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SEROLOGICAL REACTIONS WITH SIMPLE CHEMICAL COMPOUNDS (PRECIPITIN REACTIONS)

By K. LANDSTEINER, M.D., and J. van der SCHEER

(From the Laboratories of The Rockefeller Institute for Medical Research)

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Serological reactions with chemical compounds of simple constitution were demonstrated for the first time by means of the so called inhibition reaction (1, 2). This subject has been discussed (1), and the arguments given for the view that the inhibition phenomena are caused by a combination of the inhibiting substances with antibodies, thereby preventing their precipitating action. Although there is no reasonable doubt as to the validity of this explanation which is strongly supported by the close similarity in the specificity of inhibition and precipitin reactions and the analogy to the inhibition produced by an excess of precipitable substances, it has remained of value to furnish direct experimental proof of the combination of antibodies with low molecular compounds by a reaction which does not involve the use of protein antigens.

In experiments on anaphylaxis to azoproteins it was shown that previous injection with azodyes protected sensitized guinea pigs against the shocking action of azoproteins containing the same azo component (3–5). Since this protection was still evident after the dyes had practically disappeared from the circulation, the effect was attributed to a neutralization of antibodies. In some cases the injection of the dyes was even followed by typical anaphylactic symptoms. Klopstock and Selter (6) reported that they obtained complement fixation reactions with immune sera to azoproteins, and emulsions of lecithin preparations to which had been added diazotized *p*-arsanilic acid or metanilic acid. Interesting experiments were carried out by Marrack and Smith (7) who were able to show that the diffusion of an azodye prepared from *p*-arsanilic acid is interfered with by the addition of an immune serum specific for the azo component.

Attempts made in the course of studies on azoproteins to produce specific precipitin reactions with simple azodyes did not lead to consistent results. Recently, a special group of azodyes was found with

which such reactions could easily be demonstrated. These substances were made from a series of aminoanilic acids, with aliphatic side chains containing from two to eight carbon atoms, by diazotization and coupling to resorcinol or tyrosine. For the production of the immune sera, antigens were prepared by diazotizing the same aminoanilic acids and coupling the diazo compounds to protein.

Technique

Preparation of Nitroanilic Acids.—The method used was essentially the same as that described in a previous paper (8).[1] A finely ground mixture of equimolecular quantities of dicarboxylic acids (malonic, succinic, glutaric, adipic, pimelic, and suberic acids), and para-nitroaniline was melted in a paraffin bath at 170–175°C., and the mixture was stirred continuously for a period of 45 minutes. In the preparation of nitromalonic acid, the temperature of the bath was kept at only 130–135°C. In all cases except that of succinic acid it was found necessary to use fused anhydrous zinc chloride in the condensation in order to obtain satisfactory yields.

The powdered zinc chloride (8 gm. for 13.8 gm. of *p*-nitroaniline) was added to the melt in 5 portions during the first 15 minutes. After heating for 45 minutes, the mass was poured into about 10 volumes of water in a porcelain dish and heated on the steam bath until broken up. During this process, concentrated sodium hydroxide was added in small amounts until the solution remained alkaline to litmus. After filtration, the insoluble material was finely ground and extracted a second time with hot water and alkali in the same manner. The aqueous extracts were combined, made neutral to litmus, and after standing in the ice box overnight, the filtered solution was made acid to Congo red by addition of concentrated hydrochloric acid. The precipitate was washed with water and dried. Of para-nitromalonanilic acid only 20 per cent of the theoretical yield was obtained due to decomposition of malonic acid during the fusion. In the other cases the yield was 40 to 50 per cent.

The following nitroanilic acids were prepared.

Para-nitromalonanilic acid (NO_2–C_6H_4–NH–CO–CH_2–COOH): Crystallized from boiling 25 per cent alcohol. Microscopic platelets. Melting point: 157°C., with gas formation.

Titration: 0.1279 gm., dissolved in 80 per cent alcohol, required for neutralization 5.7 cc. N/10 NaOH. Formula $C_9H_8O_5N_2$ requires 5.7 cc.

Para-nitrosuccinanilic acid (NO_2–C_6H_4–NH–CO–CH_2–CH_2–COOH): Crystallized from 25 parts of boiling 25 per cent alcohol. Long, microscopic, prismatic platelets. Melting point: 194–195°C.

[1] *p*-Nitrooxanilic acid was made according to the method described by Ossian Aschan (9).

