[CONTRIBUTION NO. 1569, FROM THE STERLING CHEMISTRY LABORATORY, YALE UNIVERSITY]

A General Method for the Isolation of Antibodies

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RECEIVED JULY 27, 1959

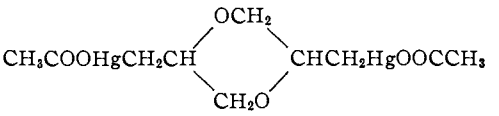
A simple and general method is described for the isolation of pure antibodies, particularly those directed against protein antigens. The essential features of the method are the use of a thiolated antigen to precipitate the specific antibody, and the subsequent removal of the thiolated antigen by cross-linking with a bifunctional organic mercurial. The details of the method have been thoroughly studied. Antibodies to three protein antigens, bovine serum albumin, ovalbumin and ribonuclease, have been isolated and shown to be at least 98% pure and active.

Introduction

The study of the chemistry of antibodies (Ab) has advanced to the point where further progress requires the ready availability of pure Ab directed to a variety of antigens (Ag). Although a number of general methods have been reported[1] for the isolation of pure Ab, none of them is without some difficulties, and the need exists for a convenient and reproducible method which is capable of good yields of Ab in its native molecular state. This is particularly true for Ab directed to protein Ag. We have developed a method which satisfies these criteria and with which we have isolated pure rabbit Ab to hen ovalbumin (OA), bovine serum albumin (BSA), and bovine pancreatic ribonuclease (RNase).

All Ab purification methods so far developed involve the following successive steps[1]: (1) combination of the Ab with its specific Ag; (2) isolation of the Ag–Ab precipitate from soluble non-specific proteins; (3) dissociation of the Ag–Ab precipitate; and (4) separation of Ag and Ab. The first two steps ordinarily present no problems. As for the third step, in general, it is possible to dissociate Ag–Ab bonds in sufficiently acid or alkaline solutions, and with Ab directed to protein Ag, these conditions are the only ones which are presently useful. Under these conditions, however, it is difficult to carry out the fourth step: protein Ag and Ab are generally not easily separated from one another by the usual physical methods, unless they happen to possess quite different properties. (For example, a large virus Ag may be centrifuged out of a solution containing its dissociated Ab.) To overcome this difficulty, protein Ag have been coupled to insoluble matrices, such as cellulose[2] and ion-exchange resins,[3] but these "immunologic adsorbents" are not entirely satisfactory for a number of reasons, particularly with respect to their capacity, their specificity and the relatively long exposure to acid which is required to effect the dissociation of the Ag–Ab bonds.

In the method described in this paper, a protein antigen is first modified by reaction with N-acetylhomocysteine thiolactone (AHT),[4,5] which places a number of sulfhydryl (SH) groups on its outer surface without seriously affecting its capacity to precipitate with Ab directed to the original protein. A specific precipitate is then prepared with the thiolated protein antigen (T–Ag) and the Ab. After the precipitate is freed from non-specific proteins, it is dissolved in a glycine–H_2SO_4 buffer at *p*H 2.4, and the appropriate amount of the bifunctional organic mercurial,[6] 3,6-bis-(acetoxymercurimethyl)-dioxane (MMD) is added.



The T–Ag is cross-linked by the mercurial through the formation of –S–Hg~Hg–S– bonds and precipitates, leaving most of the Ab in solution. In this simple way, step 4 of the purification scheme outlined above is achieved.

Preliminary accounts of these results have been reported.[7]

Materials and Methods

Reagents.—Crystalline BSA and RNase were obtained from Pentex and Armour Co., respectively. Four-times-recrystallized OA was prepared by a standard procedure.[8] CH_3HgBr was the generous gift of Dr. Walter J. Hughes, Jr. Schwarz Labs. was the source of AHT, and CfP grade glycine was obtained from the California Foundation for Biochemical Research. The bifunctional mercurial MMD was prepared by a modification of the method used by Edsall, *et al.*[6] One hundred grams of mercuric acetate was added directly and with vigorous agitation to 42.5 ml. of allyl alcohol and 0.5 ml. of glacial acetic acid over a period of about two minutes. The reaction was maintained below 70° by several immersions in an ice-bath. The reaction of mercuric acetate was quantitative as indicated by formation of only white colored products on reaction with H_2S. The MMD was recrystallized twice from hot water.

Antibody.—High titer hyperimmune rabbit antisera were prepared against BSA, OA and RNase by mixing the Ag with Freund's adjuvant and injecting subcutaneously. The antisera directed to a particular Ag were pooled, and the γ-globulin fraction containing the Ab was precipitated with 40% saturated $(NH_4)_2SO_4$ (SAS) and was washed free of other serum proteins. The γ-globulin fraction was the starting material for the Ab purification procedure and was stored under 40% SAS at 4° until needed.

Antigen-Antibody Precipitin Titrations.—These titrations were carried out by adding the same amount of an Ab containing solution to a series of dilutions of Ag. The solvent was phosphate buffer, *p*H 7.0, Γ/2 0.05, unless otherwise stated. The precipitates were allowed to form for lengths of time indicated elsewhere in the text and were then centrifuged and washed thoroughly with phosphate buffer. They were analyzed for N by nesslerization, using a Beckman Model B spectrophotometer at 425 mμ.

(1) H. C. Isliker, *Advances in Protein Chem.*, **12**, 387 (1957).

(2) D. H. Campbell, E. Luescher and L. S. Lerman, *Proc. Natl. Acad. Sci., U. S.*, **37**, 575 (1951).

(3) H. C. Isliker, *Ann. N. Y. Acad. Sci.*, **57**, 225 (1953).

(4) R. Benesch and R. E. Benesch, THIS JOURNAL, **78**, 1597 (1956).

(5) R. Benesch and R. E. Benesch *Proc. Natl. Acad. Sci. U. S.*, **44**, 848 (1958).

(6) J. T. Edsall, R. H. Maybury, R. B. Simpson and R. Straessle, THIS JOURNAL, **76**, 3131 (1954).

