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The Purification of Azoprotein Antibodies by the Dissociation of Specific Precipitates

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Antibodies can be separated from a part of the serum proteins by electrophoresis or by the fractional precipitation with ammonium sulphate. Purification by these or similar non-specific methods is limited by the fact that some of the serum proteins have properties almost identical with those of the antibodies. The separation of antibodies from such proteins is rendered possible, however, by the application of specific methods. These involve the precipitation of the antibody by the homologous antigen and the dissociation of the antibody complex into its components. The main difficulty consists in finding conditions, under which only the antibody is dissolved, while the antigen remains in the precipitate. This difficulty does not arise if cellular or bacterial antigens are used (Heidelberger & Kabat, 1938). Soluble antigens have been made insoluble by coupling them with stroma (Landsteiner & van der Scheer, 1936) or by adsorbing them on charcoal particles (Svensson, 1943). The antibody can be split off from these particles by dilute acetic acid. On the other hand, antibodies to the specific polysaccharides of pneumococci are extracted from the precipitate by lime water or baryta, while Ca or Ba compounds of the polysaccharide remain undissolved (Felton & Bailey, 1926; Heidelberger & Kabat, 1938).

Our own attempts to purify anti-azoproteins were based originally on the experience that azoproteins are precipitated by acids, while globulins (antibodies) remain dissolved (Haurowitz, 1936a). Accordingly we have attempted splitting of antibodies from the antigen-antibody precipitates by dilute acids.

EXPERIMENTAL

Preparation of antigens and immunization. Pseudo-globulin from beef or sheep serum was prepared as described previously (Haurowitz, Sarafyan & Schwerin, 1941). The protein (2 g.) was coupled with 2 mmol. of anthranilic, sulphanilic, arsanilic acid or *p*-aminobenzylamine (Haurowitz, 1936b, 1942). The azoproteins were purified by re-precipitation and dialysis and were mixed with 1% of solid NaCl. They were injected into rabbits subcutaneously as described in the papers mentioned. The immune sera were stored at -12° and thawed out shortly before the addition of the antigen.

Precipitation of anthranilic acid-azoglobulin by buffer solutions. A 5% solution of this azoprotein (0.2 ml.) was mixed with 2 ml. of the standard buffer solution of Teorell & Stenhagen (1938) and adjusted to the intended pH by the addition of the required amount of 0.1 N-HCl. The volume was adjusted to 2.5 ml. by the addition of water and the mixture kept at room temperature for 24 hr. The tubes were then centrifuged and the amount of azoprotein in the supernatant solution determined colorimetrically. The amount of precipitated azoprotein was obtained as the difference between the total and the dissolved azoprotein. The results are shown by the following figures:

pH:	2.0	2.5	3.0	3.5	4.0	4.5	5.0
Precipitated azoprotein (mg.):	0	4.2	5.4	6.2	0	0	0

Precipitation of the antibodies. Different amounts of the immune sera, varying from 3 to 55 ml., were precipitated by the addition of solutions containing from 1 to 3% of the homologous antigen. The antigen solution was added either in a single dose or fractionally, in amounts of 0.05–3.0 mg., until no further distinct precipitate was formed (Haurowitz, 1942). Some of the sera (Table 1, no. 263) were precipitated in both ways in parallel experiments. The precipitates obtained by the fractional addition of the antigen were

combined, homogenized by stirring with a glass rod and then treated as the precipitates obtained by a single dose of the antigen. Only in the exp. no. 256 (Table 1) were the combined precipitates nos. 1, 2 and 4 on the one hand and nos. 5-8 on the other, collected in two separate tubes and analyzed separately. The mixture of antigen and immune serum was kept at 38° for 30 min. and frozen solid. Having been kept at -12° over night the precipitates were thawed, homogenized and freed from soluble substances by repeated washing with 1% NaCl solution.

Dissociation of the precipitates. Preliminary experiments with anthranilic acid-azoglobulin had shown that a dissociation of the precipitate is not brought about by solutions of the Teorell & Stenhagen (1938) buffer at a pH between 4 and 7 or by concentrated solutions of NaCl (Heidelberger & Kendall, 1936). On the other hand the precipitates were completely dissolved by aqueous solutions of baryta or of HCl. The dissolution in HCl could be prevented by addition of solid NaCl. Accordingly the following procedure was adopted for the purification of antibodies.

