A COMPARISON OF HUMAN ANTISERA TO PURIFIED DIPHTHERIA TOXOID WITH ANTISERA TO OTHER PURIFIED ANTIGENS BY QUANTITATIVE PRECIPITIN AND GEL DIFFUSION TECHNIQUES*

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There are numerous studies of multiple antigen-antibody systems employing the technique of localization and separation of specific precipitates in a gel matrix (1–6). Attempts to correlate such information with that obtainable by other techniques, such as the quantitative precipitin method (*cf.* reference 7), are, however, few (6, 8). The studies described below are an attempt to provide this correlation by investigating relatively pure protein and carbohydrate antigens by gel diffusion methods and the quantitative precipitin technique and applying the results to a relatively impure system, diphtheria toxin-antitoxin.

Oudin (2), Ouchterlony (3, 4), Becker *et al.* (9), Neff and Becker (10), Preer (6) and Spiers and Augustin (11) have published theoretical treatments of diffusion of single and multiple antigen-antibody systems through agar. It has been shown that concentrations of antigen or antibody can be estimated in single diffusion (Oudin) from the rate of migration or in double diffusion (Ouchterlony, Preer) from the final location of the precipitate. Diffusion coefficients and equivalence ratios of antigen and antibody can also be estimated.

In the experiments to be reported diffusion coefficients obtained by physical methods have been used to predict the position of a toxin-antitoxin precipitate in agar at equivalence and, from this information, to help identify a particular band from among several as toxin-antitoxin. Antidextran, antipneumococcus polysaccharide, anti-blood group B antibodies, and anti-γ_2-globulin were studied in agar, and the data obtained were applied, by analogy, to estimate amounts of antibodies in human antitoxin to antigens other than toxin. Thus, from quantitative data obtained with systems precipitating as a single band in agar, the components of a com-

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plex diphtheria toxin-antitoxin system have been described quantitatively, albeit roughly.

This toxin-antitoxin system has become the subject of increasing immunochemical debate since the publication (12–18) of studies attributing skin-sensitizing properties of certain human antisera to purified diphtheria toxoid to a non-precipitating antitoxin. Since, as shown in this laboratory (19), the capacity of rabbit anti-crystalline egg albumin sera to sensitize human skin is unrelated either to precipitating or non-precipitating anti-egg albumin or to antibodies to the major antigens of egg white, but is caused by an antibody to trace impurities, it was suggested that skin sensitization in the diphtheria toxin-antitoxin system might perhaps similarly be due to some unrelated antigen-antibody system present as an impurity.

Horse antitoxin, prepared against purified toxoids, had been shown by numerous investigators (*e.g.* 20–22) to contain antibodies to antigens other than toxin. Pope *et al.* (20) have reported that a preparation of diphtheria toxin from Dr. Pappenheimer (*cf.* reference 23) stated to be 95 per cent pure contained numerous other antigens.

In immunization of Schick-negative individuals, it has been assumed (12, 17) that an anamnestic response was produced solely to toxoid. More recently Kuhns and Dukstein, however, 5 years after the original studies (24), have reported that in some human beings their purified toxoid produced antibodies to impurities. The studies described below confirm that impurities in the purified toxoid preparations, used to elicit antitoxin production (12), are antigenic in man. The amount of antibody other than antitoxin in Schick-negative individuals has been estimated by agar diffusion analysis combined with the quantitative precipitin technique. With sera from these individuals, quantitative precipitin curves comparing purified and crude toxin and toxoid are presented.

Because much of the evidence for a non-precipitating skin-sensitizing antitoxin was derived from experiments employing one serum, Hu, a sample of this serum was examined with especial care for the presence of precipitins—either to toxin or to any other antigen. The absence of antibodies other than antitoxin by the Oudin method had been reported (17) but now this antiserum has been shown to contain precipitins to antigens other than diphtheria toxin.

These results show that the diphtheria toxin–human antitoxin system, in accord with investigators who studied the system using horse antitoxin, cannot at present be considered other than a crude mixture of antigens and the antibodies formed to them and has severe limitations in the study of hypersensitivity. Indeed the system, with materials presently available, excluding crystalline diphtheria toxin (25) and the preparations of Relyveld *et al.* (26), is best used as a model for multiple antigen-antibody systems.

Materials and Methods

Purified Antigen-Antibody Systems.—The following purified antigens were employed in the gel diffusion analyses: Dextran 279, a native dextran with molecular weight (number average) of *ca.* 2,000,000 (*cf.* reference 27); dextran NRC fraction 4, lot No. 3497979 (*cf.* reference 28), molecular weight (weight average) of 91,700 supplied by Commerical Solvents Corpora-

tion, Terre Haute, Indiana; blood group B substances, horse 4–25 per cent (*cf.* reference 29) and human PM 10 per cent (*cf.* reference 30); pneumococcus Type II preparation Squibb fraction SIIB1 (*cf.* reference 31); and purified human γ_2-globulin, fraction $II_{1,2}$ obtained from E. R. Squibb and Son (*cf.* references 32, 33).

The following antisera were also employed in these analyses: Human antidextran serum 176_{D_2} (*cf.* reference 28); horse anti-pneumococcal serum No. 513 obtained through the courtesy of Dr. Jesse L. Hendry, New York State Department of Health Laboratory; human anti-horse B (4–25 per cent) serum No. 310 (*cf.* reference 34); and rabbit anti-γ_2-globulin (*cf.* reference 35).

Diphtheria Toxin-Antitoxin System.—The diphtherial antigens used in the gel diffusion analyses and quantitative precipitin determinations, kindly provided by Mr. W. S. Hammond, Lederle Laboratories, Pearl River, New York, were: purified diphtheria toxoid No. 42929-278 (1560 Lf/ml.) grown on a modified semi-synthetic medium (36) and purified by the Pillemer and Toll procedure (37); crude toxoid No. 42928-1074 (49 Lf/ml.) produced on a veal infusion medium; purified toxin No. E-2-2 (165 Lf/ml.) purified by the method described by Lepow and Pillemer (38); crude toxin No. 42286-4733A (48 Lf/ml.) grown on veal infusion; and culture filtrate prepared in a medium with a high iron concentration and containing less than 5 M.L.D./ml. Two preparations of purified toxin, one obtained from Dr. Irwin Lepow, Western Reserve University, and one prepared by Dr. Melvin Cohn,[1] Washington University, were also used in the gel diffusion studies.

Schick Testing.—Medical students were injected intradermally with 0.1 ml. (1/50 M.L.D.) of purified toxin and, as a control, 0.1 ml. of purified toxoid.[2] The sites were examined after 15 to 30 minutes, 24 hours, and periodically thereafter until 5 days had elapsed.