(7) (a) J. R. Shainoff and S. J. Singer, Abstracts Biophys. Society Meeting, Cambridge, Mass., Feb. 1958, (b) S. J. Singer, J. E. Fothergill and J. R. Shainoff, *ibid.*, **81**, 2277 (1959).

(8) R. A. Kekwick and R. K. Cannan, *Biochem. J.*, **30**, 227 (1936).

Sulfhydryl Determinations.[9]—Rough SH titrations were carried out on 1.0-ml. samples of *ca.* 1% protein solutions by adding an equal volume of carbonate buffer, *p*H 10.6, Γ/2 0.4, one drop of a 10% sodium nitroferricyanide solution and a sufficient volume of the mercurial titrant from a buret to discharge the pink color of the indicator. More accurate titrations were made by adding about 90% of the previously determined volume of titrant to the protein solution before the addition of the carbonate buffer and indicator and then completing the titration as before. This procedure is required because of the rapid oxidation of SH groups at high *p*H. Values obtained by the refined technique were therefore approximately 10% higher than the rough values and agree generally within ±3%.

All preparations of thiolated proteins were analyzed for SH content with three mercurial titrants, CH_3HgBr, MMD and $HgNO_3$, all at 1.0×10^{-3} *M*. CH_3HgBr was first dissolved in a minimal quantity of ethanol and then brought to volume with water. The $HgNO_3$ was dissolved in 0.005 *M* HNO_3. These solutions of mercurial titrants were standardized by parallel titrations with freshly prepared solutions of cysteine hydrochloride.

Other Analytical Procedures.—Van Slyke analyses were used to determine the number of free amino groups remaining on the thiolated proteins.

When the most accurate measurements of protein concentration were required, these were made on dialyzed solutions with a calibrated Brice–Phoenix differential refractometer, taking all the proteins and their thiolated derivatives to have the same specific refractive increment as BSA. For somewhat less accurate measurements, the protein was precipitated from a solution with 5% trichloroacetic acid, and the precipitate was washed and nesslerized. The factor 6.25 was used to convert from N to protein.

Sedimentation experiments were performed in a Spinco Model E Ultracentrifuge, and moving boundary electrophoresis experiments in a Perkin–Elmer Model 38A Tiselius apparatus.

Experimental Results

Thiolation of Proteins.—The thiolated derivatives of BSA, OA and RNase were prepared by closely similar methods. The following detailed preparation of T–OA will serve to illustrate the main features of the procedure.

To 4.5 ml. of an 8.8% solution of OA in H_2O was added 3.0 ml. of carbonate buffer, *p*H 10.7 (170 g. of anhyd. K_2CO_3 and 15 g. anhyd. $NaHCO_3$ per liter), and 0.30 g. of AHT in 1.5 ml. of H_2O. All three of these solutions were at 0° before mixing. The reaction was permitted to proceed for 2 hours at 0°, after which it was stopped by diluting the reaction mixture with 30 ml. of phosphate buffer, *p*H 6.8, Γ/2 0.4. The resulting solution was dialyzed with agitation against 3 liters of phosphate buffer, *p*H 7.0, Γ/2 0.05 for 24 hours at 4° with a single change of dialyzate after 12 hours. Sulfhydryl analyses of the final dialyzed solution and of samples of the protein precipitated from this solution by 5% trichloroacetic acid were less than 5% different, indicating that hydrolyzed thiolactone had been essentially completely removed by the dialysis.

Different preparations of T-OA made by essentially this procedure were found to contain an average of 5 to 9 SH groups per OA molecule. T–BSA prepared in an analogous manner, employing however a carbonate buffer of half the concentration used with OA, and a 10% BSA solution, contained an average of 13 to 20 SH groups per BSA molecule, and T–RNase prepared in the same manner as T–BSA but with a reaction time of 15 minutes instead of 2 hours, contained an average of 4 SH groups per molecule. Some of the variability in sulfhydryl content of different preparations of the same T–Ag might have been due to the rapid, and not completely controlled, hydrolysis of AHT in the course of the reaction.

After the major part of these studies was completed, an improved method for thiolating proteins was reported,[5] which does not require a reaction *p*H higher than 7.5. We have not yet determined, however, whether T–Ag prepared by this method behaves entirely similarly to our preparations in the Ab purification procedure.

SH Content of Thiolated Proteins.—From the point of view of this investigation, our primary interest in the SH content of the T–Ag was in determining an equivalence point in the reaction of T–Ag with MMD. Therefore, SH analyses were performed by titration with MMD itself. Throughout this paper, as an operational procedure, 1.0 mole of MMD is taken as equivalent to 2.0 moles of SH, as is the case in the titration of the SH groups of cysteine or mercaptalbumin.[6] That this cannot be far wrong is indicated, first, by the fact that titrations of T–Ag with MMD and Hg^{++} consistently gave the same results. (Titration results with CH_3HgBr, however, were not reproducible under these conditions, and were anomalously large, as if some CH_3HgBr were bound to protein at other sites in addition to SH.) Secondly, the number of SH groups per molecule of T–Ag determined in this way correspond reasonably well to the number of free amino groups blocked[5] (Table I). The small deficiencies in the number of SH groups observed in Table I might be attributable to some oxidation of SH at alkaline *p*H after coupling to the protein. It appears, therefore, that nearly all the MMD added to a T–Ag reacts with 2 SH groups, and that intramolecular as well as intermolecular –S–Hg∼Hg–S– crosslinks are probably formed.

TABLE I

CHEMICAL PROPERTIES OF THIOLATED PROTEINS

	T–BSA	T–OA	T–RNase
No. SH per molecule	23	8	5.5
No. NH_2 blocked per molecule	32	10	6.9

It was found that the SH content of the thiolated proteins in phosphate buffer, *p*H 7.0, Γ/2 0.05 at 4° decreased by no more than about 5% per week, if the air space above the solution was flushed with nitrogen gas after each opening of the container. In the antibody purification experiments, however, freshly prepared T–Ag was always used.

