at temperatures of 600° and above, the activity of the catalyst decreased much more rapidly than at the optimum temperature 575°. Very little difference in activity or life of the two catalysts reported in this paper was noted. Several other catalysts of different ratios of chromium and nickel were prepared; they also showed very little difference in activity. With space velocity ratio of 4:1 of hydrogen sulfide to ethylbenzene, only low yields (~5%) of thianaphthene were obtained.

Preliminary results with other alkylbenzenes indicate that the reaction may be general, however, the yields do not appear to be as good as in the case of ethylbenzene.

Acknowledgment.—The authors are very much indebted to the Research Corporation for a Frederick Gardner Cottrell grant-in-aid which supported this research.

Summary

Thianaphthene has been prepared in 18.5% conversion from hydrogen sulfide and ethylbenzene using a chromia on alumina catalyst at 575°.

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[CONTRIBUTION FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, NO. 1178]

The Purification and Properties of Antibody against *p*-Azophenylarsonic Acid and Molecular Weight Studies from Light Scattering Data

BY DAN H. CAMBPELL, ROBERT H. BLAKER AND ARTHUR B. PARDEE[1a]

It is becoming increasingly evident that many fundamental problems dealing with the structure and behavior of antibody molecules must be studied with purified antibody preparations in solution of known composition rather than in complex solutions such as serum. Methods which are devised for the isolation and purification of antibodies on a practical scale are hence of considerable interest and importance. The following report describes a method for the isolation and purification of antibody against *p*-azophenylarsonic acid in which the antibody is removed from the antiserum by specific precipitation with a polyhaptenic dye and recovered from a solution of the dissociated antigen–antibody complex.

The recovery of antibodies from specific antigen–antibody complexes has been accomplished by a variety of methods.[1b] Perhaps the best known is the one described by Heidelberger and Kendall[2] and Heidelberger and Kabat,[3] in which 15% sodium chloride solutions were used to produce a shift in the antigen–antibody ratio of specific precipitates of SSS or of intact Pneumococcus and antipneumococcus serums favoring the liberation of antibody. Liu and Wu[4] were able to obtain as good if not better yields of antibody preparations by acid dissociation of similar antigen–antibody complexes at about *p*H 4.0 with subsequent isolation of antibody by salt precipitation or removal of antigen by centrifugation if bacterial cells were used. Recently, a report has been made by Haurowitz, *et al.*,[5] which describes the isolation and purification of antibody against *p*-aminobenzylamine, anthranilic, arsanilic, and sulfanilic acids by the use of methods somewhat similar to those used by us in the present investigation. The principal difference was their use of an acid-insoluble conjugated protein for a precipitating antigen. Our own investigations of a number of antigen–antibody systems have indicated that, in general, acid dissociation is the method of choice, at least for the systems involving ovalbumin, polysaccharide, and arsanilic acid antigens. The last of these is a particularly good system since simple polyhaptenic dye antigens can be used for specific precipitating agents. The physical properties of such antigens are so different from those of the antibody proteins that the dissociated complexes can usually be separated into the antigen and antibody components without difficulty. Certain dye antigens have the added advantage that they have a low solubility under acid conditions and hence upon dissociation of the antigen–antibody precipitate the antibody dissolves and the antigen remains behind as an insoluble acid.

Purification of Antibody.—Several methods were studied for the dissociation of antibody from antigen–antibody complexes and its subsequent recovery from the dissociated mixture. For example, treatment of precipitates by alkali at *p*H 9.0–10.0 resulted in considerable dissociation, as evidenced by solution of the precipitates, but the yields of antigen-free protein were low because of the high solubility of the antigen and its tendency to remain attached to the protein. Furthermore, some denaturation of antibody protein always occurred and the purity of antibody as based on the ratio of specifically precipitable protein to total protein usually gave values of only 10 to 20%. Another method which was used with some success was dissociation of dye–antigen complexes with a simple hapten such as arsanilic acid and subsequent dialysis against the hapten until the solution was free of the dye antigen. This

(1a) Present address, McArdle Laboratory, University of Wisconsin, Madison, Wisconsin.

