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Physical-Chemical Studies of Soluble Antigen–Antibody Complexes. VIII. The Preparation and Properties of a Univalent Antigen[1,2]

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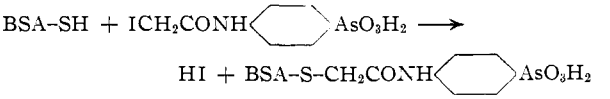
A univalent antigen has been prepared by treating bovine serum mercaptalbumin with N-(*p*-benzenearsonic acid)-iodoacetamide, under appropriate reaction conditions. A preliminary study has been made of the interaction of this antigen with antibody directed against the benzene arsonic acid group.

Physical-chemical studies of the reaction between natural protein antigens (Ag) and their antibodies (Ab) are complicated by the fact that both types of molecules are multivalent, that is, they have more than one reactive site per molecule. While precipitating antibody molecules usually have a valence of two,[3-7] antigens have valences which are larger, and which generally increase as the molecular weight of the antigen increases. As examples, ovalbumin has a valence of 5, whereas that of *Viviparus* hemocyanin is over 200.[8] Therefore, when Ag and Ab react, large aggregates may form, in which the two molecules alternate in a three-dimensional framework.[9] When the framework becomes large enough to be insoluble, the Ag–Ab precipitate forms. If a sufficient excess of Ag is present, the aggregates are relatively small and soluble, but even in these solutions the varieties of aggregates may be considerable.[6,7]

For certain types of studies, a great simplification would result if there were available a protein antigen with only one reactive site per molecule. When mixed with its specific bivalent antibody, it could form only the aggregates AgAb and $(Ag)_2Ab$. A natural protein antigen of this type is unknown. We have, however, prepared a homogeneous, structurally well-defined protein–hapten derivative, containing one hapten group per protein molecule, which should serve as a satisfactory univalent antigen.

Bovine serum mercaptalbumin[10] (BSA–SH) contains one reactive sulfhydryl group, in an apparently characteristic location,[11] per molecule. It is well known that iodoacetic acid and iodoacetamide react with sulfhydryl groups with a high order of specificity.[12] Accordingly, we have prepared a derivative of iodoacetamide, N-(*p*-benzenearsonic acid)-iodoacetamide, which contains the group benzenearsonic acid, a hapten which has been used in many immunochemical investigations. (Antibodies directed against this hapten may be isolated in a pure state.[13]) Under appropriate conditions, the following reaction may be carried out.



The product ($BSA–S–R_1$) is free of reactive sulfhydryl, and contains one gram atom of As per mole of BSA. It is a homogeneous univalent antigen.

In this paper are described the details of this preparation, and some preliminary ultracentrifuge and electrophoresis experiments with mixtures of $BSA–S–R_1$ and purified antibodies directed against the benzene arsonic acid group.

Materials and Methods

BSA and BSA–SH.—Most of the preliminary coupling experiments were performed using whole crystalline BSA[14] in order to define the most appropriate reaction conditions. The BSA contained 60.3% BSA–SH by sulfhydryl titration.

Purified BSA–SH, stored as a precipitate of its mercury dimer, was the generous gift of Dr. H. Dintzis. An aliquot of this preparation was dissolved in phosphate buffer, *p*H 7.50, $\Gamma/2$ 0.1, and examined in the ultracentrifuge (Fig. 1a). It contained about 95% of a component sedimenting with the velocity of the dimer. The preparation was therefore used without further recrystallization. It was dissolved in H_2O (producing 40 ml. of an approximately 4% protein solution) and was passed through an ion-exchange resin column containing some Amberlite IRA 400 on a thioglycollate cycle.[15] This treatment removed the mercuric ion, and converted the BSA–SH to the monomeric form. Sulfhydryl analysis indicated the presence of 98–100% monomer after conversion, and the same result was demonstrated ultracentrifugally (Fig. 1b), the diagram containing just one boundary sedimenting at the characteristic rate of BSA.