Immunization Procedure.—Schick-negative individuals who showed no sensitivity to either toxin or toxoid were bled and injected subcutaneously with 0.75 ml. (40 Lf) of purified toxoid (lot KP 28D4) obtained from Dr. James A. McComb of the Massachusetts Department of Public Health, Division of Biologic Laboratories. Kuhns and Pappenheimer (12) also used purified toxoid[3] obtained from the Massachusetts Laboratory. Seven to 9 days after immunization a sample of blood was drawn and qualitative precipitin tests using purified toxoid were made. After 24, 48, and 72 hours, the tubes were centrifuged and another portion of toxoid was added. If maximum precipitation occurred with 16 Lf or more of toxoid, a 500 ml. blood sample was obtained. All sera, pre-and post-immunization were kept in a deep freeze without preservative, following the procedure of Kuhns and Pappenheimer (12). It was also established by direct comparison that antisera could be kept in the refrigerator after addition of merthiolate and of phenol to concentrations of 0.01 and 0.25 per cent respectively without affecting their quantitative precipitin behavior.

Occasionally individuals were immunized who were hypersensitive to Schick test reagents. This was done, however, only following negative allergic reactions upon retesting with 0.1 ml. of a dilution containing 0.01 Lf/ml. of the Massachusetts purified toxoid, which was to be used for immunization.

In Vivo Titration of Antisera.—The method of Fraser (39) was followed to assay post-immunization sera for the ability to neutralize the dermonecrotic activity of toxin in the rabbit. Kuhns and Pappenheimer's (12) figure of 2 to 2.5 micrograms of nitrogen as the equivalent of one unit of antitoxin was used to determine the *in vitro* titer. Readings were made

[1] The toxin was originally given by Dr. Cohn to Dr. Merrill Chase, The Rockefeller Institute for Medical Research. Dr. Chase kindly offered us a sample of this preparation.

[2] Purified toxin and toxoid for the Schick test were also provided by Mr. W. S. Hammond.

[3] Lot KP 28D5. Personal communication from Dr. W. J. Kuhns. These represent samples from the same lot of toxoid diluted at different times.

after 3 days and again after 5 days or, occasionally, after a week or more. The standard horse antitoxic serum employed was obtained from Lederle Laboratories.

Quantitative Precipitin Determinations.—The quantitative precipitin method, as described by Heidelberger and MacPherson (40) was used to measure the total amount of antigen-antibody precipitated, but the nitrogen content was determined by the Markham micro-Kjeldahl method (41; *cf.* reference 7). In regions of extreme antigen excess, with respect to the toxin-antitoxin system, the Folin-Ciocalteu method (40, 42; *cf.* reference 7) was employed, but, because the antigenic contribution to the specific precipitate was unknown in terms of nitrogen, the optical densities obtained were not converted to micrograms of nitrogen. To prevent solubilization of precipitates, total volumes in individual precipitin tubes were kept as low as feasible, usually about 1.5 ml. for the sera with high titers as determined by the rabbit intracutaneous test, 2.0 ml. for sera with less antibody, a larger sample of antiserum being used in the latter instances.

Gel Diffusion Analyses.—The principal technique used to detect multiple antigen-antibody systems was that of Oakley and Fulthorpe (43) as modified by Preer (6). Pyrex tubes, 1.8 to 2.0 mm. inside diameter, were coated with a 0.1 per cent agar solution, cut into 5 cm. lengths, and flame-sealed at one end. To such a dry, coated tube, 0.01 to 0.02 ml. of antiserum was added with a 0.25 ml. syringe with a 2-inch 26 gauge needle. Then 0.3 per cent washed agar maintained at 50°C. was carefully layered until an agar column of 5 to 7 mm. in height was formed. The agar was made up in saline with 1:10,000 merthiolate and 0.5 M glycine. A third layer of 0.01 to 0.02 ml. antigen solution was placed on top of the agar after it had solidified. The tube was then sealed with pyseal cement[4] and placed in a horizontal position at room temperature.

To determine the number of different precipitating antibodies in a serum, stock solutions of antigens were diluted with varying amounts of borate buffer pH 7.3[5] to provide a series of concentrations with a lower limit of about 0.7 Lf/ml. With the most concentrated solution of antigen, a series of serum dilutions was also set up, using 0.6 per cent agar as the diluent.

Supernatants of quantitative precipitin determinations were also analyzed for antibody by this method (*cf.* reference 8). The usual procedure was to place an aliquot of the absorbed serum, *i.e.* the supernatant, as the topmost layer and to use as the bottom layer an antigen solution diluted with 0.6 per cent agar to a concentration known to be in slight antigen excess for antitoxin (see body of text for procedure for determining this in gels). This reversal of the usual order of addition of the reactants was necessary because the serum, which had been diluted with antigen in the quantitative precipitin determination, was not dense enough to allow the agar to form a sharp layer above. The equivalence concentration for toxin-antitoxin was chosen because at this concentration most systems other than toxin-antitoxin also formed bands within the agar column.

RESULTS

Gel Diffusion Analysis with Purified Antigens.—A comparison of three methods of analyzing antisera by diffusion of antigen and antibody through gels was made to determine the relative sensitivity of these techniques.

The three methods were those devised by Oudin (1, 2), in which an antigen solution is layered over a serum-agar mixture in a tube; by Ouchterlony (3), in which antigen and anti-

[4] Obtainable from Fisher Scientific Company.

[5] Nine parts H_3BO_3-NaCl solution (12.404 *g* H_3BO_3 + 2.905 *g* NaCl in one l.) to one part $Na_2B_4O_7 \cdot 10H_2O$ solution (19.108 *g* in one l.) 200 μg. gelatin/ml. was also added (12).

serum diffuse toward each other in a Petri dish from wells in an agar layer; and by Preer, as described above. Three highly purified carbohydrates and their respective homologous antisera (dextran-human antidextran, SII-horse anti-SII and blood group B–human anti-B) and one protein system (γ_2-globulin-rabbit anti-γ_2-globulin) were employed, and one cross-reacting system (dextran–horse anti-SII). The concentrations of antigen and antiserum were

TABLE I

Comparison of the Sensitivities of the Ouchterlony, Oudin, and Preer Methods of Gel Diffusion Analyses of Antigens and Antisera

System	Antigen	Molecular weight	Antiserum	Molecular weight	Smallest amount of antigen and antibody detectable					
					Antigen, μg/ml.			Antibody, μg. N/ml.		
					Preer	Oudin	Ouchterlony	Preer	Oudin	Ouchterlony
Dextran-human anti-dextran	D279 (27)	*ca.* 2,000,000.	176 D_2 (28)		2.5	5	>10	5	<5	>19
	NRC No. 4 (28)	91,700	176 D_2		<1.5	2.5	2.5	4	<4	9
Blood group B-human anti-B	Horse 4–25% (29)		310 (34)		9	17	>34	9	18	>35
	PM 10% (30)				7	16	>31	8	16	>32
Pneumococcus SII-horse anti-SII	SIIB1 (31)	250,000	513	*ca.* 1,000,000	<1.5	2.5	>5	<8	8	>30
Dextran-horse anti-SII	D279	>10,000,000	513			100	>100		25	>100
					μg.N/ml.					
γ_2-globulin-rabbit anti-γ_2 globulin	γ_2-globulin (32, 33)	160,000	Pool 47 (35)	160,000	1	1	>2	3	2	>6

The Oudin and Ouchterlony methods were slightly modified from the descriptions as originally presented (1, 3). In the Oudin technique tubes with an inside diameter of 1.8 to 2.0 mm. were used; in experiments in which tubes with a 6.0 mm. diameter were used no better resolution was obtained with the larger volumes. In the Ouchterlony method the wells were arranged in parallel rows instead of circumferentially, and the distance between antigen and antiserum borders was 5 to 10 mm.

adjusted to the equivalence ratio, as determined by quantitative precipitin tests, and this ratio retained throughout a dilution series until no visible precipitate appeared. The smallest amount of antigen or antibody detectable by each method could then be calculated.