Properties of Thiolated Proteins.—The electrophoretic behavior of several T–Ag preparations was investigated. In acetate buffer, *p*H 5.13, Γ/2 0.15, the electrophoretic mobilities were considerably more negative than those of the unmodified proteins. The replacement of the positively-charged α-ammonium groups of lysine by neutral groups at this *p*H is clearly responsible for this effect.[5] Electrophoresis diagrams of OA and T–OA are shown in Fig. 1. The thiolated protein exhibited a somewhat greater boundary spreading, presumably attributable to a greater charge hetero-

(9) F. A. Pepe and S. J. Singer, THIS JOURNAL, **78**, 4583 (1956). The method was worked out by H. M. Dintzis.

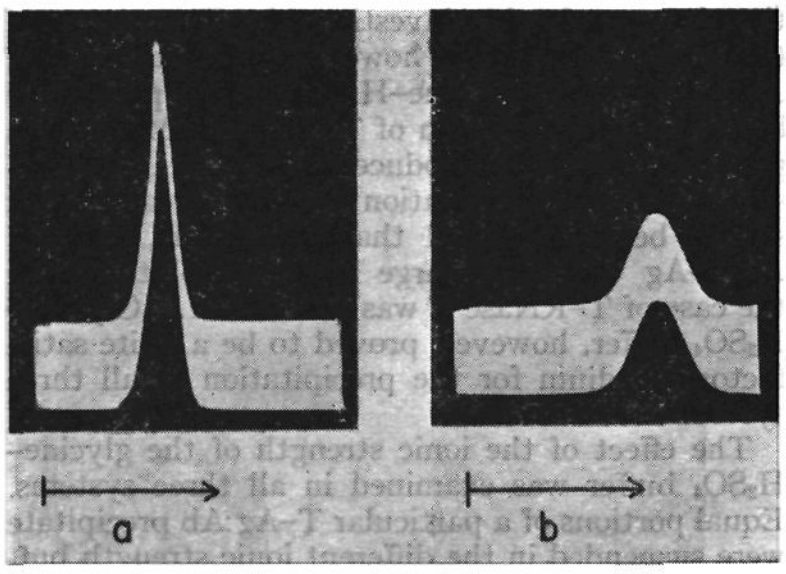


Fig. 1.—Electrophoresis diagrams of (a) ovalbumin and (b) thiolated ovalbumin (6 SH per molecule), in acetate buffer, *p*H 5.13, Γ/2 0.15, after 12,000 sec. at 7.44 volts/cm.

geneity, than the unmodified protein, but the amount of unthiolated protein in T–OA was entirely negligible. This is of importance in connection with the Ab purification procedure, since any unthiolated Ag would not be removed from the Ab upon addition of the bifunctional mercurial.

In the ultracentrifuge, the sedimentation patterns and sedimentation constants in phosphate buffer, *p*H 7.5, Γ/2 0.05 of the thiolated and respective unmodified proteins were very similar. Ultracentrifuge diagrams of BSA and T–BSA are shown in Fig. 2. When T–BSA was brought to *p*H 5 or 6, however, aggregation took place which resulted in a very highly polydisperse mixture. This was not due to –S–S– bond formation, since the effect of *p*H was at least partially rapidly reversible. T–OA did not exhibit this aggregation effect, remaining monodisperse at least down to *p*H 5. The retention of monodispersity of an Ag was found to be desirable in order to achieve reproducible and specific Ab precipitation, and the T–Ag preparative procedure was designed accordingly.

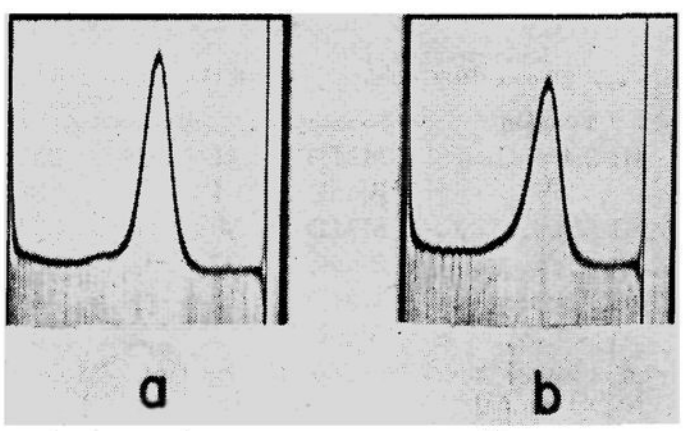


Fig. 2.—Ultracentrifuge patterns of (a) bovine serum albumin and (b) thiolated bovine serum albumin (11 SH per molecule), in phosphate buffer, *p*H 7.0, Γ/2 0.05 after 80 min. at 59, 780 r.p.m.

Parallel Ag–Ab precipitin titrations were performed with an Ag and its thiolated derivative using portions of the same solution of γ-globulin containing Ab to the native Ag. T–BSA containing about 14 SH groups per molecule, T–OA containing between 4 and 9 SH, and T–RNase containing 4 SH, all gave about 90% of the maximum precipitate obtained with their respective native Ag. A typical parallel Ab titration of OA and T–OA is reproduced in Fig. 3. In the case of T–RNase, the ability to precipitate anti-RNase Ab fell off somewhat with increasing thiolation, as is shown in Fig. 4.