The ppt. was mixed with c. 2-3 vol. of 10% (w/v) NaCl (0.5-6.0 ml.). The mixture was homogenized by stirring and cooled to 0°. Then an equal vol. (0.5-6.0 ml.) of a chilled solution of the acid indicated in Table 1 was added, the mixture homogenized immediately and centrifuged. No low-temperature centrifuge being available, the solution was centrifuged only for 2-3 min. at room temperature. The supernatant solution was poured into a centrifuge tube containing the amount of 0.1 N or 1.0 N-NaOH required for neutralization, and was adjusted to a pH of 7 by the dropwise addition of mono- or disodium phosphate, respectively (bromothymol blue being used as external indicator). The acid-treated ppt. was stirred up with 0.5-3.0 ml. of a 5% solution of NaCl and centrifuged. The supernatant liquid was added to the neutralized first extract and brought again to a pH of 7 by sodium phosphate. The bulk of the antibodies was extracted by this method. A second extraction with acid furnished only traces of antibody.

The neutralized antibody solution was clear and colourless. Previously it had been assumed, therefore, that it was free of antigen (Haurowitz & Tekman, 1946). Later experiments proved, however, that it contained an antigen-antibody complex; this was removed by diluting the solution with water to a content of 1.5% NaCl, keeping it at 38° for 30 min. and then freezing solid. The frozen solutions were thawed, homogenized and centrifuged. The precipitated antigen-antibody complex was combined with the acid-insoluble remainder. The supernatant solution, containing the purified antibody, was used for the analyses and experiments described in Table 1 and in the following sections.

The acid-insoluble remainders of the precipitates were mixed with 3-5 ml. of a 1% saline solution, homogenized by stirring, neutralized with disodium phosphate, washed with NaCl solution and organic solvents, dried, weighed and analyzed for antigen-content as described previously (Haurowitz, 1942). The results are recorded in Table 1.

Analysis of antibody solutions. The amount of total protein in the antibody solution was determined by Kjeldahl analysis on 0.5-2.0 ml. of the solution or in its trichloroacetic acid ppt. A nitrogen content of 16% was assumed for the antibody protein. The content of active antibody (Table 1) was determined by the repeated addition of 0.5-1.0 mg. of the homologous antigen to 3-5 ml. of the

purified antibody solution, until no further distinct ppt. was formed. Two to four precipitates were thus obtained. They were combined, homogenized, washed and weighed; then their content of antigen was determined colorimetrically. The difference between the wt. of the ppt. and that of its content of antigen was considered as active antibody. Its amount was recalculated for the whole volume of the solution and recorded in Table 1 as active antibody.

As an example of an analysis, 10 ml. of the serum no. 279/83, after treatment of the ppt. with N-HCl, gave 16.9 mg. of an insoluble remainder containing 7.1 mg. of the antigen and 9.8 (=16.9-7.1) mg. of antibody. Kjeldahl analysis of the antibody solution (15 ml.) gave for the total amount of dissolved protein 28.4 mg. or 74% of the total amount of antibody precipitated from the immune serum, which amounts to 9.8+28.4=38.2 mg. Five ml. of the antibody solution, upon the addition of the antigen, gave 7.1 mg. of a ppt. containing 1.3 mg. of the antigen and 5.8 mg. of antibody. This corresponds to 17.4 mg. or 61% of active antibody in the total amount of dissolved protein.

These figures are minimal values, because a small excess of antigen was present after the last addition of the antigen, so that traces of soluble antigen-antibody complexes might have been formed. The actual amount of active antibody, therefore, is slightly higher than indicated in Table 1.

Further purification of the antibody. 25 ml. of the combined antibody solutions no. 279/83a (Table 1), containing at least 24.4 mg. of active antibody/36 mg. of total protein, were precipitated by the addition of 30 mg. of the homologous antigen. The ppt. was dissociated by 1.5 ml. of a 10% solution of NaCl and 1.5 ml. of 0.1 N-HCl, as described above. The resulting solution contained at least 17.3 mg. (c. 90%) of active antibody/19.2 mg. of total protein. Similarly, an antibody solution prepared from another immune serum (no. 259) to anthranilic acid-azoglobulin, containing 25.6 mg. of total protein with 42% of active antibody, upon precipitation and dissociation of the precipitate, furnished 9.6 mg. of dissolved protein containing at least 7.7 mg. (80%) of active antibody.