(1b) Dan H. Campbell and Frank Lanni, "The Amino Acids and Proteins," edited by D. M. Greenberg, Chapt. XII, "Immunology of Proteins," Thomas Publishing Co., in press.

(2) M. Heidelberger and F. E. Kendall, *J. Exptl. Med.*, **64**, 161 (1936).

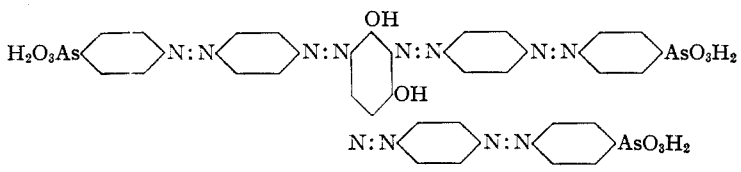
(3) M. Heidelberger and E. A. Kabat, *ibid.*, **67**, 181 (1938).

(4) S. C. Liu and H. Wu, *Proc. Soc. Exptl. Biol. Med.*, **41**, 144 (1939).

(5) F. Haurowitz, Sh. Tekman, Miervet Bilen and Paula Schwerin, *Biochem. J.*, **41**, 305 (1947).

method was limited to precipitating antigens of sufficiently small molecular size to permit diffusion through the dialysis membrane. Such antigens are rather inefficient precipitating agents, and considerable time was required to dialyze away first the dye antigen and then the arsanilic acid. The method of choice in most instances and the one used for the antibody preparation in the present study was (1) the use of a good precipitating dye antigen, (2) dissociation of the antigen–antibody complex with arsanilic acid and then acidification to about *p*H 3.5, and (3) precipitation of the dissociated antibody with salt. The purified antibody used in study of physical properties was a pool obtained by mixing several purified preparations, but all were made by essentially the same method.

Serums from a number of rabbits which had been immunized over a period of many months with sheep serum-*p*-azophenylarsonic acid were pooled and a preliminary precipitation titration was made in order to determine the antigen–antibody ratio for optimum precipitation as well as to obtain an approximate idea of the antibody concentration. The antigen used for all preparations was a trisubstituted resorcinol dye having the following structure



The antigen solutions was adjusted to *p*H 8.0 and dilutions were made which varied by a factor of 2 from 1:1000 to 1:256,000, and these were added in 0.5 ml. volumes to tubes containing 0.5 ml. of a 1:4 or 1:5 dilution of the pooled antiserum at *p*H 8.0. The mixtures were allowed to react for about two hours at room temperature and forty-eight hours at 4°. The precipitates were then washed with 1.0% sodium chloride solution and analyzed for protein by the Folin–Ciocalteu method as modified by Pressman.[6] Most of the pooled sera gave maximum precipitation in slight antigen excess with antigen dilutions around 1:40,000 under the above conditions, and antibody protein values of from about 6 mg./ml. of serum to as high as 15 mg./ml. For precipitation of antibody from an 850-ml. batch of pooled serum which showed a preliminary titration maximum for antibody precipitation of 1:10,000 (1:40,000/1:4) was adjusted to *p*H 8.0 with 0.5 *M* sodium hydroxide, diluted with one volume of saline, and mixed with an equal volume of 1:20,000 antigen solution. After several hours at room temperature and about seventy-two hours at 4° about half of the supernatant was siphoned off and the remainder centrifuged and the precipitate washed free of soluble dye and protein with 1.0% sodium chloride at room temperature. The insoluble antigen–antibody complex was usually dissociated first with sodium arsanilate and was then acidified. Although acid dissociation alone was fairly successful it was found that hapten dissociation facilitated separation and gave higher yields. Thus the precipitate was first suspended in 25 to 50 ml. of 10% sodium arsanilate at *p*H 8.0–8.5 and the mixture carefully stirred until no further solution was evident. This required from two to four hours and usually resulted in a solution with only faint turbidity. When precipitates were allowed to develop over a period of longer than seventy-two hours the dissociation with haptens required much longer time and in a few extreme instances were not complete at twelve hours. The antigen–antibody–hapten solution was then quickly adjusted to *p*H 3.2 and again carefully stirred for about one hour at room temperature. At this *p*H most of the dye antigen became insoluble and the antibody protein remained in solution. A small amount of antibody usually remained with the insoluble dye and arsanilic acid but practically all was recovered in one washing with saline and was added to the original acid extract. The small amount of antibody which remained with the insoluble dye was easily recovered by washing. The antibody was precipitated by addition of a saturated sodium chloride solution in a final concentration of 4.0 *M*. Traces of dye which remained soluble in the acid solution were removed by the careful fractional precipitation with salt solution. The dye being relatively insoluble precipitated with much less salt than was required for antibody globulin. In such instances, 10–20% of the antibody would precipitate with the dye but could be recovered by further fractional precipitation with salt at *p*H 3.2. The final salt precipitated antibody was then re-