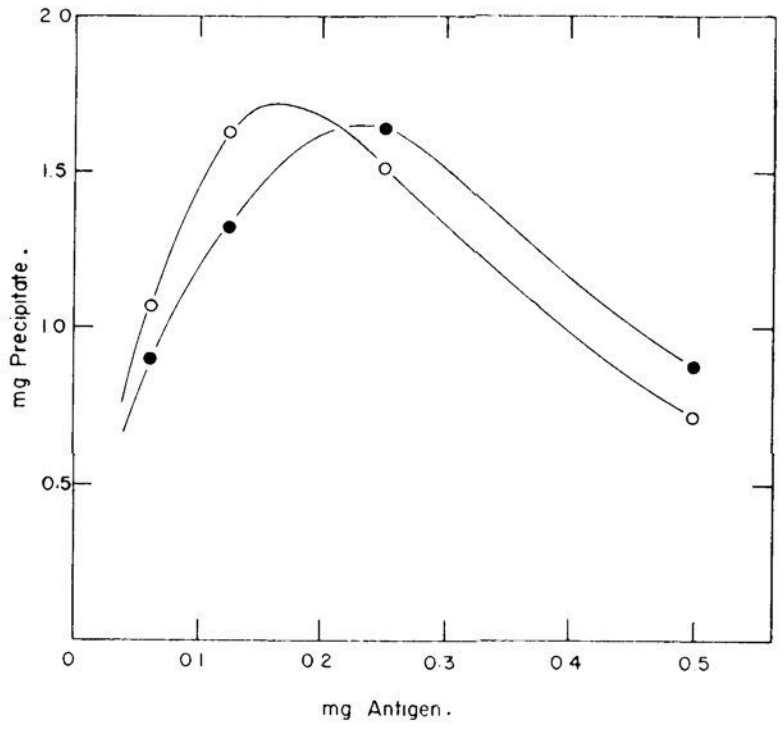


Fig. 3.—Ag–Ab precipitin titrations with: O, ovalbumin; ●, thiolated ovalbumin (9 SH per molecule), with the same anti-ovalbumin antibody.

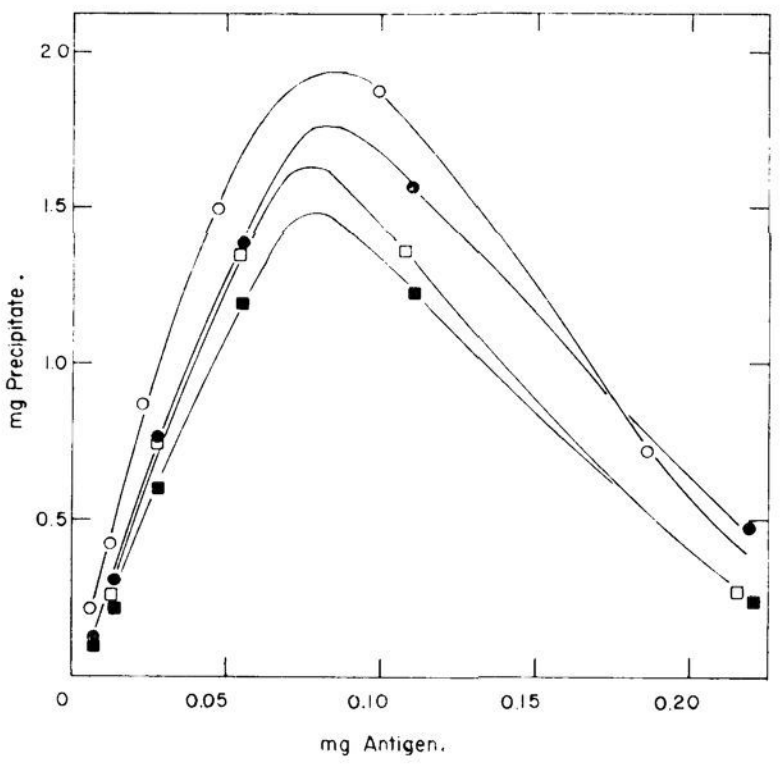


Fig. 4.—Ag–Ab precipitin titrations with thiolated ribonuclease preparations containing: O, zero; ●, 4.1; □, 4.9; and ■, 5.4; SH groups per molecule.

If monodisperse T–BSA was aggregated by exposure to *p*H below 7, the precipitin behavior with anti-BSA Ab was markedly altered. No Ag excess region was observed. Control experiments showed that some inert γ-globulin was coprecipitated with the aggregated T–BSA. No such coprecipitation occurred with monodisperse T–BSA.

The fact that the thiolated proteins retained most of the capacity to precipitate Ab to their respective unmodified Ag indicates that the structure and configuration of the protein molecules were largely

retained upon thiolation, and particularly, that the disulfide bonds of the proteins were not reduced to any significant extent during the thiolation reaction, in spite of the alkaline *p*H and the presence of some hydrolyzed AHT. This was confirmed by the finding that the presence of excess hydrolyzed AHT did not effect the rate of appearance *de novo* of protein SH.

Antibody Purification Procedure.—The Ab content of a solution of a γ-globulin fraction at a total protein concentration of about 5 mg./ml. in phosphate buffer, *p*H 7.0, Γ/2 0.05 was first determined by precipitin titration with the native or thiolated Ag. An equivalent amount of freshly-prepared T–Ag (a separate aliquot of which was titrated with MMD to determine its SH content) in the same phosphate buffer was then added to precipitate the Ab. The mixture was kept at 37° for about 15 minutes and then at 4° for another 2 hours. This short period was used in order to minimize oxidation of the SH groups of the T–Ag, but the bulk of the specific Ab at all capable of being precipitated was brought down under these circumstances. The precipitate was centrifuged and washed thoroughly with the phosphate buffer to remove nonspecific γ-globulin. It was then rapidly dissolved at 4° in a glycine–H_2SO_4 buffer, *p*H 2.4, Γ/2 0.35, and an amount of the bifunctional mercurial MMD in 10^{-3} *M* solution in water was added directly. This amount was 1.5 times that found earlier to titrate the SH groups of that particular preparation of T–Ag. A precipitate of the cross-linked T–Ag appeared almost immediately and was allowed to form for an hour at 4° before it was centrifuged. The supernatant, together with washings of the mercurial precipitate, was adjusted to neutral *p*H by the addition of a phosphate buffer, *p*H 7.2, Γ/2 1.0. After standing overnight, any remaining T-Ag was precipitated in the presence of excess Ab and was removed by centrifugation. The supernatant contained the pure Ab. To store the Ab for any considerable length of time, it was precipitated with 37% SAS until needed.

The precipitation of the T–Ag by MMD at *p*H 2.4 is the crux of the Ab purification method, and the major variables involved in this step were investigated in order to achieve maximum yields of Ab, and to determine how critical the conditions might be.