From the data presented in Table I, it is evident that in general the Ouchterlony technique was the least sensitive, with the Preer and Oudin techniques about equally sensitive. With reference to the actual amounts of antigen or antibody detectable, there was a wide variation depending on the antigen-antibody system studied. For a serum to form a band by the Preer

method, 3 to 9 micrograms antibody N/ml. were needed. Since the volume of serum used could be as small as 0.01 ml., as little as 0.03 micrograms antibody nitrogen can be detected.

It should be noted that with the native dextran–horse anti-SII cross-reaction a band did not form with the Preer and Ouchterlony techniques after 1 day, although the Oudin tubes had a visible precipitate at this time. Only after 22 days did a band appear in the Preer tubes. The diffusion rates of both of the reactants must therefore be very slow, as might be anticipated from their molecular weights.

TABLE II

Antigen-Antibody Systems Found by Gel Diffusion in Serum of Individuals Immunized with Purified Diphtheria Toxoid

Serum	*In vivo* units/ml.	No. of Bands Formed with Each Antigen				
		Purified toxoid	Purified toxin	Crude toxoid	Crude toxin	Culture filtrate
Ku	30	3	3	1	1–2	1–2
Ni	60	2–3	1–2	1	1–2	0
Sl	75	4–5	1–2	1–2	1–2	1
Sch	80	3–4	2–4	1	1–2	1–2
Hi	20	2	2–3	2	1–2	1
F	20	2–4	2–3	2–3	2	0–1
Gu	20	4	3–4	2–3	2–3	1–2
Al	50	5–6	3–4	3	3	2

In some instances it was not possible to assign a definite number of bands to a particular series of reactants owing to the possibility of "splitting" of single bands, etc. In these cases the lower and upper limits of observed bands are listed as two figures.

Gel Diffusion Studies of the Diphtheria Toxin–Human Antitoxin System.—All sera, pre- and postimmunization, obtained from Schick-negative individuals injected with purified diphtheria toxoid were analyzed by the Preer method for the presence of antibodies to toxoid, toxin, and other diphtherial antigens. Two of the pre-immunization bleedings, those from Al and Sch, possessed antibody that reacted with purified toxoid. All of the other pre-immunization sera had no diphtherial antibodies detectable by gel diffusion. From Table II it can be seen that no individual responded to the injection by the production of only a single antibody. Indeed, almost all subjects showed at least three bands by gel diffusion. Moreover, no correlation could be drawn between the antibody response as measured by the number of precipitating antibodies and the antitoxin content of a serum. For example, Gu with 20 units/ml. produced at least as many kinds of antibodies as did Sch with 80 units/ml. The largest number of bands was formed with purified toxoid, with purified toxin producing fewer, and crude toxin and toxoid producing even

fewer, and culture filtrate the least number of bands. Since purified toxoid was available in at least ten times the concentration of any other antigen and purified toxin was several times more concentrated than the crude toxin or toxoid, it is not surprising that they were able to form more bands.

Before an estimate could be made with the available material of the amount of antibody to impurities in any serum, the toxin-antitoxin band had to be identified. Each serum possessed one antibody that formed a band which could be diluted several fold and which formed the most dense precipitate at the equivalence concentrations for toxin-antitoxin. Employing the mutual dilution method of Telfer and Williams (44), it was found that this antibody was the same in all immune sera. It was therefore concluded that this band probably represented toxin-antitoxin.

Additional evidence for this identification was obtained by comparing the *in vivo* titer and position of this band formed in agar for each antiserum. As has been demonstrated by Preer (6) and by Ouchterlony (4), the position of a band reflects the relative concentrations of the reactants forming it. Thus, if the concentration of antigen is kept constant, the final position of a band formed by a number of antisera would be a measure of the antibody concentrations in the sera. Therefore, a series of antigen and antibody dilutions were diffused against each other to determine the percentage shift in band position with change in concentration. It was found that the presumed toxin-antitoxin band moved about 10 per cent for every halving of antibody concentration.

The band position and neutralizing power for toxin for each antiserum were determined. Taking the serum Sch as a standard, the position that its band would occupy when diluted to the antitoxin contents of each of the other sera was computed and found to agree closely with the observed values (Table III). Thus, there is a close correlation with each of these sera for band position and antitoxin content which provides further evidence for this being the toxin-antitoxin band.

The expected band positions were calculated by applying the following formula:

$$\text{Position of band with Sch (in per cent)} + 10 \log_2 \frac{(\textit{in vivo} \text{ units/ml. of Sch})}{(\textit{in vivo} \text{ units/ml. of serum } X)} = \text{Expected position with serum } X$$

This calculation for Hi, for example, *versus* purified toxoid would be:—

$$39 + 10 \log_2 \frac{(80)}{(20)} = 59$$

Further confirmation was obtained by diluting several of the sera to the same neutralizing power and noting that the band formed with each serum appeared in the same position.

Finally, the band position at equivalence for toxin-antitoxin was compared with the position as predicted from known diffusion coefficients. The equivalence position for several sera with all antigen preparations is about 50 per cent (Table IV). Using the diffusion constant of

6.2×10^{-7} cm.²/sec. for diphtheria toxin (45) and that for human anti-pneumococcal antibody of 3.6×10^{-7} (46) and substituting in the equation used by Preer

$$P = \frac{\sqrt{D_1}}{\sqrt{D_1} + \sqrt{D_2}}$$

gives the expected equivalence position of 57 per cent as compared with the observed value of 50 per cent. With the reported value for horse antitoxin (45), P in per cent would be 54,

TABLE III

Comparison of Observed Position of Toxin-Antitoxin Band with That Calculated from Neutralizing Power of Antisera

Serum	*In vivo* units/ml.	Antigen	Purified toxoid		Purified toxin		Crude toxoid		Crude toxin	
		Lf/ml	12.4		10.3		12.0		12.0	
			Observed	Calculated	Observed	Calculated	Observed	Calculated	Observed	Calculated
Sl	75		37	40	41	39				
Hi	20		62	59	61	58	60	61	54	65
F	20		62	59	63	58			66	65
Gu	20		59	59	63	58	60	61	67	65
Ku	30		51	53	45	52	49	55	47	55
Al	50		39	46	47	45	38	48	48	48
Ni	60		47	43	44	42	44	43	44	45
Sch	80		39	—	38	—	41	—	45	—

The observed location of a band was determined by measuring the distance from the bottom of the agar-antigen meniscus and from the bottom of the serum-agar meniscus to the middle of the band (X_1 and X_2 respectively). The positions are given in per cent down the agar column

$$\frac{X_1}{X_1 + X_2} \times 100$$

By the Preer method with decreasing concentration of antibody, the band appears further down the column. The procedure for calculating a band position from the *in vivo* titer of a serum is given in the text.

in which P is the proportion of the total agar length represented by the distance from the band to the antigen-agar interface and D_1 and D_2 are the diffusion coefficients of the antigen and antibody respectively.