Preparation of N-(*p*-Benzenearsonic acid)-iodoacetamide.—This compound has been briefly described previously.[16] We prepared it by the reaction of iodoacetyl chloride and *p*-arsanilic acid, following the procedure of Giesma and Tropp[17] for the preparation of the chlorine analog. It melted with decomposition at 195–196°.[16]

Anal. Calcd. for $C_8H_9O_4NIAs$: C, 25.0; H, 2.4; N, 3.6; I, 33.0; As, 19.5. Found: C, 25.2; H, 2.7; N, 3.8; I, 32.4; As, 19.8.

Purified Antibenzenearsonic Acid (Anti-R) Antibodies.—The purified anti-RAb was kindly donated by Dr. S. I.

(1) This work was supported in part by grants from the Rockefeller Foundation, and from the National Microbiological Institute, United States Public Health Service.

(2) The previous paper in this series is M. C. Baker, D. H. Campbell, S. I. Epstein, and S. J. Singer, THIS JOURNAL, **78**, 312 (1956).

(3) H. N. Eisen and F. Karush, *ibid.*, **71**, 363 (1949).

(4) M. E. Carsten and H. N. Eisen, *ibid.*, **77**, 1273 (1955).

(5) F. Karush, paper presented before the meeting of the American Chemical Society, Minneapolis, September, 1955.

(6) S. J. Singer and D. H. Campbell, *ibid.*, **77**, 3499 (1955).

(7) S. J. Singer and D. H. Campbell, *ibid.*, **77**, 4851 (1955).

(8) W. C. Boyd, in "The Proteins," edited by H. Neurath and K. Bailey, Vol. IIB, Academic Press, New York, N. Y., 1954, p. 777.

(9) J. R. Marrack, "The Chemistry of Antigens and Antibodies," Med. Research Council Brit. Special Rept. Series, No. 230, 1938.

(10) W. L. Hughes, Jr., *Cold Spring Harbor Symposia Quant. Biol.*, **14**, 79 (1950).

(11) B. W. Low, THIS JOURNAL, **74**, 4830 (1952).

(12) F. W. Putnam, in "The Proteins," edited by H. Neurath and K. Bailey, Vol. IB, Academic Press, New York, N. Y., p. 917.

(13) D. H. Campbell, R. H. Blaker and A. B. Pardee, THIS JOURNAL, **70**, 2496 (1948).

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(15) H. M. Dintzis, Ph.D. Thesis, Harvard University, 1952.

(16) *Chem. Zentr.*, **85**, I, 588 (1914).

(17) G. Giesma and C. Tropp, *Ber.*, **59**, 1776 (1926).

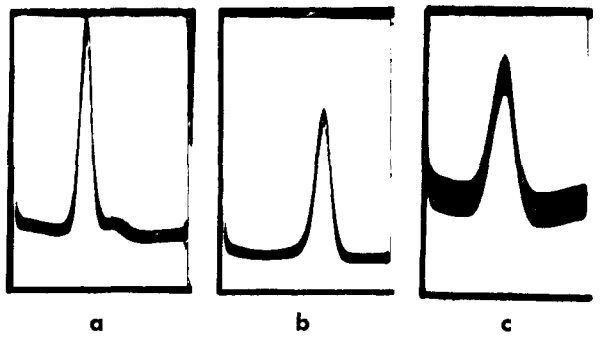

Fig. 1.—Ultracentrifuge diagrams, sedimentation proceeding to the left: (a) Hg dimer of BSA–SH, 14 mg./ml. in phosphate buffer, *p*H 5.9, Γ/2 0.13, after 4822 sec. at 59,780 r.p.m.; (b) BSA–SH (monomer), 15 mg./ml. in phosphate buffer, *p*H 7.5, Γ/2 0.1, after 4800 sec. at 59,780 r.p.m.; (c) BSA–S–R_1, 8.8 mg./ml. in barbital buffer, *p*H 8.70, Γ/2 0.1, after 7920 sec. at 50,740 r.p.m.