Titration: 0.119 gm., dissolved in 80 per cent alcohol, required for neutralization 5 cc. N/10 NaOH. Formula $C_{10}H_{10}O_5N_2$ requires 4.96 cc.

Para-nitroglutaranilic acid (NO_2–C_6H_4–NH–CO–CH_2–CH_2–CH_2–COOH): Crystallized from 40 parts of boiling 25 per cent alcohol. Long, thin, microscopic crystals. Melting point: 170–171°C.

Titration: 0.2199 gm., dissolved in 80 per cent alcohol, required for neutralization 8.7 cc. N/10 NaOH. Formula $C_{11}H_{12}O_5N_2$ requires 8.72 cc.

Para-nitroadipanilic acid (NO_2–C_6H_4–NH–CO–CH_2–CH_2–CH_2–CH_2–COOH): Crystallized from 25 parts of boiling 50 per cent alcohol. Narrow microscopic platelets. Melting point: 174–175°C.

Titration: 0.133 gm., dissolved in 80 per cent alcohol, required for neutralization 5 cc. N/10 NaOH. Formula $C_{12}H_{14}O_5N_2$ requires 5 cc.

Para-nitropimelanilic acid (NO_2–C_6H_4–NH–CO–CH_2–CH_2–CH_2–CH_2–CH_2–COOH): Crystallized from 50 parts of boiling 25 per cent alcohol. Long, narrow, microscopic platelets. Melting point: 147–148°C.

Titration: 0.1530 gm., dissolved in 80 per cent alcohol, required for neutralization 5.45 cc. N/10 NaOH. Formula $C_{13}H_{16}O_5N_2$ requires 5.46 cc.

Para-nitrosuberanilic acid (NO_2–C_6H_4–NH–CO–CH_2–CH_2–CH_2–CH_2–CH_2–CH_2–COOH): The crude substance often contained some suberic acid from which it was easily freed by redissolving in water with sodium hydroxide and partial precipitation from this solution with hydrochloric acid which caused the nitro compound to separate first. It was then dissolved in boiling 50 per cent alcohol and crystallized upon cooling of the hot solution as microscopic platelets. Melting point: 158–159°C.

Titration: 0.147 gm., dissolved in 80 per cent alcohol, required for neutralization 5.05 cc. N/10 NaOH. Formula $C_{14}H_{18}O_5N_2$ requires 5 cc.

Preparation of Aminoanilic Acids.—The nitroanilic acid, dissolved in about 3 parts of water by addition of a slight excess of ammonium hydroxide and heating, if necessary, was added to a hot solution of ferrous sulfate, 7 aq. (6.5 mols for each mol of nitroanilic acid) in 2.5 parts of water. A 28 per cent ammonia solution (10 cc. for each 12 gm. of ferrous sulfate, 7 aq.) was added in 5 equal portions over a period of 10 minutes, shaking well with each addition. After 15 minutes heating on the steam bath, the ferric hydroxide was removed by filtration and to the clear filtrate enough 10 per cent hydrochloric acid was added to obtain maximum precipitation of the amino compound. The precipitate was filtered off on a Buchner funnel after standing in the ice box overnight. It was freed from a very small amount of acid-insoluble material by redissolving in a small amount of water with a slight excess of dilute hydrochloric acid and reprecipitating from the filtrate by addition of the required amount of dilute sodium hydroxide. After standing in the ice box overnight, the substance was filtered off and dried *in vacuo* at 50° over calcium chloride. 70 to 80 per cent of the theoretical yield was obtained.

Para-aminomalonanilic acid (NH_2–C_6H_4–NH–CO–CH_2–COOH): Crystallized from hot water using norit for decolorizing. White microscopic platelets. Melting point: 175–176°C., with gas formation.

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Kjeldahl determination after drying at 100° *in vacuo* over sulfuric acid: $C_9H_{10}O_3N_2$ calculated N 14.43 per cent, found 14.31 per cent.

Para-aminosuccinanilic acid (NH_2–C_6H_4–NH–CO–CH_2–CH_2–COOH): Crystallized from 15 parts of boiling water, using norit for decolorizing. Long, narrow, microscopic platelets. Melting point: 183–184°C., with darkening.

Kjeldahl determination after drying at 100° *in vacuo* over sulfuric acid: $C_{10}H_{12}O_3N_2$ calculated N 13.46 per cent, found 13.30 per cent.