Multiplicity of the antibodies. The antibody solution recovered from the immune serum no. 270 (Table 1) was exhausted at first by the addition of beef serum globulin, then by arsanic acid-azo-ovalbumin and, finally by arsanic acid-azoglobulin. The amount of added antigen and of the recovered precipitates is recorded in the Table. Since the first test antigen (beef serum globulin) was not coloured, its amount could not be determined analytically. An antigen/antibody ratio of 1/3 was accepted as fairly probable.

RESULTS

According to the experiments described in Table 1 a considerable purification of azoprotein antibodies is accomplished by dissociation of the precipitates with dilute acids containing 5% NaCl. In most of the experiments 0.1 N-HCl was used, but equally good results were obtained with 0.1 M-phosphoric acid. A certain minimal acidity is required for a successful dissociation. The optimal pH depends upon the isoelectric point of the azoprotein. While antibodies to the feebly acid anthranilic acid-azoglobulin and to *p*-aminobenzylamine-azoglobulin

Table 1. *Dissociation of precipitated azoprotein-antibody complexes by acids*

(Acids and buffer solutions were 0.1 M unless otherwise stated. Serum no. 279/83 is a mixture of the four sera nos. 279, 281, 282 and 283. The figures recorded for the dissolved protein and for the active antibody in the experiments nos. 256 and 262 are slightly too high, because the small amount of dissolved antigen-antibody complex was not removed from the antibody solution. The following abbreviations are used throughout the Table: BGI = beef serum globulin, SGI = sheep serum globulin; Buffer = mixture of 0.1 M-phosphoric acid and 0.1 M-KH₂PO₄. The ratio phosphoric acid/phosphate was 3/1, 1/1 and 1/3 in the buffer solutions A, B and C respectively. Buffer D is 0.1 M-KH₂PO₄. The pH of the solutions was determined by a quinhydrone electrode.)

No.	Immune serum		Dissociating medium*		Insoluble remainder		Dis-solved protein (mg.)	Active antibody (as % of dissolved protein)	Special remarks
	Homologous antigen	Vol. (ml.)	Added antigen (mg.)	Vol. (ml.)	Total wt. (mg.)	Wt. of antigen (mg.)			
237	Anthranilic acid-SGI	21.0	2 × 10	1.5	H ₃ PO ₄	30.7	12.5	24.5	Active serum
237	Anthranilic acid-SGI	21.0	2 × 10	1.5	H ₃ PO ₄	21.7	8.3	18.4	Inactivated serum
256	Anthranilic acid-SGI	25.0	4 × 3	5.0	HCl	23.2	8.0	17.7	Fraction 1 to 4
			4 × 3	5.0	HCl	22.6	10.0	15.2	Fraction 5 to 8
262a	Aminobenzylamine-SGI	7.5	3.5	0.75	CH ₃ COOH	11.1	5.7	7.6	(a) 11. Dec. 1945; (b) 20 Sept. 1946. The ppt. of 262b was extracted first with buffer C, then with n-HCl
262b	Aminobenzylamine-SGI	9.0	2 × 3.5	0.5	Buffer C	—	—	3.2	
			0.5	0.5	n-HCl	5.9	3.3	2.7	
263	Anthranilic acid-SGI	4.5	3 × 2	0.5	HCl	8.0	3.1	7.8	Precipitated fractionally
263	Anthranilic acid-SGI	4.5	6	0.5	HCl	8.5	3.3	8.6	Precipitated in a single dose
270	Arsanilic acid-BGI	8.0	3 × 9	1.0	HCl	18.5	8.0	29.4	Precipitated by BGI
									Precipitated by arsanic acid-ovalbumin
									Precipitated by arsanic acid-BGI
276a	Sulphanilic acid-SGI	5.0	2.5	0.5	HCl	5.4	2.1	9.5	(a) 1 July 1946; (b) 29 July 1946. The ppt. of 276b was extracted first with buffer, then with HCl
276a	Sulphanilic acid SGI	5.0	2.5	0.5	n-HCl	5.8	1.5	8.5	
276b	Sulphanilic acid-SGI	6.5	4	0.5	Buffer C	—	—	3.95	
				0.5	n-HCl	6.9	0.7	3.7	
278	Arsanilic acid-SGI	9.3	4 × 1	0.75	0.1 n-HCl	7.5	3.0	12.5	(a) 3 Sept.; (b) 5 Sept.; (c) 15 Sept. 1946
278	Arsanilic acid-SGI	9.3	4 × 1	0.75	0.3 n-HCl	8.7	3.0	15.6	
278	Arsanilic acid-SGI	9.3	4 × 1	0.75	1.0 n-HCl	7.8	3.0	16.8	
279/83a	Anthranilic acid-BGI	10.0	12	1.0	n-HCl	16.9	7.1	28.4	Buffer pH = 1.3 Buffer pH = 1.8 Buffer pH = 2.3 Buffer pH = 3.7
279/83a	Anthranilic acid-BGI	10.0	12	1.0	HCl	15.4	7.1	26.2	
279/83b	Anthranilic acid-BGI	10.0	12	1.0	H ₃ PO ₄	15.2	6.0	19.0	
279/83b	Anthranilic acid-BGI	10.0	12	1.0	Buffer A	17.2	6.0	18.0	
279/83b	Anthranilic acid-BGI	10.0	12	1.0	Buffer B	17.3	5.5	16.2	
279/83c	Anthranilic acid-BGI	10.0	12	1.0	Buffer C	15.8	10.0	15.0	
279/83c	Anthranilic acid-BGI	10.0	12	1.0	Buffer D	26.0	9.0	4.6	