Having identified one band as due to toxin-antitoxin, the information obtained from gel diffusion analyses of systems precipitating as a single band could now be used to assign to individual sera antibody nitrogen values reflecting the amount of antibody to impurities. Each serum was diluted until all the bands, aside from toxin-antitoxin, disappeared. The amount of antibody at the dilution at which the band was last seen was taken as being 3 to 9 μg. N/ml. (*cf.* Table I). Multiplying this quantity by the dilution gave an

estimate of the amount of this impurity. When the values for each antigen-antibody system were summed, a range for total amount of antibody to impurities in each serum was obtained. The figures listed in Table V indicate

TABLE IV

*Position of Toxin-Antitoxin Band in Agar at Equivalence**

Serum	Antigens used in gel diffusion and position, *per cent*			
	Purified toxoid	Purified toxin	Crude toxoid	Crude toxin
Ku	51	45	49	47
Ni	53	55		
Sl	49	54	48	49
Sch	49	55	49	49
Hi	52	51	49	45
Gu	45	51	47	53
F	49	55	53	
Al	50	53	58	59
Average.....	50	52	50	50

* Equivalence ratios determined by quantitative precipitin analyses.

TABLE V

Per Cent of Antibodies to Impurities in Antitoxic Sera Estimated by Gel Diffusion

Serum	Antibody N/ml. to impurities	Total antibody N/ml.	Antibody to impurities
	μg.	*μg.*	*per cent*
Sl	12–36	175	7–21
Sch	9–27	182	5–15
Ku	6–18	73	8–25
Ni	6–18	98	6–18
Hi	3–9	36	8–25
Gu	9–27	38	24–71
F	9–27	40	23–68
Al	15–45	156	9–27

To simplify the calculation of total antibody, the antigen contribution was calculated assuming that all of the antigen in the precipitate was toxoid and that 1 Lf = 0.46 μg. N (*cf.* reference 12). The lower and upper values in the first and last columns of figures represent estimated limits based on the finding that for a band to appear in agar there must be from 3 to 9 μg. N/ml. serum.

that most of the antisera contain considerable quantities of antibodies to impurities, and, in a few instances, more than half of the antibody may not be antitoxin.

Quantitative Precipitin Determinations.—The amount of nitrogen precipitated, upon the addition of each of four different antigen preparations, from

sera of eight subjects was determined by the quantitative precipitin technique. Three sample curves for individuals with 75, 30, and 20 antitoxin units/ml. respectively are presented in Figs. 1, 2, and 3.

Although sera possessing the same neutralizing power varied with respect to total amount of nitrogen precipitated by a given antigen (Table VI), two patterns of behavior are evident. With all of the sera the precipitating efficiency of the purified toxoid preparation was greater than the crude toxoid solution. The differences are generally slight, but nevertheless reproducible. Secondly, the toxoid preparations, crude or purified, almost without exception

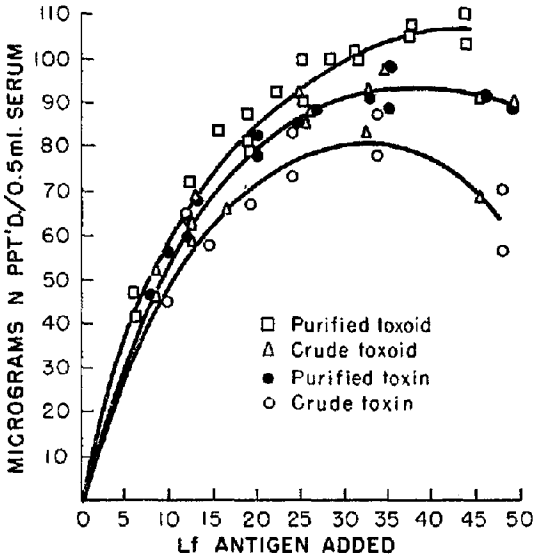

FIG. 1. Precipitin reactions of purified and crude diphtheria toxoid and toxin with 0.5 ml. antiserum S1 (75 units/ml.).

precipitated more antibody than did the crude or purified toxin respectively. Also, purified toxin precipitated more nitrogen than did crude toxin from five of the sera.

Quantitative precipitin determinations were also made with large amounts of antigen, well into the region of excess toxin. The data obtained were consistent with those expected for multiple antigen-antibody systems if the secondary antigens were present in small amounts. It was difficult to form any estimate of the relative amounts of nitrogen precipitated by any particular antigen or the number of systems present. However, when these sera were first absorbed with amounts of antigen sufficient to remove the antitoxin and large amounts of antigen were added to these supernatants (*cf.* reference 47), unambiguous curves were obtained which show that small amounts of non-toxoid antigens were present in the purified toxoid preparation (Fig. 4).

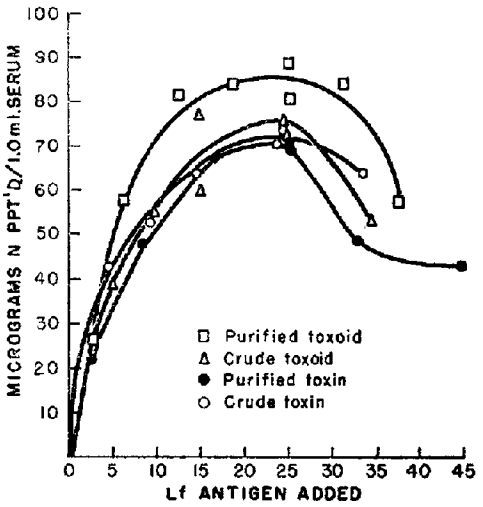

FIG. 2. Precipitin reactions of purified and crude diphtheria toxoid and toxin with 1.0 ml. antiserum Ku (30 units/ml.).

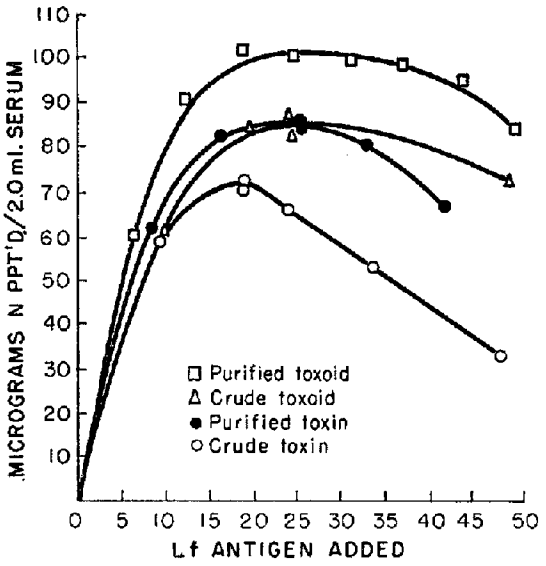

FIG. 3. Precipitin reactions of purified and crude diphtheria toxoid and toxin with 2.0 ml. antiserum F (20 units/ml.).