Para-aminoglutaranilic acid (NH_2–C_6H_4–NH–CO–CH_2–CH_2–CH_2–COOH): Crystallized from boiling water, using norit for decolorizing. Microscopic platelets. Melting point: 186–187°C., with darkening.

Kjeldahl determination after drying at 100° *in vacuo* over sulfuric acid: $C_{11}H_{14}O_3N_2$ calculated N 12.61 per cent, found 12.20 per cent.

Para-aminoadipanilic acid (NH_2–C_6H_4–NH–CO–CH_2–CH_2–CH_2–CH_2–COOH): Crystallized from 15 parts of boiling water, using norit for decolorizing. Long, narrow, microscopic platelets. Melting point: 165–166°C.

Kjeldahl determination after drying at 100° *in vacuo* over sulfuric acid: $C_{12}H_{16}O_3N_2$ calculated N 11.86 per cent, found 11.57 per cent.

Para-aminopimelanilic acid (NH_2–C_6H_4–NH–CO–CH_2–CH_2–CH_2–CH_2–CH_2–COOH): Crystallized from boiling water, using norit for decolorizing. Long, narrow, microscopic platelets. Melting point: 178–179°C., with darkening.

Kjeldahl determination after drying at 100° *in vacuo* over sulfuric acid: $C_{13}H_{18}O_3N_2$ calculated N 11.20 per cent, found 11.06 per cent.

Para-aminosuberanilic acid (NH_2–C_6H_4–NH–CO–CH_2–CH_2–CH_2–CH_2–CH_2–CH_2–COOH): Crystallized from 100 parts of boiling water, using norit for decolorizing. Long, narrow, microscopic platelets. Melting point: 162–163°C.

Kjeldahl determination after drying at 100° *in vacuo* over sulfuric acid: $C_{14}H_{20}O_3N_2$ calculated N 10.61 per cent, found 10.38 per cent.

Para-aminooxanilic acid (NH_2–C_6H_4–NH–CO–COOH): Prepared by condensation of para-phenylenediamine and oxalic acid following the methods described by G. Koller (10) and by W. A. Jacobs and M. Heidelberger (11). The product thus obtained was redissolved in about 30 parts of water with a slight excess of dilute sodium hydroxide. After making neutral to litmus, the solution was heated and decolorized with norit. The colorless filtrate was made faintly acid to Congo by addition of hydrochloric acid and the precipitated para-aminooxanilic acid was filtered and dried *in vacuo* at 50°.

Preparation of Azodyes.—Resorcinoldisazo-*p*-anilic acids: 2 millimols of aminoanilic acid were dissolved in 50 cc. of water and 6 cc. of normal hydrochloric acid and diazotized at 0–5°C. by slow addition of 2 cc. of normal sodium nitrite. After the diazotization was complete (test with starch iodide paper), 1 millimol of resorcinol (110 mg.) dissolved in 40 cc. N/2 sodium carbonate solution, cooled to 0–5°, was rapidly added with stirring, and the mixture was kept at 0–5° for ½ hour. Enough dilute hydrochloric acid was added to make the solution weakly acid to Congo and bring about complete precipitation of the dye, which was then separated from the liquid by centrifugalization. After washing twice

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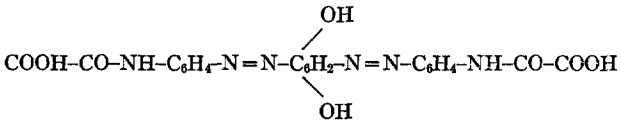
in the centrifuge tube with 50 cc. of water, the dye was redissolved in 40 cc. of water by addition of a slight excess of normal sodium hydroxide and was reprecipitated by the addition of hydrochloric acid. It was again washed three times with 50 cc. of water and dried at 75° *in vacuo* over calcium chloride. The yield was from 97 to 99 per cent of the theory.

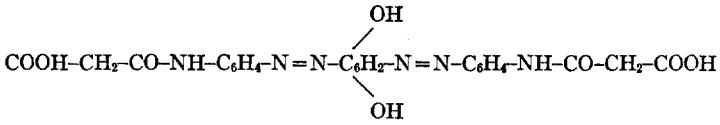
The following values for nitrogen found by analysis are calculated for ash-free substance. The content of ash in the dyes varied between 1 and 2 per cent with the exception of the resorcinoldisazo-*p*-oxanilic acid which contained 6.2 per cent ash.