* An equal vol. of 10% (w/v) NaCl is added to the acid solution given.

are split off from the specific precipitates by the buffer solution C ($\text{pH}=2.3$), antibodies to the strongly acid sulphanilic acid-azoglobulin are not extracted from the precipitate by this buffer solution (Exp. nos. 262, 276 and 279/283), and require the addition of HCl.

About 50–80 % of the precipitated antibody is split off from the precipitate by acids; on the other hand nearly all of the antigen and a part of the antibody remain undissolved. This method was applied successfully to nineteen different immune sera against anthranilic acid, arsanilic acid, sulphanilic acid and *p*-aminobenzylamine-azoglobulins. For brevity only some of these experiments are shown in Table 1.

A negative result in regard to arsanilic acid-azoglobulin, reported in a preliminary communication (Haurowitz & Tekman, 1946), was due to the very low titre of antibodies in the serum employed, as was stated later.

Only a part of the protein split off from the precipitate by acids is active antibody, precipitable by the homologous antigen. It was assumed, at first, that the inactivity of a part of the extracted antibody is due to its denaturation by the acid used for its dissociation. Our experiments (nos. 276, 278, 279/283) indicate, however, that the yield of active antibody is not reduced, when 0.1 N-HCl is replaced by 1.0 N-HCl. This does not indicate a strong denaturing effect of the acid. Possibly a part of the dissociated antibodies is composed of 'low-grade' antibodies which are not precipitated in the absence of more reactive antibodies (Heidelberger & Kendall, 1935). The presence of non-precipitable proteins cannot be attributed to non-specific proteins of the immune serum, since these proteins had been removed by repeated washing of the specific precipitates with saline solution. But one has to consider the possibility that a part of the complement protein is adsorbed to the precipitate and is split off by the acid. Attempts to reduce the amount of non-precipitable protein by keeping the immune sera at 56° for 50 min. and thus destroying the complement, had no success (Exp. no. 237, Table 1).

The yield of active antibody does not depend on the consistency of the precipitate, for it is not altered markedly by keeping the precipitate at 38° instead of freezing it or by the addition of the antigen in a single dose instead of adding it fractionally (serum no. 263). Higher yields of antibody were obtained, however, from the first than from the last fractions of the precipitate (Exp. no. 256, Table 1).

The experiments carried out with the immune serum no. 270 (Table 1) and similar experiments with other immune sera demonstrate that the solution of the purified antibody does not contain one or other of the antibodies present, but a multiplicity of antibodies adapted to the different determinant groups of the homologous antigen.

The dissociation of the specific precipitate by the acid-salt mixture had been ascribed previously (Haurowitz & Tekman, 1946) to the insolubility of the antigen at low pH values. This explanation is not satisfactory because anthranilic acid-azoglobulin is soluble at pH values <2 , as shown in the experimental part. Moreover, a part of the antibody cannot be extracted from the precipitate, even if the treatment with acid is repeated. An explanation is furnished, however, by recent experiments of Haurowitz, Schwerin & Tunç (1946), according to which azoproteins give non-specific precipitates with normal serum globulins at pH values under 5.0. We assume, therefore, that the specific precipitate, in which both components, antigen and antibody, are linked to each other by means of specifically adapted groups, on the addition of acid, is converted into a non-specific precipitate, in which azoprotein anions are bound to antibody cations by salt-like linkages. While specific precipitates contain 70–90 % of antibody, non-specific precipitates contain less than 50 % of globulin (Haurowitz, Schwerin & Tunç, 1946). Accordingly, considerable amounts of antibody are released from the precipitate by the addition of acid.