(6) David Pressman, *Ind. Eng. Chem., Anal. Ed.*, **15**, 357 (1943).

TABLE I

DATA ON THE PURIFICATION OF ANTIBODY FROM RABBIT ANTI-SHEEP SERUM-*p*-AZOPHENYLARSONIC ACID

Volume of pooled serum, ml.	Type of antigen	Maximum[c] antibody pptd., mg./ml.	Total protein recovered,[d] mg.	Yield, %	Purity[e]
25	R^1_3[a]	14.81	368	97	98
850	R^1_3	6.27	4487	84	87
230	R^1_3	14.81	3390	99	96
400	R^1_3	9.05	3158	87	93
750	XXX[b]	8.68	4100	63	71
100	XXX	8.68	685	79	83

[a] The trisubstituted resorcinol dye described in text. [b] A chromotropic acid derivative containing two azophenyl-azo-arsonic acid groups. These antibody preparations were not used in the present study. [c] Subsequent experiments with the purified antibody indicated that less precipitate was obtained in the presence of serum proteins, hence these values may represent only relative amounts of antibody. [d] Protein based on microkjeldahl analysis. [e] Purity = specific precipitable protein/total protein in solution.

suspended in 0.9% saline and dialyzed against saline until the *p*H became practically neutral.

Representative values for several batches of pooled serum are given in Table I. It will be seen that the preparations obtained by use of the trisubstituted dye antigen were better than those obtained by use of a chromotropic acid derivative. This was due largely to the fact that the latter antigen showed an appreciable solubility at *p*H 3.5 and hence tended to complex with the soluble protein. Serums with lower titers always gave smaller yields.

Electrophoretic Pattern.—Electrophoretic studies of the purified preparations in the Tiselius apparatus indicated a very high degree of homogeneity. The experiments were made with approximately 1.0% protein solutions in 0.15 *M* sodium chloride plus 0.04 *M* phosphate buffer at *p*H 7.2. The current used was approximately 15 ma. and the pattern allowed to develop for two to three hours. The electrophoretic mobility was very similar to that of the gamma globulin fraction of serum.

Molecular Weight Determination. (a) From Osmotic Pressure.—The molecular weight determinations by osmotic pressure were made with simple osmometers of the static rise type with a Visking cellophane bag used for the membrane. The protein concentration was 2.0% in 0.15 *M* sodium chloride and 0.04 *M* phosphate buffer at *p*H 7.3. The values obtained varied from 136,000 to 144,000, as compared to the currently accepted values of 158,000. The slightly lower values were probably a reflection of the *p*H at which the determinations were made.

(b) **From Light Scattering Data.**—Measurements of the turbidity, refractive index, and depolarization of a protein solution can, under certain conditions, be used to calculate the molecular weight of the dissolved protein. The theoretical bases of these calculations are due principally to Rayleigh,[7] Von Smoluchowski,[8] Einstein,[9] Raman,[10] and Debye.[11]

If the dissolved particles are small compared with the wave length of light the following equation gives a relation between the turbidity of the solution, its concentration, refractive index, depolarization, and the molecular weight of the solute.

$$h = \left\{ \frac{\frac{32\pi^3 n^2}{3\lambda^4 N_0}\left(\frac{\partial n}{\partial c}\right)^2 c}{\left(1/M + \frac{2Bc}{RT}\right)} \right\} \left(\frac{6+3\rho}{6-7\rho}\right) \qquad (1)$$

where

h is the extinction coefficient due to scattering
n is the refractive index of the solution
c is the concentration
$\partial n/\partial c$ is the refractive index increment of the solute
λ is the wave length of the incident light
N_0 is Avogadro's number
M is the molecular weight of the solute
B is a constant which describes the deviation of the system from van't Hoff's law
R is the gas constant
T is the absolute temperature
ρ is the depolarization of the scattered light.