The choice of MMD was indicated by early experiments which showed that while Hg^{++} could rapidly precipitate T–Ag at *p*H 7, it did not do so at *p*H 2.4. This is not surprising in view of the studies of Kay and Edsall[10] on the effect of *p*H on the relative rates of dimerization of mercaptalbumin with Hg^{++} and MMD. The electrostatic repulsion and steric hindrance of the protein molecules is less pronounced the larger their separation. Also, CH_3HgBr did not precipitate T–Ag under any conditions that were investigated, which is to be expected since it cannot cross-link the T–Ag.

The *p*H 2.4 was chosen as the least acid *p*H at which Ag–Ab bonds in the BSA and OA systems are sufficiently dissociated.[11,12] This variable was therefore not further investigated. The nature of the buffer at this *p*H, however, was found to be critical. With glycine–HNO_3 and phosphate buffers, no precipitation of T–Ag by MMD in the presence of Ab was produced, although in the absence of Ab, precipitation occurred readily. It should be remembered that the weight ratio of Ab:T–Ag was quite large in these solutions: in the case of T–RNase it was about 20:1. Glycine–H_2SO_4 buffer, however, proved to be a quite satisfactory medium for the precipitation in all three systems.

The effect of the ionic strength of the glycine–H_2SO_4 buffer was examined in all three systems. Equal portions of a particular T–Ag:Ab precipitate were suspended in the different ionic strength buffers. To two aliquots at a given ionic strength were added either: (a) 1.5 times the amount of MMD equivalent to the SH of the T–Ag or (b) an equal volume of water. The latter samples are referred to in Tables II, III and IV as blanks. The per cent. of the total protein in each aliquot which was (a) precipitated at *p*H 2.4, (b) subsequently precipitated at *p*H 7, after the supernatant of the *p*H 2.4 precipitate was adjusted to *p*H 7; and (c) left in the supernatant at *p*H 7 (presumably all pure Ab); was determined by Nessler analyses. The data are given in Tables II, III and IV.

TABLE II

PRECIPITATION OF T–BSA AT *p*H 2.4 BY MMD[a]

Ionic strength of glycine–H_2SO_4 buffer		0.11	0.19	0.35	0.56	0.70
Fraction	Reagent	% Total protein precipitated				
*p*H 2.4 ppt.	MMD	2	7	26	31	36
	Blank	3	6	20	24	27
*p*H 7 ppt.	MMD	72	62	32	20	14
	Blank	83	82	64	59	56
*p*H 7 *supernat.*	*MMD*	*26*	*31*	*43*	*49*	*50*
	Blank	14	12	17	17	17

[a] Total protein concentration 8.3 mg./ml. Mole ratio MMD to SH = 1.5.

TABLE III

PRECIPITATION OF T–OA AT *p*H 2.4 BY MMD[a]

Ionic strength of glycine–H_2SO_4 buffer		0.11	0.19	0.35
Fraction	Reagent	% Total protein precipitated		
*p*H 2.4 ppt.	MMD	1	25	32
	Blank	1	0	8
*p*H 7 ppt.	MMD	80	46	33
	Blank	87	90	82
*p*H 7 supernat.	MMD	19	29	35
	Blank	12	10	10

[a] Total protein concentration 12 mg./ml. Mole ratio MMD to SH = 2.0.

Below a threshold value of ionic strength, the yields of pure Ab were poor, but above this value, the yields were adequate and largely independent of ionic strength. A glycine–H_2SO_4 buffer, *p*H 2.4, Γ/2 0.35[13] was used satisfactorily for all three systems. These data also reveal the efficacy of

(10) C. M. Kay and J. T. Edsall, *Arch. Biochem. Biophys.*, **65**, 354 (1956).

(11) S. J. Singer and D. H. Campbell, THIS JOURNAL, **77**, 3504 (1955).

(12) S. J. Singer, L. Eggman and D. H. Campbell, *ibid.*, **77**, 4855 (1955).

(13) Prepared from 32 g. of glycine and 230 ml. of 1 *N* H_2SO_4 in 1 liter of solution.

TABLE IV

PRECIPITATION OF T–RNase AT *p*H 2.4 BY MMD[a]

Fraction	Reagent	Ionic strength of glycine–H_2SO_4 buffer 0.06	0.11	0.19	0.35	0.56
		% Total protein precipitated				
*p*H 2.4 ppt.	MMD	1	52	59	60	58
	Blank	0	13	42	39	37
*p*H 7 ppt.	MMD	88	22	9	7	7
	Blank	90	76	45	46	33
*p*H 7 supernat.	MMD	11	26	32	33	35
	Blank	10	11	13	15	30

[a] Total protein concentration 6 mg./ml. Mole ratio MMD to SH = 1.5.

the mercurial precipitation of the T-Ag: a much larger fraction of protein was precipitated at *p*H 2.4, and a much smaller fraction was precipitated at *p*H 7, with the mercurial than with the blank.

The influence of other variables was similarly investigated but the results will only be summarized. The final yield of Ab was independent of the ratio of mercurial to SH groups in the T–Ag: Ab solution at *p*H 2.4, provided the mercurial was added in equivalent or greater amount. In the recommended procedure, it is therefore adequate to assume that the SH content of the solution at *p*H 2.4 is determined by the amount of T–Ag originally used to precipitate the Ab, without performing another SH analysis, and to add an amount of MMD 1.5 times that equivalent to the calculated SH content.

The total protein concentration in solution at *p*H 2.4 was varied from 6 to 26 mg./ml. without any effect on the yield of Ab. The yield was also unaffected if the entire procedure was carried out at 4 or 25°, and the lower temperature is therefore recommended in order to minimize acid denaturation of Ab.

Several large scale preparations of pure Ab were made, and by analyzing small samples at each stage of procedure, the material balances and overall yields given in Table V were obtained. These preparations were used for the characterization experiments described below.

TABLE V

ANTIBODY PURIFICATION DATA

	Antibody	OA	BSA (A)	BSA (B)	RNase (A)	RNase (B)
		Grams protein				
1.	*p*H 2.4 ppt.	204	174	22	544	294
2.	*p*H 7 ppt.	13	7	7	224	66
3.	*p*H 7 supernat. (pure Ab)	146	371	43	197	144
4.	Total protein[a]	363	552	72	965	504
5.	Total original Ag	30	80	16	63	28
6.	Total original Ab[b]	333	472	56	902	476
7.	Pure Ab yield[c]	44%	78%	77%	22%	30%

[a] Sum of (1), (2) and (3). [b] Difference between (4) and (5). [c] 100 × ratio of (3) and (6).