Epstein, and was part of a preparation utilized in other studies.[18] It was originally produced by injection of rabbits with horse serum proteins coupled with diazotized *p*-arsanilic acid, and antibodies specifically directed against the benzenearsonic acid (R) group were isolated from the resultant anti-sera.[18] By electrophoretic analysis, this preparation was essentially pure Ab γ-globulin.[19]

Sulfhydryl Analyses.—The following procedure is due to Dr. H. M. Dintzis.[20] One ml. of the protein solution to be analyzed, containing approximately 1 μmole –SH, is denatured with 1 ml. of a guanidine bromide–sodium carbonate solution. Upon the addition of a drop of a 1% solution of sodium nitroferricyanide, a deep pink-colored complex forms with labile –SH, which is then titrated with a standard solution of mercuric nitrate to the disappearance of the color. The color also fades on exposure to air, and in order to minimize this effect, the solution is kept at 0° during the titration. A rough preliminary titration on a separate aliquot of the solution is also advisable.

Arsenic Analyses.—The procedure of Magnuson and Watson,[21] as modified by Maren,[22] was utilized.

Protein Analyses.—Unless otherwise stated, the protein concentration of a solution was determined by measurement of its refractive increment relative to the buffer against which it had been dialyzed to equilibrium. A modified Brice-Phoenix differential refractometer, calibrated with standard KCl solutions, was used.

Ultracentrifugation and Electrophoresis.—A Spinco Model E ultracentrifuge and a Perkin-Elmer Model 38 Tiselius electrophoresis apparatus were employed for these experiments. Measurements of sedimentation constants and relative areas in the schlieren diagrams were carried out as indicated previously.[6]

***p*H Measurements.**—These were performed near 25° with a Beckman Model G instrument.

Experimental Results and Discussion

Preparation of the Univalent Antigen.—Iodoacetic acid and its derivatives are known to react with amine as well as with sulfhydryl groups, although more rapidly with the latter.[12] Therefore, in order to prepare the univalent antigen, reaction conditions had to be found in which the sulfhydryl group of BSA–SH was completely reacted, but no significant attack at the many amine groups of BSA had occurred. Arsenic analyses yielded the total number of hapten groups bound to BSA, and sulfhydryl analyses determined the number of –SH groups which had reacted.

For the exploratory experiments, equal volumes of a buffered solution containing 150 mg./ml. of whole BSA, and a buffered solution containing 3.0 mg./ml. of N-(*p*-benzenearsonic acid)-iodoacetamide, were mixed and allowed to react at room temperature for approximately 45 minutes. The solution was then set to dialyze at 4° overnight against distilled H_2O, to remove most of the buffer salts and excess amide derivative. It was then thoroughly de-ionized by passing it through an ion-exchange column similar to that used to de-ionize the BSA–SH mercury dimer, but lacking the thioglycolate cycle.[15] The eluent from the column was then analyzed for protein, –SH, and As content. The results depended on the buffer medium in which the reaction was carried out (Table I). In barbital buffers, the extent of reaction of the –SH groups increased linearly with *p*H up to *p*H 8.17; at this and higher *p*H values, reaction was complete. The number of As atoms coupled per –SH group originally available also increased linearly with *p*H, rising above 1.0 at *p*H > 8.17. This suggests that at *p*H > 8.17, coupling occurred not only at –SH, but to some extent at amine groups as well. Optimal reaction conditions therefore were achieved in barbital buffer, *p*H 8.17, Γ/2 0.045.

TABLE I

Type[a]	Buffer Γ/2	*p*H	Fraction of SH reacted	Arsenic[b] bound
(A) Reactions with whole BSA, containing 60.3% BSA–SH				
P	0.10	7.52	0.2_0	...
P	.10	8.08	$.3_8$	...
V	.020	7.90	$.5_8$	...
V	.026	8.00	$.7_2$	0.8_3
V	.030	8.08	$.8_8$	1.0_0
V	.045	8.17	1.0_0	1.0_8
V	.045	8.18	1.0_0	1.0_8
V	.060	8.37	1.0_0	1.2_8
V	.086	8.52	1.0_0	1.4_8
V	.140	8.72	1.0_0	1.8_5
(B) Reactions with pure BSA–SH				
V	0.045	8.18	1.0_0	1.0_5
V	0.045	8.17	1.0_0	1.0_8

[a] P, Na phosphate; V, Na barbital. [b] Expressed as no. of gram atoms As bound per mol–SH originally available for reaction.