Resorcinoldisazo-*p*-oxanilic acid:



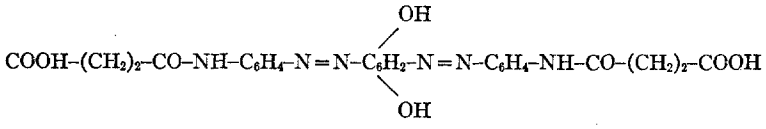
Calculated N 17.01 per cent, found 16.89 per cent.

Resorcinoldisazo-*p*-malonanilic acid:



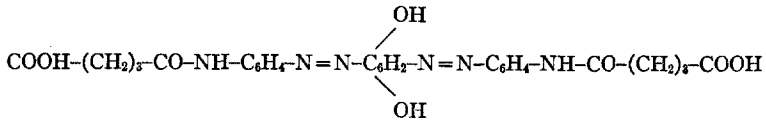
Calculated N 16.16 per cent, found 16.01 per cent.

Resorcinoldisazo-*p*-succinanilic acid:



Calculated N 15.33 per cent, found 15.56 per cent.

Resorcinoldisazo-*p*-glutaranilic acid:



Calculated N 14.58 per cent, found 14.35 per cent.

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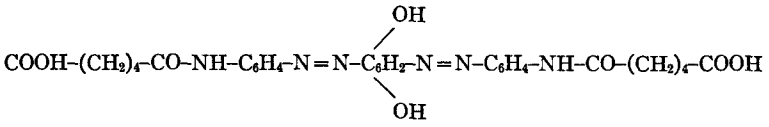
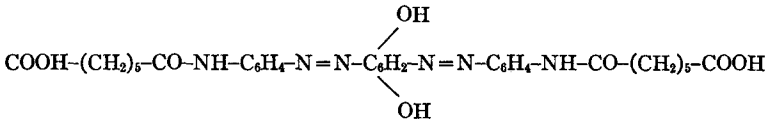
Resorcinoldisazo-*p*-adipanilic acid:



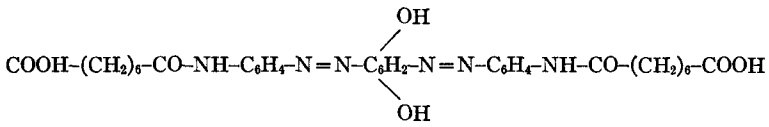
Calculated N 13.91 per cent, found 14.03 per cent.

Resorcinoldisazo-*p*-pimelanilic acid:



Calculated N 13.29 per cent, found 13.10 per cent.

Resorcinoldisazo-*p*-suberanilic acid:



Calculated N 12.73 per cent, found 12.81 per cent.

Preparation of Azoproteins.—1 millimol of aminoanilic acid was diazotized as described before at 0–5° and the diazo solution added to a cold solution containing 540 mg. of horse serum-globulin (prepared from diluted horse serum by half-saturation with ammonium sulfate and subsequent dialysis to remove the salt) and 13 cc. of normal sodium carbonate. The total volume of the mixture was approximately 65 cc. Coupling was allowed to proceed for ½ hour at 0–5° and the azoprotein was precipitated by addition of enough hydrochloric acid to make the solution weakly acid to Congo. The precipitate was separated from the liquid by centrifugalization and was washed several times with weakly acidulated saline. It was redissolved in saline by means of dilute sodium carbonate, making the solution neutral to litmus. As a preservative, 0.25 per cent phenol was added. The solutions used for the injections contained 5 mg. of azoprotein pro cc.

Immunization.—Four azoproteins were used for immunization; namely, those prepared from *p*-aminooxanilic acid, *p*-aminosuccinanilic acid, *p*-aminoadipanilic acid, and *p*-aminosuberanilic acid by coupling to horse serum-globulin as described above. For the immunization with each antigen four rabbits were used, each receiving daily intravenous injections of 2 cc. of the antigen solution for 6

days. One or two more courses of injections were given at intervals of 1 week,[2] and the sera were tested 7 days after the last injection. Two to three active sera were obtained in each series. For convenience the sera will be referred to as oxanilic, succinanilic, adipanilic, and suberanilic immune sera.

EXPERIMENTAL

Precipitin Reactions with Azodyes.—The immune sera obtained in the manner described, with the exception of the oxanilic immune sera, gave distinct precipitin reactions when added to solutions of the sodium salts of the corresponding resorcinol-azodyes (Table I).[3]

Results practically identical with those recorded in the table were obtained with the other sera at our disposal.