SUMMARY

1. Specific precipitates containing 10–30 % of azoprotein and 70–90 % of antibody are, on the addition of acids, converted into non-specific azoprotein-globulin precipitates, containing less than 50 % of antibody. About 50–80 % of the antibody is thus released from the precipitate and passes into the acid solution. Hydrochloric acid or phosphoric acid, containing 5 % of sodium chloride, was used for the dissociation of the precipitates.

2. Antibodies to anthranilic, arsanilic and sulphanilic acids and *p*-aminobenzylamine-azoglobulins were purified by this method. About 40–70 % of the dissolved protein was active antibody, precipitable by the homologous antigen. A repetition of the same procedure furnished antibody preparations of a high degree of purity containing at least 90 % of precipitable antibody.

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The Fate of Nicotinamide Methochloride and the Effect of Liver Poisons on its Elimination Rate in the Rat*

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The object of this work was to investigate two obscure aspects of the metabolism of nicotinamide, namely the fate of nicotinamide methochloride in the body and the effect of liver poisons on its formation and elimination. An important factor determining the rate of methylation of nicotinamide is probably the methylating efficiency of the liver. There are indications that in certain liver diseases in humans the urinary elimination of nicotinamide methochloride is affected (Najjar, Hall & Deal, 1945; Ellinger, 1945). The effect of carbon tetrachloride poisoning on the elimination of ingested nicotinamide methochloride by the rat has been studied by Perlzweig, Huff & Rosen (1946), who found elimination to be increased during the first days of poisoning. Ellinger & Coulson (1944) have shown that the recovery of nicotinamide methochloride from human urine is about 15% after oral and about 60% after parenteral administration; the remainder is unaccounted for. These findings have been confirmed by Perlzweig & Huff (1945), who postulated partial destruction in the liver (Perlzweig, Huff & Rosen, 1946), while Najjar & Deal (1946) suggested that hydrolysis of nicotinamide methochloride took place in the liver to yield methyl groups and nicotinamide.

To investigate these factors, the following experiments were carried out. The possible destruction of nicotinamide methochloride in the liver was studied (a) with liver slices and (b) with perfused surviving livers; (c) faeces and (d) bile were tested for nicotinamide methochloride; (e) the effect of

faecal bacteria on the compound was examined and (f) the effect of liver poisons on the elimination of nicotinamide methochloride and protein nitrogen was investigated.

METHODS

(a) *Liver slices.* Albino rats of 250–400 g. of both sexes from our own stock, kept since weaning on a mixed diet and with a known daily nicotinamide methochloride output, were stunned and killed by bleeding. The liver was removed immediately after death and kept in a covered glass bowl. Pieces of 300–500 mg. wet weight were cut off, weighed, sliced into slices of 0.2–0.4 mm. thickness and suspended in 10 ml. of modified Ringer solution (Krebs & Henseleit, 1932) in sufficient weighing bottles to form a duplicate series; the solutions contained in addition 0, 1, 3, or 10 µg. of nicotinamide methochloride per ml. Suspensions were aerated with a mixture of 5% CO₂ and 95% O₂ before stoppering and controls prepared of each concentration without the addition of liver. All bottles were agitated at 37°. Never more than 30 min. elapsed between the death of the animal and commencement of shaking. Pairs of bottles containing liver were opened after 2, 30, 60, 120 or 240 min. respectively; after 4 hr. the blanks were opened. The supernatant liquids were decanted into graduated centrifuge tubes, the residues washed twice with 0.5 ml. water which was added to the centrifuge tubes. 1.0 ml. of 20% (w/v) trichloroacetic acid was added. After heating to 75° for 30 min. and adjusting the volume to 12 ml., the precipitate was centrifuged off and nicotinamide methochloride in the liquid determined by the method of Coulson, Ellinger & Holden (1944). On two occasions liver slices were agitated in Krebs-Ringer solution at 37° for 2 hr. before 3 µg./ml. nicotinamide methochloride were added. Determinations of this compound were made 2, 30, 60, and 120 min. subsequently.

(b) *Liver perfusion.* Rats of the same type were killed as in (a); the peritoneal cavity was opened, a cannula inserted

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