With whole BSA as the starting material, of course, the product obtained under these optimal reaction conditions is a mixture of BSA–S–R_1 and BSA originally without an available –SH group. In order to prepare pure BSA–S–R_1, purified BSA–SH must be used. Two such preparations were therefore made in this buffer, starting with a solution containing 28 mg./ml. BSA–SH. In both cases (Table I), the composition of the product was exactly that predicted from the exploratory experiments, confirming the reproducibility of the preparative procedure. The product was essentially homogeneous electrophoretically (Fig. 2) and ultracentrifugally (Fig. 1c).

(18) S. I. Epstein, P. Doty and W. C. Boyd, THIS JOURNAL, **78**, 3306 (1956). The Ab used in our studies is the same as that designated pool 54 in this reference.

(19) The absence of inert γ-globulin in this preparation is demonstrated in ref. 2, Fig. 3. In this reference, the same Ab preparation is referred to as anti-R-II.

(20) H. M. Dintzis, personal communication.

(21) H. J. Magnuson and E. B. Watson, *Ind. Eng. Chem., Anal. Ed.*, **16**, 339 (1944).

(22) T. H. Maren, *ibid.*, **18**, 521 (1946).

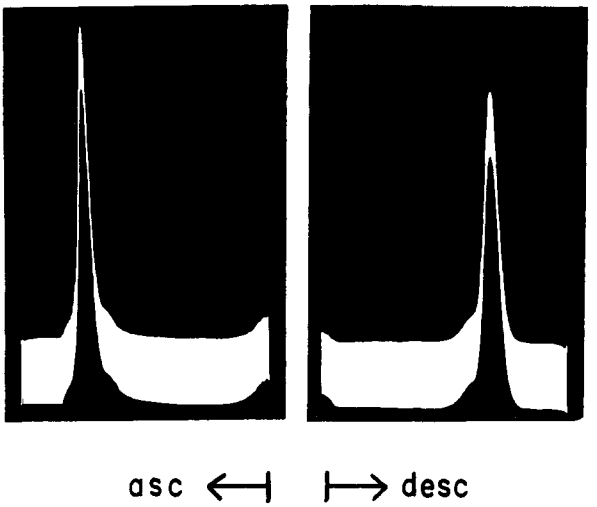

Fig. 2.—Electrophoresis diagrams of BSA–S–R_1, 10.9 mg./ml. in barbital buffer, *p*H 8.70, Γ/2 0.1, after 3600 sec. migration at a potential gradient of 12.9 volts/cm. The starting positions, and the directions of migration, in the ascending and descending limbs are indicated by the arrows.

The Reaction of the Univalent Antigen and its Specific Antibody.—As final proof of the structure of BSA–S–R_1, some preliminary experiments were performed which demonstrate the reaction of BSA–S–R_1 with antibodies directed against the R group. Ultracentrifuge experiments were performed in barbital buffer, *p*H 8.70, Γ/2 0.1, with three mixtures of weight ratio of BSA–S–R_1 to anti-R Ab of 1.00, 3.13, and 4.15, and of total protein concentrations of 10.5, 12.1 and 12.2 mg./ml., respectively. No precipitation occurred in any of these mixtures. Patterns from the first two experiments are shown in Fig. 3a and 3b, respectively. Three major peaks are discernible, although they are not well resolved. The slowest (Ag) and intermediate (Ab) peaks sediment at rates expected of the free Ag and free Ab, respectively, while the fastest peak (**a**) must be due to aggregates formed between the two[23] (Table II). However, no more rapidly sedimenting boundaries are observed, which indicates the absence of larger aggregates such as are present in the system (BSA:anti-BSA) containing the multivalent antigen, BSA, and its rabbit antibodies.[24a]