The reactions with the succinanilic sera were very specific whereas the action of the immune sera for the antigens with longer side chains extended to the compounds next in the series. The same sort of specificity appeared in tests with azoproteins. These observations will be described in a subsequent communication.

The strongest reactions occurred with the suberanilic immune sera and the homologous dye. Upon the addition of the antiserum, a distinct turbidity appeared almost immediately and after $\frac{1}{2}$ hour small flakes, and later larger ones were formed, the phenomenon resembling in all respects a common precipitin reaction. The highest dilutions in which a reaction was still noticeable corresponded to a content of 0.001 mg. per 1 cc.; consequently, the sensitivity of the tests is of the same order as precipitin reactions with proteins or bacterial carbohydrates. In the tests with the succinanilic and adipanilic immune sera the reactions were similar, but the precipitation developed more slowly and was less intense. Whilst in the case of the adipanilic sera this difference is probably due to the fact that the immune sera happened to be less active, as evidenced by the reactions with azoproteins, this explanation does not apply to the succinanilic sera which were almost as active as those for the suberanilic compound. Probably the reason for the weaker reactions is a lower precipitability of the dyes due to the shorter side chain. This assumption is borne out by the observation that other azodyes examined were far less precipitable

[2] *Cf.* Reference 12.

[3] Reactions were also obtained by complement fixation.

TABLE I

1/50 millimol of the dye (for instance 13.2 mg. of the resorcinoldisazo-*p*-suberanilic acid) was dissolved in 3 cc. of N/50 sodium hydroxide and 1 cc. N/50 hydrochloric acid and 1 cc. distilled water was added. The solution was centrifuged to remove traces of insoluble material. In the case of resorcinoldisazo-*p*-oxanilic acid, 1 cc. water was added in place of hydrochloric acid since it was found that the greater alkalinity was necessary to prevent flocculation upon subsequent dilution with saline.

For the precipitin tests given in Table I, 1 to 3 capillary drops of immune serum were added to 0.2 cc. of the dye solutions (prepared as described above), diluted 1:20, 1:100, and 1:500, with saline. Readings were taken after 1 and 2 hours at room temperature and after standing overnight in the ice box. The intensity of the reactions is indicated as follows: 0, f. tr. (faint trace), tr. (trace), tr. (strong trace), ±, ∔, +, +±, ++, etc.

Control tests with normal rabbit sera and some other immune sera such as precipitins for human or pig serum gave no reactions.

Immune sera for azoproteins made from	Readings taken after	Dyes made from: *p*-Aminooxanilic acid, dilution			*p*-Aminomalonanilic acid, dilution			*p*-Aminosuccinanilic acid, dilution			*p*-Aminoglutaranilic acid, dilution			*p*-Aminoadipanilic acid, dilution			*p*-Aminopimelanilic acid, dilution			*p*-Aminosuberanilic acid, dilution		
		1:20	1:100	1:500	1:20	1:100	1:500	1:20	1:100	1:500	1:20	1:100	1:500	1:20	1:100	1:500	1:20	1:100	1:500	1:20	1:100	1:500
p-Aminosuccinanilic acid, 2 drops	1 hr.	0	0	0	0	0	0	±	+	0	0	0	0	0	0	0	0	0	0	0	0	0
	2 hrs.	0	0	0	0	0	0	+	+±	0	0	0	0	0	0	0	0	0	0	0	0	0
	Night in ice box	0	0	0	0	0	0	+±	++	±	0	0	0	0	0	0	0	0	0	0	0	0
p-Aminoadipanilic acid, 3 drops	1 hr.	0	0	0	0	0	0	0	0	0	0	0	0	+	+	f. tr.	±	tr.	0	±	f. tr.	0
	2 hrs.	0	0	0	0	0	0	0	0	0	0	0	0	+	+	tr.	±	tr.	0	±	tr.	0
	Night in ice box	0	0	0	0	0	0	0	0	0	0	0	0	++±	++	∔	++	±	f. tr.	+±	±	f. tr.
p-Aminosuberanilic acid, 1 drop	1 hr.	0	0	0	0	0	0	0	0	0	0	0	0	∔	∔	0	+	+	tr.	+±	++	±
	2 hrs.	0	0	0	0	0	0	0	0	0	0	0	0	∔	∔	f. tr.	+	+±	tr.	++	++	∔
	Night in ice box	0	0	0	0	0	0	0	0	0	tr.	0	0	++	++	±	+++±	+++±	+	++++	+++	+±

The dilutions are in terms of a solution containing 1/50 millimol of the dye in 5 cc.

