin-sensitizing antitoxin, with a preponderance of activity localized in the

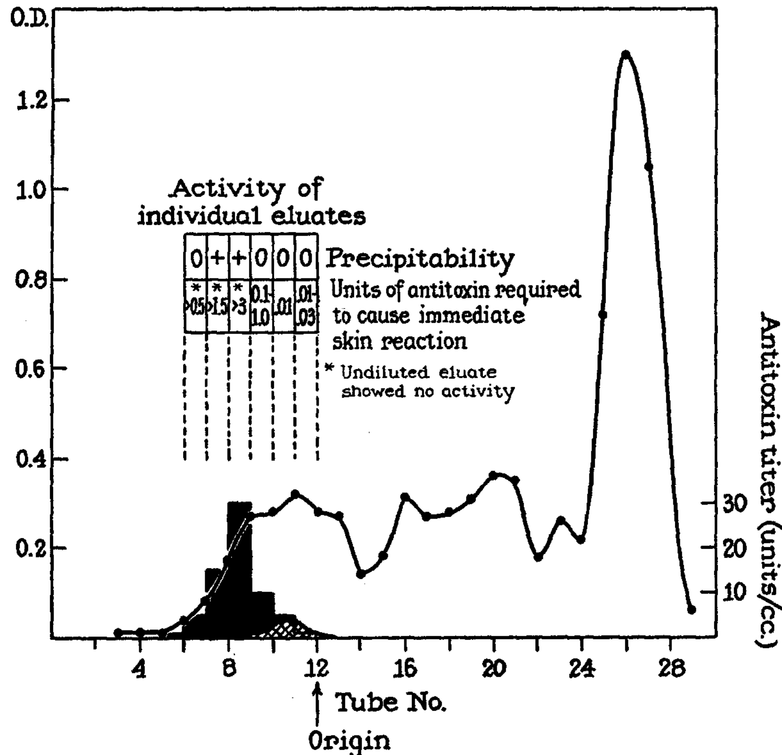

FIG. 8. Electrophoretic pattern of whole serum Reb containing mixture of precipitating and non-precipitating skin-sensitizing antitoxins showing distribution of activity in gamma globulin. Skin-sensitizing antitoxin is depicted in cross-hatched portion under gamma globulin curve. Characteristics of individual eluates are presented as described above.

slowest moving portions of the gamma globulin (γ_2). Upon examination of these fractions using the capillary technique, visible precipitation was found to occur in those eluates which contained 10 or more units of antitoxin per cc. Passive transfer to the skin of human recipients showed little or no wheal and erythema reactivity (Figs. 5 and 6).

Serums Containing Mixtures of Precipitating and Non-Precipitating Skin-Sensitizing Antitoxin.—Three specimens were analyzed, and are designated as Pag (240 units per cc.), Reb (225 units per cc.), and T. J. (140 units per cc.).

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All showed marked skin reactivity upon challenge with purified diphtheria toxoid at the time of the postimmunization bleeding, and the sera from these subjects also contained demonstrable precipitating antitoxin. Qualitative precipitin tests showed that heavy and rapid precipitation occurred when the appropriate amounts of toxin were added. In the case of Pag, the amount of antitoxic N in 1 cc. of serum which was specifically precipitable by toxin was 342 μg. per cc. The properties of fractions obtained by electrophoresis of sera Pag and Reb are shown in Figs. 7 and 8.

It will be observed in Figs. 7 and 8 that most of the antitoxin migrated with the slower moving portions of the gamma globulin and behaved as precipitating antitoxin. However, the antitoxin present in lesser amounts in the faster migrating gamma globulin was not precipitable. Passive transfer of pooled or individual eluates obtained from starch segments in proximity to the origin (segments 10 to 13 for sera Pag and Reb and 13 to 15 for serum T. J.) showed that they were capable of causing wheal reactions upon toxoid challenge when amounts as small as 0.008 to 0.015 unit were injected. On the other hand, eluates from the slower migrating precipitating antitoxin either caused no reactions (Reb eluates 6 to 9) or mild reactions when relatively large amounts of antitoxin were used (Pag 4 to 9 and T. J. 8 to 12). As the figures indicate, in the case of serum Pag, pooled eluates were used for test purposes (Fig. 7) whereas individual eluates of serum Reb were tested (Fig. 8).

DISCUSSION

Kekwick and Record (14) have used chemical fractionation and electrophoresis to show that diphtheria antitoxic horse sera contain two antitoxins associated with the beta and gamma globulins, but electrophoretic differences between diphtheria antitoxins have not previously been studied in human sera. The present work shows that protein components of human diphtheria antitoxic sera can be effectively separated by the method of zone electrophoresis so that it is possible to differentiate between precipitating and skin-sensitizing antitoxins. Precipitating antitoxin migrates largely with a slower moving portion of the gamma globulin (γ_2) as contrasted with non-precipitating skin-sensitizing antitoxin, which migrates largely with a faster moving gamma globulin (γ_1 or β_2) component.

The properties inherent in both varieties of human antitoxin appear to remain unimpaired by the procedure of starch electrophoresis. It is of particular interest that antitoxic fractions from skin-sensitizing sera behave similarly to whole sera in the ability to cause wheal reactions. Thus, 0.01 unit of antitoxin was found to be reactive in human skin upon toxoid challenge. An additional related finding of interest is the fact that subject Hu maintained a high titer of skin-sensitizing antitoxin two years after receiving a booster dose of toxoid. This is in contrast to the usual secondary antitoxin response in human

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subjects which is characterized by a comparatively rapid rise and fall in titer (15, 1).