In view of the low yields of anti-RNase Ab, some preliminary attempts were made to extract more Ab from the mercurial precipitate at *p*H 2.4, without success, however.

Characterization of Purified Ab.—The Ab prepared by this procedure was investigated by ultracentrifugation and electrophoresis; some typical results are shown in Figs. 5 and 6, respectively. The main component had the sedimentation constant and average electrophoretic mobility usually associated with γ-globulin. In the ultracentrifuge pattern, there is evidence of a small amount of a component which is probably a dimer of the main component and which is often found in γ-globulin preparations. The ultracentrifuge pattern of anti-BSA Ab also shows a small amount of an unidentified component sedimenting more slowly than the main one. Electrophoretically, a mixture of purified anti-OA and anti-BSA Ab was indistinguishable in boundary spreading from either Ab separately.

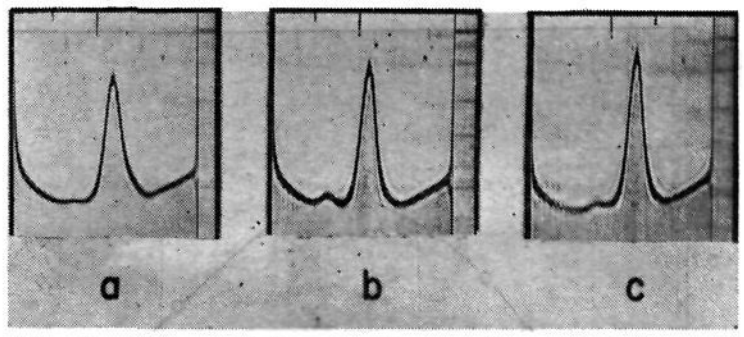


Fig. 5.—Ultracentrifuge patterns of purified Ab to (a) bovine serum albumin; (b) ovalbumin; and (c) ribonuclease, in barbital–NaCl buffer, *p*H 8.53, Γ/2 0.30, after 64 min. at 59,780 r.p.m.

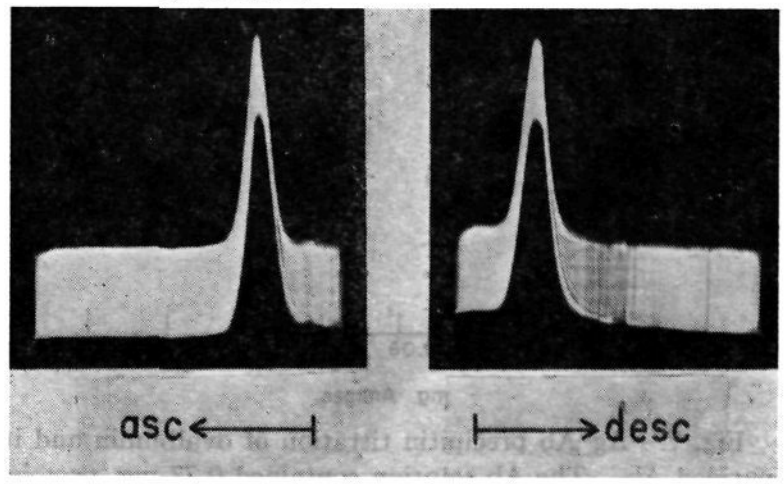


Fig. 6.—Electrophoresis patterns of purified Ab to ovalbumin, in barbital–NaCl buffer, *p*H 8.53, Γ/2 0.30, after 14,300 sec. at 4.14 volts/cm.

The purity of the Ab was tested by careful precipitin titrations with the specific unmodified Ag. The Ab and Ag were dissolved in a barbital–NaCl buffer, *p*H 8.53, Γ/2 0.3 in connection with electrophoretic studies to be described below. About 90% of the protein in purified anti-OA and anti-BSA solutions was specifically precipitable by Ag; typical data are given in Fig. 7. With purified anti-RNase Ab, the precipitin curve was markedly dependent on the concentration of the Ab solution. At a concentration of 0.32 mg./ml., a maximum of 45% of the protein was specifically precipitable, whereas at 4.0 mg./ml., 69% was precipitable (Fig. 8). This curious equilibrium behavior is probably attributable to the relatively small number of antigenic sites on the RNase molecule.

The only important contaminant of the purified Ab which might have been present was inactive γ-globulin. The most exacting test for the presence of this impurity is an electrophoretic analysis of a mixture of the Ab in an excess of the Ag.[14,15]

(14) M. C. Baker, D. H. Campbell, S. I. Epstein and S. J. Singer, THIS JOURNAL, **78**, 312 (1956).

(15) F. A. Pepe and S. J. Singer, *ibid.*, **81**, 3878 (1959).