Supernatants from quantitative precipitin curves were also tested for antibody by allowing them to diffuse against various antigen solutions in agar (*cf.* reference 8). Of the sera examined by the Preer method, four could be shown to possess antibody to diphtherial antigens after removal of antitoxin. As seen from Table VII, when a precipitin is being removed by absorption

with increasing amounts of antigen, the band moves down the column (indicated by the increase in per cent) as would be expected from previous studies with other systems (6). When a precipitin is not being absorbed or is only

TABLE VI

Quantitative Precipitin Determinations with Purified and Crude Toxin and Toxoid

Serum	Antibody N precipitated/ml. serum			
	Purified toxoid	Crude toxoid	Purified toxin	Crude toxin
	μg.	μg.	μg.	μg.
Sl	216	188	196	164
Ni	113	102	91	86
Hi	42	35	27	40
F	51	44	43	35
Sch	206	190	210	188
Gu	45	40	39	40
Al	180	176	184	172
Ku	85	75	71	74

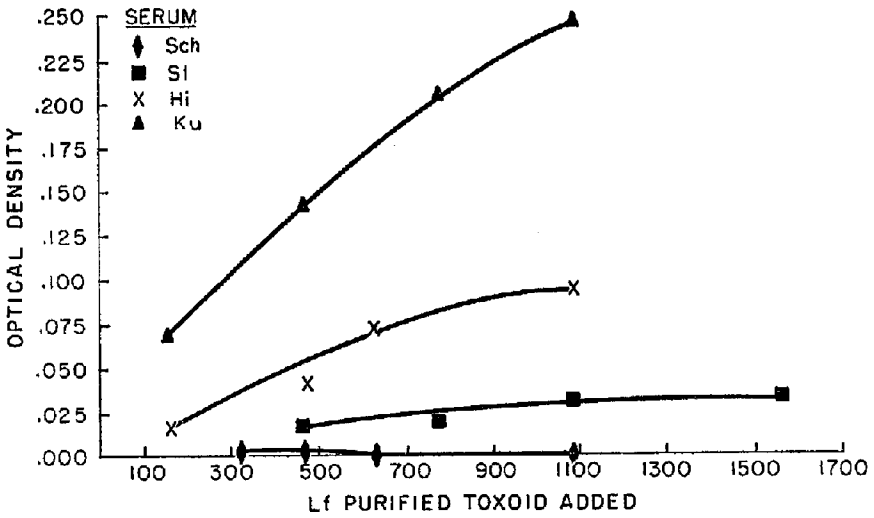

FIG. 4. Precipitin reactions of purified toxoid solution with antisera Sch, Sl, Hi, and Ku, previously absorbed with amounts of purified toxoid in slight excess for toxoid.

gradually removed, then the position of the band remains constant. The antibody involved in a stationary antigen-antibody band must be formed against an antigen present only in small quantities in the antigen solution used for absorption.

The four sera which contained no antibodies demonstrable by gel diffusion

TABLE VII

Gel Diffusion Analyses of Sera Sl, Ku, and Hi Supernatants of Quantitative Precipitin Determinations

Antigen	Quantitative precipitin data			Tests on supernatants Band position in agar with various antigens											
	Serum			Purified toxoid			Purified toxin			Crude toxoid			Crude toxin		
	Sl	Ku	Hi	Sl	Ku	Hi	Sl	Ku	Hi	Sl	Ku	Hi	Sl	Ku	Hi
μg. N ppid./ml.															
0.0				50	62	59	29	42	34	64	63	59	19	39	46
Lf purified toxoid added														*64*	
6.2	82	57	33	31	84		58	72		58	87		30	*57*	*49*
12.5		81	45											*66*	*45*
18.7	162	84												*66*	
25.0	180	88												*71*	
31.2	198	84												*70*	
43.7	220														
62.4	172														
Lf purified toxin added															
8.3	92	48	27	24	81		40	74		28	93		36	80	*41*
				56						*36*				*55*	
12.4	118														
16.5	132	66	28							*34*				*56*	*43*
25.3	170	71		*58*						*34*				*56*	
45.4	138	43		*58*						*33*				*64*	
Lf Crude Toxoid added															
4.9		39	25		72			61			81			68	*44*
9.8	112		36	78			59								*45*
19.6	156		32												*50*
24.5	170	75													
34.3	178														
Lf crude toxin added															
4.8		43	35		71			70			75			75	
9.6	90			72			58			79			37		
19.2	134		12												
24.0	146	74													
33.6	156														

Italicized band position = impurity–anti-impurity band. All other band positions refer to toxin-antitoxin. Blank spaces in quantitative precipitin determinations indicate that no data were obtained with that particular serum. Blank spaces in gel diffusion data indicate that no band was formed.

after absorption with toxin or toxoid had several antibodies prior to absorption. Probably the curves of the individual antigen-antibody systems reach equivalence with about the same amounts of the several antigens, and consequently there would be no differential absorption.

Studies with Hu Serum.—A sample of the serum Hu, obtained through the kindness of Dr. Colin M. MacLeod and Dr. A. M. Pappenheimer, Jr., containing 10 units of antitoxin/ml. was tested with several concentrated antigen preparations for the presence of precipitins. Serum samples from Hu had been extensively employed by Kuhns and Pappenheimer (13) to determine the immunological properties of "non-precipitating skin-sensitizing diphtheria antitoxin." In the original experiments, the bleeding available was reported to contain as much as 80 units/ml. It failed to form any precipitate with 35 Lf of purified toxin after mixing and standing in an ice box for 3 months and did not form a band by the Oudin technique of gel diffusion with amounts of toxoid ranging from 55 to 115 Lf/ml. (17). Consequently it was concluded that all of the experimental findings could be ascribed to non-precipitating antitoxin.

Hu was set up by the gel diffusion method of Preer. Fig. 5 is a photograph of a series of such tubes from one of several experiments in which undiluted Hu was tested with highly purified toxoid. A definite band has formed, and, as expected, the band moves upward with dilution of the antigen preparation. The band is faint and diffuse with an antigen concentration of 156 Lf/ml. and disappears entirely in lower concentrations. A band similar in appearance was formed with concentrated purified toxin and culture filtrate although with both of these it was more diffuse. A second preparation of concentrated purified toxin, prepared by Dr. M. Cohn in Dr. Pappenheimer's laboratory, did not form a precipitate. This preparation of purified toxin possessed at least three or four antigens, aside from toxin, as determined by gel diffusion with human antitoxin.