Fractionation of sera containing skin-sensitizing antibody has been accomplished in earlier studies using several methods, including ammonium or sodium sulfate, cold ethanol, and free electrophoresis. Sherrer (16) and Stull, Sherman, and Cooke (17) applied the technique of salting out of fractions from sera containing reagin. They found that the pseudoglobulin fraction possessed more skin-sensitizing activity than did euglobulin or albumin which were relatively inactive. Subsequent separation experiments of Cooke and his coworkers (18) using free electrophoresis showed that purified gamma globulin from skin-sensitizing sera was about ten times less active in its ability to be passively transferred than was whole serum at comparable gamma globulin strengths. Newell and coworkers (19), on the other hand, previously applied the same technique and found skin-sensitizing antibody in the gamma globulin but not in the beta globulin fraction. Other work on sera containing skin-sensitizing antibody by Campbell and his coworkers (20), using the method of electrophoresis convection, indicated that reagin activity was associated with alpha and beta globulins particularly when the serum was from a patient with immediate type skin sensitivity to a single substance. However, it was shown by Campbell, *et al.* (20) that reaginic activity occurred in all globulin fractions when the serum was from a patient who was sensitive to numerous materials. Loveless and Cann (21) used the same method to fractionate sera from insulin-sensitive and ragweed-sensitive patients. Their results suggested that skin-sensitizing activity is concentrated in beta globulin. Menzel and coworkers (22) used combined chemical fractionation and electrophoresis and found that reagin occurred in the gamma globulin in some, but not all sera, and that beta globulin was present in most specimens which possessed the ability to give passive transfer reactions. Vaughan, Favour, and Jaffee (23) fractionated reaginic sera by means of cold ethanol and demonstrated that fraction II (almost pure gamma globulin) was weak in its ability to sensitize human skin as compared with the reactivity of whole serum or of serum fractions containing beta or alpha globulins.

Our own experience using the techniques of electrophoresis and of cold ethanol precipitation (5) in separating components of sera containing skin-sensitizing antitoxin suggests that the method employed may be of considerable importance in the interpretation of biological reactivity related to a given fraction. Thus, fast migrating gamma globulin (γ_1) separated by electrophoresis and containing skin-sensitizing antitoxin was equivalent to whole serum in its ability to yield wheal reactions upon toxoid challenge. On the other hand, highly purified gamma globulin obtained by cold ethanol fractionation was 50 times less active than whole serum. This fact, in addition to evidence that marked spread of antitoxin through other globulin fractions occurred follow-

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ing alcohol fractionation, indicates that the ethanol procedure causes changes in the distribution and activity of skin-sensitizing antitoxin which do not occur when the more gentle method of starch electrophoresis is used.

The ability to separate diphtheria antitoxins by electrophoresis affords certain advantages in a study of their biological properties. The fact that the skin-sensitizing antibodies which were investigated in the present study are also antitoxic enables one to relate skin-sensitizing titers to antitoxin titer which in turn can be related to protein concentration. Similar investigation of hay-fever pollen antibodies has of necessity utilized skin-sensitizing activity as sole index of antibody activity and this has been related to the protein concentration of a given fraction as a means of expressing its degree of potency. In the case of diphtheria antitoxin, the technique of passive transfer may indicate the presence of only a small portion of the total antitoxin present, since our studies have demonstrated that certain sera contain precipitating antitoxin in addition to skin-sensitizing antitoxin.

The use of immediate wheal reactivity as an index of total antibody in fractionated allergic sera may introduce an additional error in view of the finding that "blocking" antibody is demonstrable in gamma globulin fractions separated by starch electrophoresis (4) and by electrophoresis convection (21). Thus, mixtures of different types of non-precipitating antibody may not be detectable on the basis of a single biological property and it is possible that previous differences in skin activity in fractions from allergic sera separated by electrophoresis (18, 22) may have been due in part to the presence of varying amounts of blocking antibody.

We have examined sera containing non-precipitating antitoxin which does not sensitize human skin. This variety of antitoxin appears to occur by itself and also in mixtures with other varieties of antitoxin. Certain specimens of non-precipitating, non-skin-sensitizing sera cause inhibition of wheal reactions in appropriately prepared recipients. The electrophoretic mobility of this antitoxin is similar to that of precipitating antitoxic sera. The properties of these sera will be described in a future report.

SUMMARY

Electrophoresis on a starch-supporting medium was used to fractionate sera containing human diphtheria antitoxin of the following varieties (*a*) precipitating antitoxin, (*b*) non-precipitating skin-sensitizing antitoxin, and (*c*) mixtures containing precipitating and skin-sensitizing antitoxins. Aliquots of the protein fractions thus separated were tested for activity using the rabbit toxin neutralization test, precipitin techniques, and passive transfer tests in human skin.

Non-precipitating, skin-sensitizing diphtheria antitoxin migrated largely as a fast moving gamma (γ_1) globulin. Passive transfer studies of isolated

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antitoxic fractions showed that they were as potent as whole serum in the ability to cause immediate wheal reactions. These fractions were not precipitable using appropriate quantities of purified toxoid.

Precipitating diphtheria antitoxin migrated largely as a slow moving gamma (γ_2) globulin. Isolated antitoxic fractions of appropriate strength obtained from representative sera were precipitable by toxin and were unable to cause immediate wheal reactions upon toxoid challenge in human recipients.

Mixtures of skin-sensitizing and precipitating antitoxins were separable by the technique of starch electrophoresis. The individual components removed from mixtures by this method retained the properties by which they could be characterized in whole serum.

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