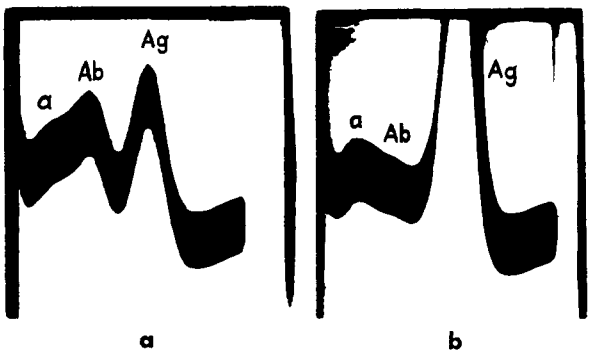

Fig. 3.—Ultracentrifuge diagrams of mixtures of BSA–S–R_1 and anti-R Ab. Sedimentation proceeds to the left. (a) Weight ratio of BSA–S–R_1 to anti-R Ab = 1.00. Total protein concentration 10.5 mg./ml., after 6060 sec. at 50,740 r.p.m. (b) Weight ratio = 3.13, 12.1 mg. protein/ml., after 6720 sec. at 50,740 r.p.m. Both experiments in barbital buffer, *p*H 8.70, Γ/2 0.1. Peaks labeled Ag, Ab and a correspond to free BSA–S–R_1, free anti-R Ab and aggregates, respectively.

An electrophoresis experiment was performed with the solution of weight ratio of BSA–S–R_1 to anti-R of 1.00, at a total protein concentration of 18.8 mg./ml. in barbital buffer, *p*H 8.70, Γ/2 0.1, at a field strength of 12.9 volts/cm. for 3620 sec. (Fig. 4). Three major peaks are apparent in both the ascending and descending patterns. The fastest and slowest peaks in each pattern have mobilities corresponding to the free BSA–S–R_1 and free Ab, respectively, and the intermediate peak is clearly due to aggregates of the two. The two limbs do not give mirror-image patterns, which is mostly ascribable to re-equilibration of the components during the experiment, but this will not be discussed in detail here.

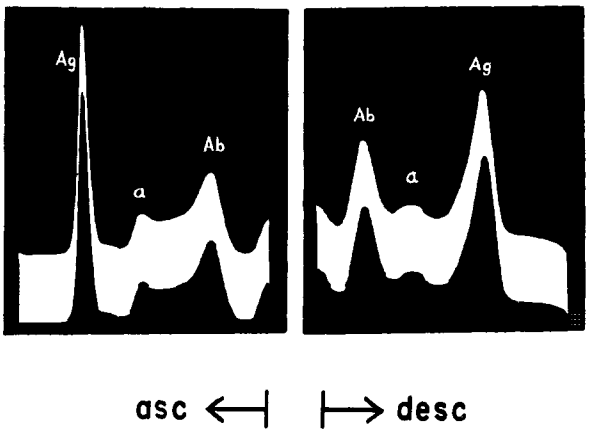

Fig. 4.—Electrophoresis diagrams of a solution of BSA–S–R_1 and anti-R Ab at a weight ratio of 1.00. For conditions of experiment, see text. The starting positions, and the directions of migration in the ascending and descending limbs are indicated by the arrows. The peaks labeled Ag, Ab, and **a** correspond to free BSA–S–R_1, free anti-R Ab and aggregates, respectively.

We may calculate a preliminary value for an equilibrium constant characterizing the BSA–S–R_1:anti-R system from this one electrophoresis experiment, by methods which we have previously developed and utilized.[25,6,7] For this purpose, the equilibrium concentration of free Ag is determined from the relative area under the fastest peak in the ascending pattern of Fig. 4. For this preliminary calculation, no correction need be made for electrophoretic anomalies.[6] The apparent free Ag concentration is 37.4% by weight of the total protein. Knowledge of the free Ag, total Ag and total Ab concentrations, together with the assignment of univalence to the Ag and bivalence to the Ab,[3] and the assumption that all Ag–Ab bonds in this system are intrinsically equivalent, fixes the equilib-

(23) The fact that only one peak appears due to the AgAb and $(Ag)_2Ab$ aggregates is consistent with a similar finding in our previous studies of the BSA:anti-BSA system.[24]

(24) (a) S. J. Singer and D. H. Campbell, THIS JOURNAL, **74**, 1794 (1952); (b) **77**, 3504 (1955).