In practice it is difficult to measure h accurately so instead the amount of light which is scattered at an angle of 90° to the incident beam is measured. For solutions of particles which are small compared with the wave length of light the angular distribution of intensity of scattered light obeys a $(1 + \cos^2\theta)$ relation where θ is the angle between the direction of the incident beam and the scattered beam. The relation between h, I_0, the intensity of the original beam and i, the intensity of the light scattered at 90° to the incident beam, is

$$h = \frac{16\pi}{3} i/I_0 \qquad (2)$$

The direct measurement of the quantity, i/I_0, is a time consuming task so that routine measurements in this Laboratory are made by comparing the light scattered from a solution with that scattered from a sealed tube of purified carbon disulfide. Various investigators have reported values of i/I_0 for carbon disulfide and in addition the value has been redetermined in this Laboratory.[12] The value of i/I_0 for carbon disulfide which has been used in this investigation is $4.4 \cdot 10^{-5}$ for light of the wave length of 5461 Å.

The instrument which was used for the measurement of the scattered light is one which was designed and built in this Laboratory. A slightly convergent beam of monochromatic light from a mercury arc (GE-AH-4) is passed up through the bottom of a cylindrical glass cell. The light which is scattered in directions near 90° to the incident beam is focused on a 931-A electron multiplier phototube. A small fraction of the incident beam is reflected to another phototube and the outputs of the two tubes are balanced against one another by means of a potentiometer arrangement. A constant voltage transformer reduces fluctuations in the mercury arc and in the supply of a voltage regulator and rectifier which provides a source of high potential for the plates of the phototubes.

A diaphragm arrangement is installed in the path of the scattered beam which permits sections of polaroid film with known orientations to be switched in and out of the light path. This device gives a convenient way of measuring the depolarization of the scattered light.

The refractive index increment is measured with a differential refractometer similar in design to one which has been described in the literature.[13,14]

(7) Lord Rayleigh, *Phil Mag.*, **12**, 81 (1881).
(8) M. Von Smoluchowski, *Ann. Physik*, **25**, 205 (1908).
(9) A. Einstein, *ibid.*, **33**, 1275 (1910).
(10) C. V. Raman, *Indian J. Phys.*, **2**, 1 (1927).
(11) P. Debye, *J. Applied Phys.*, **15**, 338 (1944).

(12) A more complete description of the light scattering apparatus and technique which have been developed in this Laboratory will soon be published.
(13) D. Rau and W. Roseveare, *Ind. Eng. Chem., Anal. Ed.*, **8**, 72 (1936).
(14) P. Debye, *J. Applied Phys.*, **17**, 392 (1946).

Four solutions of the protein were made with a dilute salt solution (0.15 *M* sodium chloride) and were dialyzed against the same solution for two weeks at 4°. The *p*H of the protein solution at the end of the dialysis was 7.5. The solutions were then centrifuged for twenty minutes in a field 32,000 times that of gravity to remove any suspended dust, placed in a scattering cell, and the intensity of the scattered light compared with that scattered from carbon disulfide for a wave length of 5461 Å. Depolarization measurements were made. The refractive index increment was computed from the difference between the refractive indices of the solution and the solvent. Two of the solutions were slightly colored. Optical density measurements were made on these solutions with a spectrophotometer at the wave length used so that the magnitude of the scattering could be corrected for the true absorption. Concentrations were determined as described in the previous section.