Another lot (PT64) of toxoid from the Massachusetts Antitoxin Laboratory recently provided by Dr. Kuhns also failed to show a band. Dr. Kuhns has also been able to demonstrate a band in the Hu serum using Lederle toxoid.[6]

When the purified toxoid, diluted to 520 Lf/ml. with 0.6 per cent agar, was layered on the bottom of a tube and Hu diluted in saline used as the upper layer, a band formed with concentrations as low as 1:4 of serum. Utilizing the values obtained with known antigen-antibody systems, it would be expected that this sample of Hu (with one-eighth the antitoxin content of the original bleeding) should have at least 12 μg. N/ml. of precipitating antibody. A quantitative precipitin determination with 1.0 ml. of serum and 1560 Lf of purified toxoid yielded 23 micrograms N of specific precipitate. From the relative density changes of the precipitate in the gel dilution series, it was estimated that the equivalence concentration of purified toxoid with undiluted serum would be expected to be more than 1000 Lf/ml.

From the information obtained with the Preer technique, it appeared that with the usual Oudin method a band would fail to form and migrate because even with the most concentrated antigen solution on hand Hu possessed excess antibody against the antigen forming the band. Consequently, antigen, in-

[6] Personal communication (added in proof.)

stead of serum, was incorporated into agar and undiluted Hu allowed to diffuse directly. A band formed and migrated.

If the position of a band in the Preer tubes is a resultant of both precipitating and non-precipitating antibody, then it is highly unlikely that this band with Hu represents toxin-antitoxin. Other sera with the same *in vivo* titers, but with an *in vivo / in vitro* ratio of about 1.0, would not form a band in the agar column with the concentrations of antigen used with Hu. Furthermore the estimated equivalence position of this antigen-antibody system appears to be about 80 per cent or greater. Since the equivalence position of

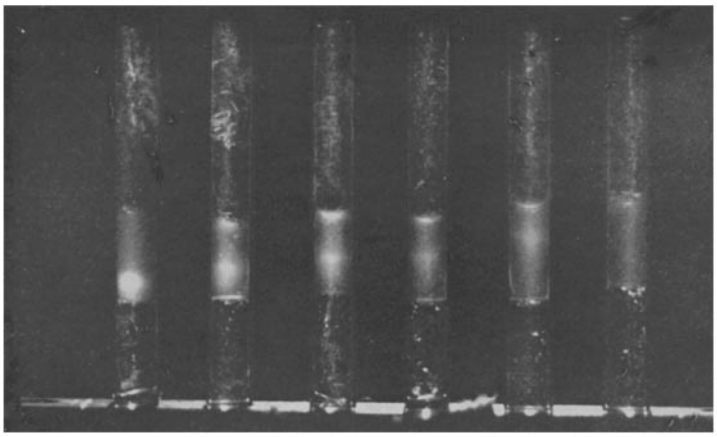

FIG. 5. Gel diffusion analyses of the Hu by Preer method. Bottom layer, Hu undiluted (10 units/ml.); middle layer, 0.3% agar; top layer, left to right.

Tube No.	1	2	3	4	5	6
Purified toxoid, *Lf/ml.*	1560	780	520	312	156	0 (control)

toxin-antitoxin is about 50 per cent (*cf.* Table IV), it must be concluded, therefore, that Hu possesses at least one precipitating antibody, and that this is antibody to an impurity. It is possible, of course, that earlier bleedings with eight times as much antitoxin would have revealed additional precipitins when studied by this method.

Immediate and Delayed Skin Reactions to Toxin and Toxoid.—In the course of Schick testing prospective subjects, it was observed that the generally expected reactions were not encountered in most individuals. If there is an immediate or delayed reaction to toxin, then a similar reaction should have been noted with toxoid, and *vice versa*. However, it was observed that most individuals manifested a more intense immediate reaction to toxoid than to toxin and a more intense delayed hypersensitivity to toxin than to toxoid (Table VIII). Essentially the same results were observed among the Schick-

negative individuals. It can be concluded, therefore, in agreement with Kuhns (17, 18) that the presence of antitoxin in a serum does not assure skin-sensitizing ability.

TABLE VIII

Reactions to Skin Tests with Purified Diphtheria Toxin and Toxoid

	Immediate skin reactions				Delayed skin reactions			
	All subjects		Schick-negatives		All subjects		Schick-negatives	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
No reaction to toxoid or toxin...	10	18	4	16	7	13	6	24
Equal reaction to toxoid and toxin.....	7	13	4	16	3	5	1	4
Greater sensitivity to toxoid....	33	60	15	60	0	0	0	0
Greater sensitivity to toxin.....	5	9	2	8	45	82	18	72

DISCUSSION

In a complex mixture in which most of the constituent antigens may not be available in comparatively pure concentrated form or when the nature of a suspected contaminating antigen is unknown, two parameters for a serum with a multiplicity of antibodies can be established. By the combined use of quantitative precipitin and agar diffusion methods, the equivalence concentration of the antigen in terms of the solution on hand and a rough estimate of the amount of antibody to this antigen may be obtained. For the diphtheria toxin-antitoxin system it has been possible, by reference to values for known purified antigen-antibody systems studied by gel diffusion, to estimate the amounts in a serum of the various antibodies other than antitoxin. With some sera, these antibodies to impurities could make up as much as 50 per cent of the total antibody.

Quantitative precipitin curves have shown slight differences between purified toxin and toxoid in precipitating power, suggesting a possible difference in immunological specificity of these antigens. Moreover, application of the quantitative precipitin method, after addition of large amounts of purified antigens to supernatants of sera whose antitoxin had previously been removed, has provided additional evidence for the presence of impurities in the original antigen preparations. Also the presence of precipitins in a serum, Hu, previously thought to contain none, has been demonstrated by gel diffusion and quantitative precipitin techniques.

These results emphasize the need for recognition of both the limitations and flexibility of gel diffusion techniques and of the quantitative precipitin method. For instance, to detect antibodies to impurity in a serum prepared against

purified diphtheria toxin when the suspected impurity is available in concentrated form and relatively free from toxin, only small amounts of the impurity need be employed. If the amount of these antibodies is assumed to represent 10 per cent of the antibody of an antitoxic serum, which has been considered a reasonable estimate (21, 48), then about one-tenth as much of this atoxic protein as of toxin should be sufficient to precipitate the antibody completely. Pappenheimer and Yoneda (49), in an experiment designed to demonstrate the specificity of toxin-antitoxin flocculation, precipitated 995 μg. of unlabelled toxin and almost none of 1940 μg. of S^{35}-labelled P-protein added to a horse antitoxin. From the absence of label in the precipitate, it was concluded that there were no antibodies to impurities in this antitoxic serum. If, however, such antibodies comprised only a small fraction of all the antibodies,[7] then instead of 1940 μg. about 100 μg. of the P-protein and preferably less should have been added in order to obtain an insoluble impurity-antibody complex in the equivalence zone or in antibody excess. The amounts actually used were in such antigen excess for antibody to impurity that complete inhibition of precipitation would have occurred, assuring the presence of all label in the supernatant.