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than the resorcinoldisazo-suberanilic acid.[4] Azodyes made by coupling 2 mols diazotized dextro-para-aminotartranilic acid or para-arsanilic acid with 1 mol resorcinol, in dilutions of 1:100 and 1:500 of 1/50 millimol of the dye in 5 cc., gave only very weak reactions with the homologous immune sera (1, 8) upon standing for 2 hours at room temperature and overnight in the ice box. On subsequent

TABLE II

0.2 cc. of a solution of the sodium salts of the resorcinol dyes in saline (containing 1/2500 millimol in 10 cc.) were mixed with 0.05 cc. of a neutral solution of the sodium salts of the nitroanilic acids, containing 1/32 millimol in 10 cc. for the tests with the suberanilic immune serum and 1/64 millimol in 10 cc. for the tests with the succinanilic and adipanilic immune sera. 1 to 2 drops immune serum were added. The control tube contained the dye solution, immune serum and 0.05 cc. saline. The readings were taken after 1 and 3 hours at room temperature and after standing overnight in the ice box.

Immune sera for azoproteins made from	Readings taken after	Substances				
		p-Nitro-oxanilic acid	*p*-Nitrosuc-cinanilic acid	*p*-Nitro-adipanilic acid	*p*-Nitro-suberanilic acid	Control
p-Aminosuccinanilic acid, 1 drop	1 hr.	+	0	±	±	+
	3 hrs.	+	0	+	+	+
	Night in ice box	++±	0	++±	++±	++±
p-Aminoadipanilic acid, 2 drops	1 hr.	±	±	0	tr.	±
	3 hrs.	+	±	0	tr.	+
	Night in ice box	+±	+	0	±	+±
p-Aminosuberanilic acid, 1 drop	1 hr.	+±	+±	+	0	++
	3 hrs.	++	+±	+	0	++±
	Night in ice box	+++±	++±	++	0	+++±

spinning the reactions became much more evident, the precipitate appearing as large, thin flakes upon shaking up the sediment.

The tests presented in the table were made with freshly prepared solutions of the sodium salts of the dyes. When the solutions (con-

[4] In this connection the results of Hartley (13) on the precipitation of defatted proteins are worth mentioning.

taining 1/50 millimol of the dye in 5 cc.) were kept in the ice box for several days up to a week, the precipitability with immune serum increased gradually, probably owing to a lowered dispersion of the dissolved substance. These solutions were made as described above and contained only a very small quantity of inorganic salt. For the tests they were diluted with saline in the usual way.

From the results reported one would anticipate the possibility of eliciting anaphylactic shock in animals sensitized with azoprotein, by administration of those dyes which are distinctly precipitated by immune sera. Such was indeed the case; it is intended to describe these experiments later in detail.

Inhibition of the Precipitin Reaction.—The similarity between the precipitin reaction with dyes and those with azoproteins extends to the phenomenon of inhibition by low molecular substances with corresponding groupings. Thus neutral solutions of the sodium salts of nitroanilic and aminoanilic acids inhibited the precipitin reactions with the dyes specifically. Such results are presented in Table II. The solutions of nitro- or aminoanilic acids were not precipitated by the immune sera.

COMMENT AND SUMMARY

Experiments are described demonstrating the precipitation of azodyes by immune sera prepared by the injection of azoproteins containing the same azo component. These precipitin reactions prove conclusively the view already advanced on the basis of inhibition reactions that antibodies combine specifically with substances of small molecular weight. Although in this respect both phenomena have the same significance, the precipitin reactions with dyes are simpler in that the aid of a protein antigen is eliminated.

That specific serological precipitin reactions can take place with substances other than proteins has been amply demonstrated by studies on bacterial antigens (14) (polysaccharides (15)). The present findings show that for the precipitation with immune sera not even a high molecular weight of the reactive substance is required. Factors determining the tendency to separate out from the liquid upon combination with antibody seem to be the colloidal state of the solution and the chemical composition of the substance. With regard to the

influence of chemical composition, a striking example is provided by the suberic acid dye which gives particularly strong precipitin reactions, most probably on account of its long aliphatic side chains.

The results reported may be of use for studies on the mechanism of serological precipitation.

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