The molecular weight of the dissolved protein is given by

$$M = \frac{\lambda^4 N_0}{2\pi^2 n^2 \left(\frac{\partial n}{\partial c}\right)^2 (c/i/ics_2)_{c\to 0}(I_0/ics_2)} \qquad (3)$$

which follows from (1) and (2)

$c/i/ics_2$ is the concentration of the solution divided by the ratio of the intensity of the light scattered from the solution to that scattered from carbon disulfide. This quantity is corrected for the depolarization of the scattered light and is extrapolated to zero concentration.

A plot of $c/i/ics_2$ *vs.* c is given in Fig. 1. The refractive index increment of this protein is 0.171. The depolarization of the solution is 0.032 and apparently is independent of concentration.

The value of the molecular weight which is calculated from light scattering measurements, 158,000 ± 10,000 compares favorably with previously published data from sedimentation and osmotic pressure studies.

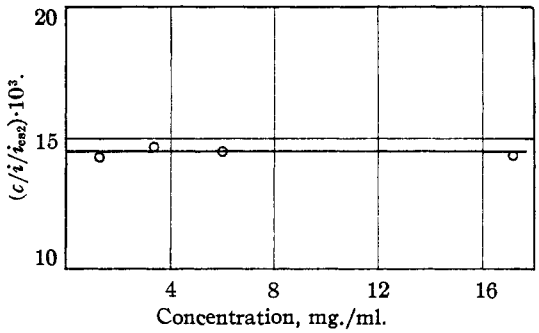


Fig. 1.—Light scattering data for purified rabbit antibody.

There is evidence, however, that the turbidity, depolarization, and refractive index of a protein solution change somewhat with *p*H and perhaps with salt content.[15,16] Not enough work has yet been done to understand how these changes should be taken into account when a value of the molecular weight is to be calculated.

We wish to express our thanks to Professor R. M. Badger and Dr. Stanley Swingle for their suggestions and assistance.

This work was supported in part by a Grant from the Rockefeller Foundation.

Summary

Methods are described for the isolation and purification of rabbit antibody against *p*-azophenylarsonic acid. The purified preparations were electrophoretically homogeneous and similar to gamma globulin.

Molecular weight studies from osmotic pressure and light scattering data gave values of approximately 140,000 and 158,000, respectively.

(15) Unpublished work on solutions of human serum albumin, human serum globulin, and blood group A-Specific substance.

(16) S. Armstrong and others, THIS JOURNAL, **69**, 1747 (1947).

PASADENA, CALIF. RECEIVED FEBRUARY 6, 1948

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE UNIVERSITY OF COLORADO]

The Inhibition of Microbiological Growth by Allylglycine, Methallylglycine and Crotylglycine[1,2]

BY KARL DITTMER, HARLAN L. GOERING,[3] IRVING GOODMAN AND STANLEY J. CRISTOL

The interchange of an aromatic sulfide for a vinylene group in thiamin,[4] nicotinic acid[5] and phenylalanine[6] has led to the formation of specific metabolite antagonists. Because of these effects and because of the theoretical basis for the similarity of the vinylene group (—CH=CH—) and a divalent sulfur atom (—S—),[7] it has been possible to assume that substituting a sulfur for a vinylene group or *vice versa* may be the basis for the preparation of one type of specific metabolite antagonist.[8,9,10]

Since all of these examples are of aromatic com-

(1) This work was supported in part by a research contract with the Office of Naval Research.

(2) This paper, which is Number 1 of the Unsaturated Amino Acid Series, was presented in part at the 111th meeting of the American Chemical Society at Atlantic City, April, 1947.

(3) American Cyanamid Company Fellow.

(4) Woolley and White, *J. Exp. Med.*, **78**, 489 (1943).

(5) Erlenmeyer, Block and Kiefer, *Helv. Chim. Acta*, **25**, 1066 (1942).

(6) Dittmer, Ellis, McKennis and du Vigneaud, *J. Biol. Chem.*, **164**, 761 (1946).

(7) Neuhaus, *Die Chemie*, **57**, 33 (1944).

(8) Wagner-Jauregg, *Naturwissenschaften*, **31**, 335 (1943).

(9) Woolley, *Physiol. Rev.*, **27**, 308 (1947).

(10) Roblin, *Chem. Rev.*, **38**, 255 (1946).