Similarly, if the toxin-antitoxin curve is used as the sole guide in choosing concentrations of antigens for gel diffusion experiments, certain features of quantitative precipitin and gel diffusion methods may be overlooked. Antigens present as impurities in a solution, 95 per cent of which is toxin, would be present in such small quantities in an aliquot of solution containing amounts of toxin suitable for maximum precipitation of antitoxin that the amount of a non-toxin antigen-antibody precipitate formed would be extremely small. To make optimum use of gel diffusion, the antigen preparation should be in extreme antigen excess in terms of Lf unitage so that significant amounts of impurity are added. This is especially important for the usual Oudin tubes since a band only appears and migrates in antigen excess. Indeed, the only way precipitins could be detected in Hu using the Oudin method was to incorporate the antigen, rather than the antiserum in agar since, from observations with the Preer method, even the most concentrated antigens available would cause the system to be in antibody excess.

Kuhns and Dukstein have reported (24) that in eleven of fifty-three Schick-negative subjects who were immunized with purified toxoid several precipitating antibodies in addition to antitoxin were found. This relatively infrequent detection of individuals with multiple antibodies in comparison with our finding that all eight of our subjects have antibodies to impurities may be ascribed to the insensitivity of the gel method used by these workers and the utilization of antigens or antiserum in too low a concentration. Further-

[7] This same serum was analyzed by gel diffusion in our laboratory and estimated to contain 13 per cent antibodies to impurities.

more, the antigen primarily employed was P-protein, a fraction of *Corynebacterium diphtheriae* assumed to possess all antigens found in crude toxin, excluding toxin itself. Limitations to the use of P-protein for this purpose have been pointed out by Vaughan and Kabat (47). Kuhns and Dukstein do not remark on the pertinence of antibodies to impurities in large amounts in antitoxic sera to the earlier findings regarding the specificity of the reported skin-sensitizing properties of antitoxin.

Finally, it should be noted that, in his original papers, Kuhns (17) presented as evidence for the specificity of the skin sensitization the correlation of antitoxin titer and flocculating units of toxin with neutralization of wheal and erythema. This correlation, however, was quantitatively inexact in that larger amounts of toxin were needed to elicit reactions than would be required by the titers of the passively transferred antitoxin. Our demonstration that along with toxoid purification there is apparently a coincident concentration of impurity stresses the need for caution in interpreting such qualitative correlations. Indeed, none of the experiments of Kuhns and Pappenheimer (12, 13) and of Kuhns (17, 18) eliminate the possibility that antigens other than toxin are the allergens. If antibodies to impurities are the sensitizing agents, as has been shown for crystalline egg albumin and conalbumin (47), then there is no evidence to associate non-precipitating antitoxin with skin-sensitizing capacity.

The findings show that most, if not all, sera with appreciable antitoxin titers possess antibody to impurities, that the experiments devised to demonstrate the specificity of skin sensitization to the toxin-antitoxin human system are inconclusive, and that the methods used were either of insufficient sensitivity (17), or happened not to be in the right range to detect antibodies against impurities (49).

This discussion should not be construed as establishing that skin sensitization to diphtheria toxin is caused by antibody to impurities. Rather, the studies reported here and the analyses of other workers' results support the idea that the presence of antibodies to impurities offers a reasonable, simple alternative explanation for the skin-sensitizing capacity of human diphtheria antitoxic sera other than that of postulating three kinds of antitoxin for a system whose complexity was unrecognized.

SUMMARY

The limits of sensitivity of three gel diffusion methods are compared and their utilization in the detection of small amounts of antibody to antigens present in traces in a preparation is illustrated with the diphtheria toxin-human antitoxin system. The Preer modification of the Oakley-Fulthorpe technique and the Oudin tube method were found more sensitive than the Ouchterlony plate method, and permitted the detection of as little as 3 μg. antibody N/ml. of serum.

Antisera from eight Schick-negative individuals immunized with purified diphtheria toxoid have all been shown to contain, in addition to antitoxin, antibodies to substances present as impurities in the purified toxoid injected. The amounts of these antibodies in a serum and a partial characterization of their antigen-antibody curves have been determined through the combined use of quantitative precipitin and gel diffusion methods.

Different amounts of antibody have been precipitated by toxin and toxoid from individual sera. Evidence is presented that this may have been due to slight differences in antigenic specificity.

A serum, Hu, which had been held to contain no precipitating antibody, has now been shown, by the Preer and Oudin techniques, to contain at least 12 μg. of precipitating antibody N (per ml. serum) against an impurity in the toxoid preparation. This estimate has been confirmed by quantitative precipitin determinations. The presence of antibodies to impurities in all human antitoxins examined in the present work brings into question the assumption that human antitoxin as such has a skin-sensitizing capacity.

BIBLIOGRAPHY

1. Oudin, J., L'analyse immunochimique du sérum de cheval par précipitation spécifique en milieu gélifié: Premiers résultats, *Bull. Soc. chim. biol.*, 1947, **29**, 140.
2. Oudin, J., *Methods Med. Research*, 1951, **5**, 335.
3. Ouchterlony, O., Antigen-antibody reactions in gels, *Ark. Kemi*, 1949, **B26**, 1.
4. Ouchterlony, O., Antigen-antibody reactions in gels: II. Factors determining the site of the precipitate, *Ark. Kemi*, 1950, **1**, 43.
5. Grabar, P., and Williams, C. A., Jr., Methode immuno-électrophorétique d'analyse de mélanges de substances antigéniques, *Biochim. et Biophysica Acta*, 1955, **17**, 67.
6. Preer, J. R., Jr., A quantitative study of a technique of double diffusion in agar, *J. Immunol.*, 1956, **77**, 52.
7. Kabat, E. A., and Mayer, M. M., Experimental Immunochemistry, Springfield, Illinois, Charles C. Thomas, 1948.
8. Kaminski, M., Studies on egg white and its constituents by immunochemical techniques in gelified media. Specific precipitation by double diffusion and immuno-electrophoretic analysis, *J. Immunol.*, 1955, **75**, 367.
9. Becker, E. L., Munoz, J., Lapresle, C., and LeBeau, L., Antigen-antibody reactions in agar. II. Elementary theory and determination of diffusion coefficients of antigen, *J. Immunol.*, 1951, **67**, 501.
10. Neff, J. C., and Becker, E. L., Antigen-antibody reactions in agar. III. Rate of change of band migration with antigen concentration, *J. Immunol.*, 1957, **78**, 5.
11. Spiers, J. A., and Augustin, R., Antigen-antibody reactions in gel single diffusion. Theoretical considerations, *Tr. Faraday Soc.*, 1958, **54**, 287.
12. Kuhns, W. J., and Pappenheimer, A. M., Jr., Immunochemical studies of antitoxin produced in normal and allergic individuals hyperimmunized with

diphtheria toxoid. I. Relationship of skin sensitivity to purified diphtheria toxoid to the presence of circulating, non-precipitating antitoxin, *J. Exp. Med.*, 1952, **95,** 363.