(25) S. J. Singer and D. H. Campbell, *ibid.*, **75**, 5577 (1953).

rium constants.[25-27] For example, the equilibrium constant K for the reaction Ag + AgAb $\rightleftarrows$ $(Ag)_2Ab$, in the Goldberg notation,[25,27] is given by

$$K = \frac{M_A p}{4C_A(1-p)\left(1-p\frac{C_G M_A}{2C_A M_G}\right)}$$

where C_A and C_G are the concentrations in g./liter of total Ab and total Ag, respectively; M_A and M_G are the molecular weights, 160,000 and 70,000, of Ab and Ag, respectively; and p is determined from the relation $C_{01} = C_G(1-p)$, where C_{01} is the concentration of free Ag. For this single experiment $K = 0.2 \times 10^4$. The present purpose is merely to show that this preliminary value is consonant with those obtained in other Ag–Ab systems. In order to compare K with the corresponding value in the BSA:anti-BSA system, for example, we must first multiply the former by 6 to take into account the difference in Ag valence. The value 1.2×10^4 then compares favorably with the value $2.5 \pm 0.5 \times 10^4$ for the BSA system.[6] More extensive studies of the thermodynamics of the BSA–S–R_1:anti-R system are contemplated, and hence we defer further discussion of the significance of K.

It is clear from the foregoing discussion, therefore, that the electrophoretic as well as ultracentrifugal experiments strongly support the analytical evidence that BSA–S–R_1 is a univalent antigen.

TABLE II

SEDIMENTATION CONSTANTS OF COMPONENTS IN BSA–S–R_1: ANTI-R MIXTURES

Weight[a] ratio	Component	S^{*}_{20}, svedbergs
1.00	Free Ag	4.2
	Free Ab	6.6
	a complexes	8.0
3.13	Free Ag	3.9
	Free Ab	6.4
	a complexes	7.7

[a] Weight ratio of BSA–S–R_1 to anti-R Ab in original solution. The solution of weight ratio 4.15 was not analyzed because of the low concentrations and poor resolution of the free Ab and a components.

Significance of These Results.—The feasibility of the preparation of derivatives such as BSA–S–R_1 makes possible the exploration of several problems of general interest in protein chemistry. For example, a similar derivative might be used to investigate the sequence of amino acids in the vicinity of the –SH group in BSA–SH. In another connection, relatively unambiguous studies can be made of the effect of charge increments on the electrophoretic mobilities of proteins, by varying the number of charges on the R_1 residue. Furthermore, since these charges are located at a fixed position on the BSA molecule, they might have an effect on the dipole moment of the molecule and on the dielectric increment of its solutions, which it would be of considerable interest to correlate.

In immunochemical studies, the availability of a univalent antigen might make feasible studies of the kinetics of Ag–Ab reactions by light scattering measurements. Light scattering studies with natural protein Ag–Ab systems in the past[28,29] have been severely limited by the multivalence of Ag, and hence, by the large number of simultaneously occurring reactions. Furthermore, by means of chemical reactions, such as the acetylation of free amino groups, the net charge on BSA–S–R_1 may be radically altered without affecting the R_1 group itself, and this should permit unambiguous studies to be made of the effect of the net charge on a protein Ag molecule on the Ag–Ab bond strength.

With proteins other than BSA–SH containing definite small numbers of available –SH groups, similar derivatives might be made with Ag valences greater than one, and the increasing complexity of the Ag–Ab reaction might be studied in a controlled manner.

These and other studies with BSA–S–R_1 and similar derivatives are in progress or are contemplated in our laboratory.

We wish to acknowledge our indebtedness to Dr. Howard M. Dintzis for his generosity in making available to us the mercaptalbumin and the techniques he has developed for its study.

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(26) L. Pauling, D. Pressman, D. H. Campbell and C. Ikeda, THIS JOURNAL, **64**, 3003 (1942).

(27) R. J. Goldberg, *ibid.*, **74**, 5715 (1952).

(28) B. Gitlin and H. Edelhoch, *J. Immunol.*, **66**, 67 (1951).

(29) R. J. Goldberg and D. H. Campbell, *ibid.*, **66**, 79 (1951).