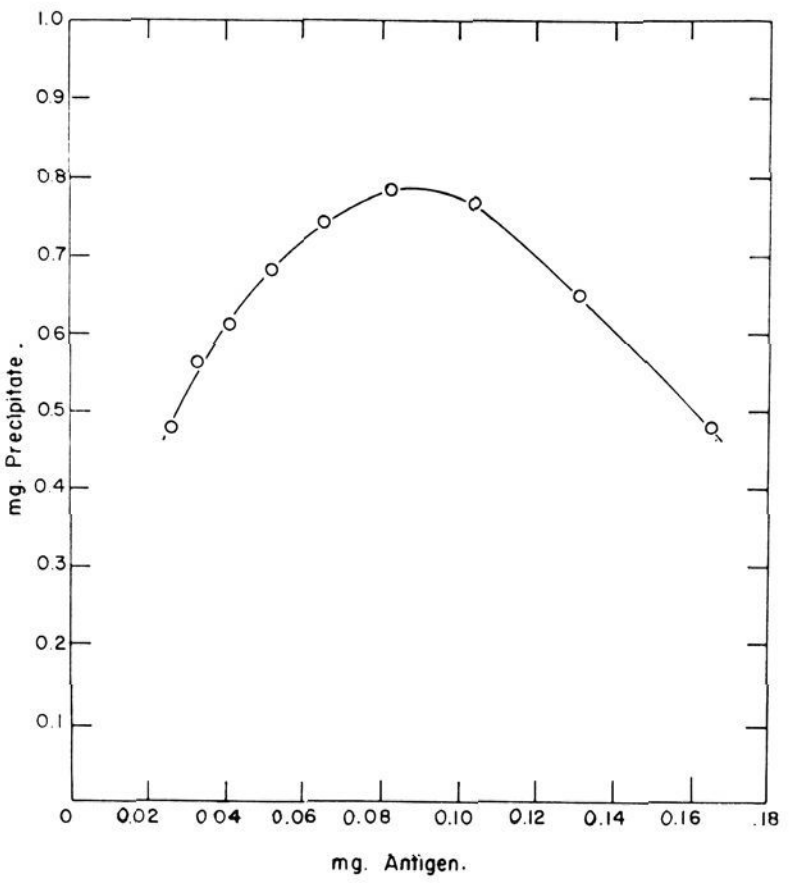


Fig. 7.—Ag–Ab precipitin titration of ovalbumin and its purified Ab. The Ab solution contained 0.77 mg. protein/ml.; at the maximum point the amount of Ag–Ab precipitate after 40 hours at 4° was 0.79 mg./ml., including 0.090 mg. ovalbumin.

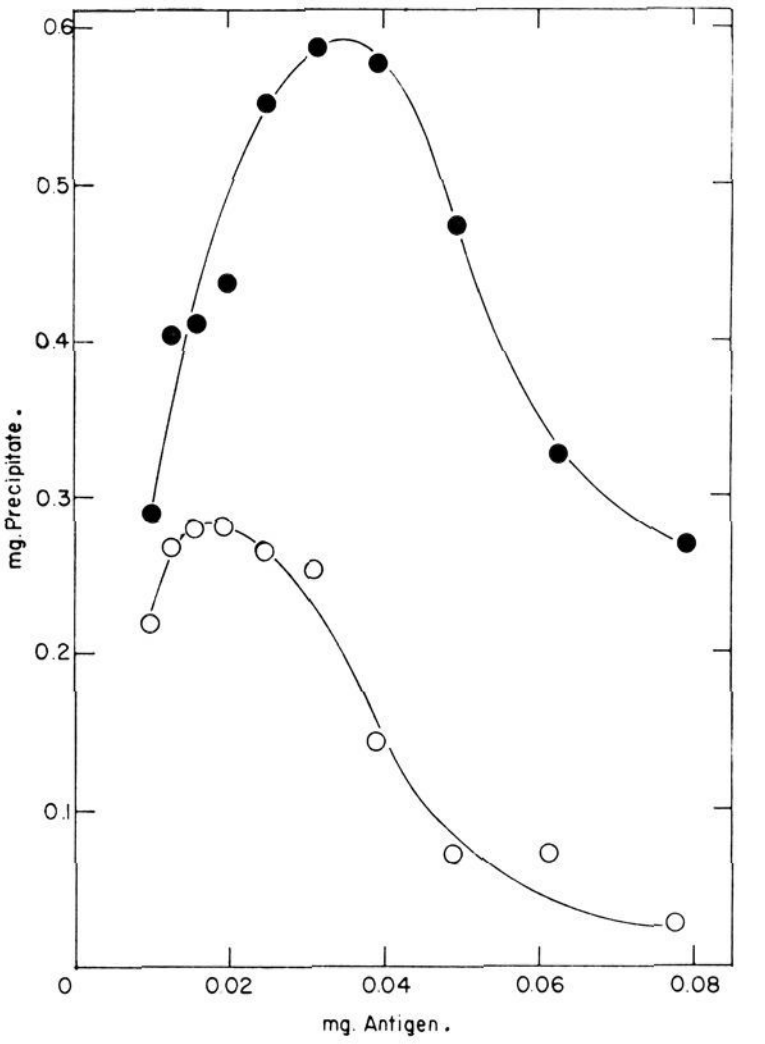


Fig. 8.—Ag–Ab precipitin titrations with ribonuclease and its purified Ab, using: O, 2.0 ml. of solution containing 0.319 mg./ml. Ab protein; ●, 0.20 ml. of solution containing 4.04 mg./ml. of the same Ab protein. All in acetate buffer, *p*H 5.7, Γ/2 0.1.

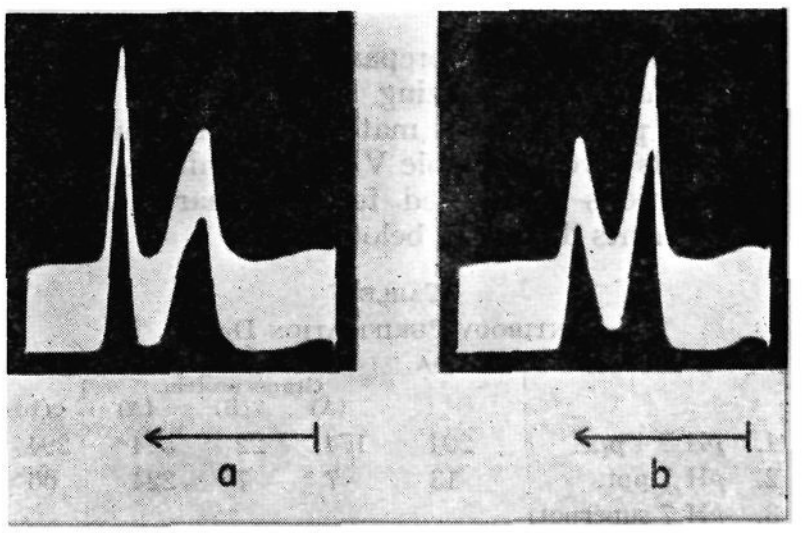


Fig. 9.—Ascending electrophoresis patterns of soluble Ag–Ab complexes made by mixing Ag with purified Ab. (a) Bovine serum albumin system, 60.1% total Ag, 15.1 mg. protein/ml., after 13,500 sec.; (b) ovalbumin system, 55.0% total Ag, 14.0 mg. protein/ml., after 14,300 sec. Both experiments in barbital-NaCl buffer, *p*H 8.53, Γ/2 0.30 at 4.14 volts/cm.