13. Kuhns, W. J., and Pappenheimer, A. M., Jr., Immunochemical studies of antitoxin produced in normal and allergic individuals hyperimmunized with diphtheria toxoid. II. A comparison between the immunological properties of precipitating and non-precipitating (skin-sensitizing) antitoxins, *J. Exp. Med.*, 1952, **95,** 375.
14. Kuhns, W. J., Immunochemical studies of antitoxin produced in normal and allergic individuals hyperimmunized with diphtheria toxoid. III. Studies of passive Arthus reaction in guinea pigs using human precipitating and non-precipitating diphtheria antitoxin, *J. Exp. Med.*, 1953, **97,** 903.
15. Kuhns, W. J., 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, *J. Exp. Med.*, 1954, **99,** 577.
16. Kuhns, W. J., Immunochemical studies of antitoxin produced in normal and allergic individuals hyperimmunized with diphtheria toxoid: V. Peculiar electrophoretic configuration of serum proteins and protein-bound polysaccharides in certain antitoxic sera. Demonstration of serum changes in certain severe manifestations of allergy, *J. Exp. Med.*, 1954, **100,** 485.
17. Kuhns, W. J., Immunochemical studies of antitoxin produced in normal and allergic individuals hyperimmunized with diphtheria toxoid. VI. Further investigations on the identity and specificity of non-precipitating skin-sensitizing antitoxin, *J. Exp. Med.*, 1955, **101,** 109.
18. Kuhns, W. J., Immunological properties of a form of non-precipitating diphtheria antitoxin which does not sensitize human skin. Studies of its relation to the inhibition reaction, *J. Immunol.*, 1955, **75,** 105.
19. Vaughan, J. H., and Kabat, E. A., An unidentified antibody in anti-egg albumin sera revealed by the agar diffusion technic. Methods for its immunochemical analysis, *J. Immunol.*, 1956, **73,** 205.
20. Pope, C. G., Stevens, M. F., Caspary, E. A., and Fenton, E. L., Some new observations on diphtheria toxin and antitoxin, *Brit. J. Exp. Path.*, 1951, **32,** 246.
21. Bowen, H. E., The homogeneity of purified diphtheria toxins and toxoids as investigated by the semi-solid precipitin technic, *J. Immunol.*, 1952, **68,** 429.
22. Relyveld, E. H., Grabar, P., Raynaud, M., and Williams, C. A., Jr., Étude par la méthode immuno-électrophorétique de la toxine diphtérique purifiée, *Ann. Inst. Pasteur*, 1956, **90,** 688.
23. Pappenheimer, A. M., Jr., and Robinson, E. S., A quantitative study of the Ramon diphtheria flocculation reaction, *J. Immunol.*, 1937, **32,** 291.
24. Kuhns, W. J., and Dukstein, W., Multiple antibody responses following immunization of human subjects with diphtheria toxoid, *J. Immunol.*, 1957, **79,** 154.
25. Pope, C. G., and Stevens, M. F., Isolation of a crystalline protein from highly purified diphtheria toxin, *Lancet*, 1953, ii, 1190.

26. Relyveld, E. H., Turpin, A., Laffaille, A., Paris, C., and Raynaud, M., Purification de la toxine diphtérique contrôle qualitatif des fractions par la méthode de précipitation spécifique en gel, *Ann. Inst. Pasteur*, 1954, **87,** 301.
27. Kabat, E. A., and Berg, D., Dextran—an antigen in man, *J. Immunol.*, 1953, **70,** 514.
28. Kabat, E. A., and Bezer, A. E., *Arch. Biochem. and Biophysics*, 1958, in press.
29. Baer, H., Kabat, E. A. and Knaub, V., Immunochemical studies on blood groups. X. The preparation of blood group A and B substances and an inactive substance from individual horse stomachs and of blood group B substances from human saliva, *J. Exp. Med.*, 1950, **91,** 105.
30. Leskowitz, S., and Kabat, E. A., Immunochemical studies on blood groups. XIV. The separation and quantitative estimation of glucosamine and galactosamine in blood group substances, *J. Am. Chem. Soc.*, 1954, **76,** 4887.
31. Beiser, S. M., Kabat, E. A., and Schor, J. M., Immunochemical studies on the specific polysaccharide of type II pneumococcus, *J. Immunol.*, 1952, **69,** 297.
32. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., Preparation and properties of serum and plasma proteins. IV. System for separation into fractions of protein and lipoprotein components of biological tissues and fluids, *J. Am. Chem. Soc.*, 1946, **68,** 459.
33. Kabat, E. A., and Murray, J. P., A comparison of human γ-globulins in their reactivity with rabbit anti-γ-globulin by the quantitative precipitin method, *J. Biol. Chem.*, 1950, **182,** 251.
34. Allen, P. Z., and Kabat, E. A., unpublished data.
35. Kabat, E. A., Glusman, M., and Knaub, V., Quantitative estimation of the albumin and gamma globulin in normal and pathologic cerebrospinal fluid by immunochemical methods, *Am. J. Med.*, 1948, **4,** 653.
36. Mueller, J. H., and Miller, P. A., Production of diphtheric toxin of high potency (100 Lf) on a reproducible medium, *J. Immunol.*, 1941, **40,** 21.
37. Pillemer, L., and Toll, D., The purification of diphtheria toxoid, *Science*, 1947, **105,** 102.
38. Lepow, I. H., and Pillemer, L., Studies on the purification of diphtherial toxin, *J. Immunol.*, 1952, **69,** 1.
39. Fraser, D. T., The technique of a method for the quantitative determination of diphtheria antitoxin by a skin test in rabbits, *Tr. Royal Soc. Canad.*, 1931, sec. **V25,** 175.
40. Heidelberger, M., and MacPherson, C. F. C., Quantitative microestimation of antibodies in the sera of man and other animals, *Science*, 1943, **97,** 405; **98,** 63.
41. Markham, R., A steam distillation apparatus suitable for micro-Kjeldahl analysis, *Biochem. J.*, 1942, **36,** 790.
42. Folin, O., and Ciocalteu, V., Tyrosine and tryptophane determinations in proteins, *J. Biol. Chem.*, 1927, **73,** 627.
43. Oakley, C. L., and Fulthorpe, A. J., Antigenic analysis by diffusion, *J. Path. and Bact.*, 1953, **65,** 49.
44. Telfer, W. H., and Williams,C. M., Immunological studies of insect metamorphosis, *J. Gen. Physiol.*, 1953, **36,** 389.

45. Pappenheimer, A. M., Jr., Lundgren, H. P., and Williams, J. W., Studies on the molecular weight of diphtheria toxin, antitoxin and their reaction products, *J. Exp. Med.*, 1940, **71,** 247.

46. Kabat, E. A., and Pedersen, K. O., The molecular weights of antibodies, *Science*, 1938, **87,** 372.

47. Vaughan, J. H., and Kabat, E. A., Studies on the antibodies in rabbit antisera responsible for sensitization of human skin: I. The role of impurities in crystalline egg albumin in stimulating the production of skin-sensitizing antibody, *J. Exp. Med.*, 1953, **97,** 821.

48. Pappenheimer, A. M., Jr., Edsall, G., Lawrence, H. S., and Banton, H. J., A study of reactions following administration of crude and purified diphtheria toxoid in an adult population, *Am. J. Hyg.*, 1950, **52,** 353.

49. Pappenheimer, A. M., Jr., and Yoneda, M., A quantitative study of the diphtheria toxin-antitoxin flocculation reaction using S^{35}-methionine-labelled toxin, *Brit. J. Exp. Path.*, 1957, **38,** 194.