Any inactive γ-globulin migrates slowly as a separate peak, since it is incapable of forming Ag–Ab complexes. Anti-BSA and anti-OA preparations were at least 98% pure Ab by this criterion (Fig. 9). From these electrophoresis patterns it was also possible to obtain values for the equilibrium constant K characterizing each Ag–Ab system, as completely described elsewhere.[16,17] For the BSA: purified anti-BSA system $K = 0.6 \times 10^4$ moles/liter, whereas the value obtained with an independent batch of anti-BSA Ab which was never isolated was[16] $K = 2.5 \pm 0.5 \times 10^4$. In the OA system, the corresponding values[17] were 1×10^4 and $3.1 \pm 0.5 \times 10^4$, respectively. The differences are barely significant in view of the large experimental error, and they might reflect differences between independent batches of antiserum rather than a partial selection or inactivation of Ab by the purification procedure. Electrophoretic studies of mixtures of RNase and its purified Ab will be reported separately.

Discussion

The procedure for purifying Ab described in this paper is convenient, simple and reproducible. It is not critically dependent on any of the variables in the method and can readily by scaled up or down as desired. It requires no elaborate apparatus or difficult syntheses. (It is perhaps worth noting that 1 gram of MMD is enough for the preparation of about 100 grams of pure Ab.) The method has functioned well with three protein Ag systems and should be readily applicable to others. The pure Ab has been obtained in yields equal to or higher than by previous methods, and as a consequence of having been only minimally exposed to acid conditions, was found to have retained essentially full binding capacity for Ag.

The essential feature of the method should be applicable to the isolation of anti-hapten as well as anti-protein Ab, and investigation of this possibility is contemplated.

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It might also be possible to turn the method around, and use it to isolate a pure protein Ag.[18] Assuming that there is available a γ-globulin fraction containing Ab reactive to the desired Ag, one might thiolate it, use the thiolated Ab to precipitate the Ag free of contaminating proteins, and then remove the thiolated Ab from the Ag by MMD precipitation at acid *p*H.

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We gratefully acknowledge the interest and support of Professor J. M. Sturtevant, from whose National Science Foundation grant G-2855, the postdoctoral research assistantship held by one of us (J.E.F.) was provided. This research was also supported in part by U. S. Public Health Service grants E-1204 (C2,3) to Professor S. J. Singer.

NEW HAVEN, CONN.

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Tyrosyl Hydrogen Bonds in Insulin[1,2]

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RECEIVED JUNE 8, 1959

The ultraviolet absorption spectra of zinc- and zinc-free insulins at *p*H 8 are modified either by acidification or by tryptic digestion. The character of the difference spectra produced by either treatment is very similar and appears to be due to the breaking of tyrosyl hydrogen bonds. Since the acid-induced shift still persists after tryptic digestion and is of undiminished magnitude, it is concluded that there are two tyrosyl residues which are hydrogen bonded as donors to two acceptor groups. The *p*H-dependence of the acid-induced shift suggests that one of the acceptors is a carboxylate ion side chain. In a 6000 mol. wt. insulin model, involving α-helices, the only tyrosyl-carboxylate ion hydrogen bond which can be formed is one between the B13 glutamic acid residue and the B16 tyrosyl residue. For the spectral shift induced by tryptic digestion, the B26 tyrosyl residue is implicated. It has been shown that trypsin splits native insulin at the same two bonds as in isolated B-chain, *viz.*, at the B22-B23 and the B29-B30 peptide bonds. The former splitting releases one of the four tyrosyl residues in insulin as a heptapeptide fragment. The rate of splitting follows the rate of spectral change during tryptic digestion. Removal of the terminal B-30 alanyl residue by carboxypeptidase digestion has no effect on the spectrum. It is concluded that the B26 tyrosyl residue is hydrogen bonded to an acceptor which does not ionize the *p*H range 1.5 to 8.0. The validity of Beer's Law and the effect of dioxane and added salt on the difference spectra have been examined. It is not yet possible to decide whether the two tyrosyl hydrogen bonds are within the 6000 or 12,000 mol. wt. units of insulin.

Introduction

When the amino acid sequence and the positions of the disulfide cross links in a protein are known, it should be possible to determine the folding of the molecule in solution by locating a few specific interactions (*e.g.*, hydrogen or hydrophobic bonds) between side chains. The present work is an attempt to apply this approach to the determination of the configuration of insulin[4] in solution by searching for possible side chain hydrogen bonds involving tyrosyl residues as donors.

Titration data[5] on zinc- and zinc-free insulins have been interpreted to indicate that the tyrosyl groups ionize normally. However, it should be kept in mind that titration curves give only average *pK* values over several groups and, in the case of insulin, it has not thus far been possible to distinguish between the four tyrosyl groups and the single ϵ-amino group[6] from titration data.[5] Furthermore, the detailed interpretation of insulin titration data is considerably complicated by *p*H-dependent changes in the degree of aggregation. Since titration curves provide no evidence for tyrosyl hydrogen bonds in insulin, the more sensitive and group-specific technique of difference spectrophotometry was employed in this investigation.

Difference spectrophotometry has been used to determine the *pK* values of ionizable groups in compounds of tyrosine and tryptophan.[7-10] It has also been used to determine the *pK* values of the acceptor groups involved with tyrosyl groups in hydrogen bonds in insulin[11] and in ribonuclease.[12,13] A number of workers have also observed the difference spectra arising from shifts of the tyrosyl absorption band in proteins, without investigating the nature of the groups responsible for the perturbation.[14-21] In particular it has not always

(1) This investigation was supported by research grant No. E-1473 from the National Institute of Allergy and Infectious Diseases, of the National Institutes of Health, U. S. Public Health Service.

(2) Presented in part before the Division of Biological Chemistry at the 131st meeting of the American Chemical Society, Miami, Florida, April, 1957.

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