bromide,[16] dissolved in 15 ml. of dry benzene, was added to a solution of 12.0 g. of acetamidomalonic ester dissolved in 100 ml. of magnesium-dried ethanol containing 1.2 g. of sodium. The reaction mixture was heated under reflux for 30 hr., the salt was filtered and the filtrate was reduced to dryness *in vacuo*. The residue was recrystallized from 5 liters of boiling water to yield 5.2 g. of white silky flakes, m.p. 84°.

Anal. Calcd. for $C_{16}H_{27}NO_5$: C, 61.32; H, 8.68; N, 4.47. Found: C, 61.38; H, 8.63; N, 4.73.

Hydrolysis of this intermediate in the presence of 6 *N* hydrochloric acid gave a quantitative yield of cyclohexanealanine, identical in R_f value with a product obtained *via* catalytic reduction of phenylalanine.

α-Aminocyclohexanevaleric Acid.—To a mixture of 18.5 g. of cyclohexanevaleric acid and 0.15 g. of phosphorus trichloride, 19.5 g. of bromine was added dropwise with stirring, while the reaction mixture was cooled in an ice-bath. After addition of bromine was completed, the solution was allowed to stir 1 hr. at room temperature and finally heated to reflux for 48 hr. The reaction mixture was then distilled *in vacuo* to yield 15.5 g. of material, b.p. 155–160° (1.5 mm.). A mixture of 6.5 g. of the crude α-bromocyclohexanevaleric acid and 60 ml. of concentrated ammonium hydroxide was shaken at room temperature for five days. The solvent was removed, the residue was taken up in water and neutralized with concentrated hydrochloric acid. The solid which formed on cooling was filtered and washed with ether. After recrystallization from water there was recovered 0.78 g. of material which starts decomposing at about 200°, melts at 250° dec.

Anal. Calcd. for $C_{11}H_{21}NO_2$: C, 66.29; H, 10.62; N, 7.03. Found: C, 66.56; H, 10.76; N, 7.07.

R_f values of this substance in 95% methanol, 65% pyridine, and butanol:acetic acid:water (4:1:1) were 0.70, 0.83 and 0.82, respectively.

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AUSTIN, TEXAS

[CONTRIBUTION FROM THE MARY IMOGENE BASSETT HOSPITAL (AFFILIATED WITH COLUMBIA UNIVERSITY)]

The Isolation of Serum Albumin from Specific Precipitates of Serum Albumin and its Rabbit Antibodies[1]

BY THEODORE PETERS, JR.

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When specific precipitates of either bovine serum albumin or chicken serum albumin, each with its respective rabbit antibody, are redissolved at *p*H 2.4, addition of ethanol to 70–80 vol. % at 0° causes precipitation of the antibody but allows most of the serum albumin to remain in solution. At 71 vol. % alcohol and 0.4% protein, recovery of albumin, determined by ultracentrifugation or by the use of I^{131}-labeling, was 88–92%. Purity of the albumin was 82–97%, representing removal of all but 0.4–2.8% of the original antibody. The effects of alcohol concentration and *p*H on the degree of separation were studied. The recovered serum albumin appeared unaltered as determined by ultracentrifugation, electrophoresis, precipitation with specific antiserum and double diffusion in agar. Eight per cent. or less of the antibody could be recovered in a state which would still precipitate with the specific antigen, but the remainder apparently was irreversibly altered.

Introduction

The separation of antigens and antibodies from specific immunological precipitates has been attempted for many years by a variety of procedures.[2] Investigations have been directed mainly at recovery of antibody, and yields have been low. In the present studies, the separation of serum albumin from its rabbit antibodies has been undertaken for a different purpose: (1) to determine the incorporation of C^{14}-labeled amino acids into the serum albumin and (2) to study the nature of apparent albumin precursor compounds which can be precipitated from liver extracts with rabbit antiserum.

That specific precipitates will redissolve in dilute acid or alkali has been known since 1922.[3] More recently, electrophoretic and ultracentrifugal studies have shown that specific precipitates of bovine serum albumin (BSA)[4] and ovalbumin[5] not only redissolve but that the antigen and antibody dissociate completely below *p*H 2.4 or above *p*H 11.7–12.3. This finding suggested that it might be feasible to effect the separation of antigen and antibody by working at high or low *p*H. Little success was achieved in preliminary trials using ammonium sulfate, sodium sulfate, sodium acetate or low concentrations of calcium or in attempted denaturation of antibody by heating. Fair separation was obtained with paper electrophoresis at either *p*H 2.4 or 11.7, but adsorption of albumin limited the recovery. Electrophoresis on starch at *p*H 2.4 effected some separation, but elution of the albumin and antibody from the starch was always incomplete.

The report of Levine[6] that serum albumin is soluble in the presence of high concentrations of alcohol after precipitation with trichloroacetic acid suggested that simple addition of alcohol at *p*H 2.4 might leave the albumin in solution while precipitating the antibodies. Use of *p*H 2.4 was preferred over *p*H 11.7 since the properties of serum albumin have been studied extensively at acid conditions and its antigenic activity, intrinsic viscosity, optical rotation and molecular volume have been shown to be unaffected by exposure of the protein to *p*H 2 and return to neutrality.[7]

At an ethanol concentration of 71 vol. % at *p*H 2.4 and 0.4% protein, it was found that 88–92% of the serum albumin of a specific precipitate is soluble. Purity of the albumin recovered was

(1) Supported by a Grant (H-2751) from the National Heart Institute, U. S. Public Health Service.

(2) *Cf.* (a) W. C. Boyd, "Fundamentals of Immunology," Interscience Publishers, Inc., New York, N. Y., 1956, pp. 70-73; (b) E. A. Kabat and M. M. Mayer, "Experimental Immunochemistry," C. C. Thomas, Springfield, Ill., 1948, pp. 478–488.

(3) V. R. Mason, *Bull. Johns Hopkins Hosp.*, **33**, 116 (1922).

(4) (a) D. H. Campbell, E. Leuscher and L. S. Lerman, *Proc. Natl. Acad. Sci.*, **37**, 575 (1951); (b) S. J. Singer and D. H. Campbell, THIS JOURNAL, **77**, 3504 (1955).

(5) (a) W. J. Kleinschmidt and P. D. Boyer, *J. Immunol.*, **69**, 247 (1952); (b) S. J. Singer and D. H. Campbell, THIS JOURNAL, **77**, 4855 (1955).

(6) S. Levine, *Arch. Biochem. Biophys.*, **50**, 515 (1954).

(7) (a) J. T. Yang and J. F. Foster, THIS JOURNAL, **76**, 1588 (1954); (b) M. Champagne, *J. Polymer Sci.*, **23**, 863 (1957).

82–97%, representing removal of all but 0.4–2.8% of the antibody initially present. The separation was followed both by ultracentrifugal analysis and by the use of serum albumin and antiserum lightly labeled with I^{131}. The effects of alcohol concentration and *p*H were studied, and the separated albumin and antibody were tested for antigenic activity and for homogeneity on paper electrophoresis.

Experimental

Materials.—Bovine serum albumin was Armour 3 times crystallized albumin. Chicken serum albumin (CSA) was a preparation described in a previous work.[8] NaI^{131} was obtained from Abbott Laboratories, specified "for chemical use only." Antisera were prepared by injection of alum-precipitated BSA or CSA into rabbits,[9] and crude γ-globulin fractions were isolated by precipitating with 0.5 volume of saturated ammonium sulfate, dialyzing and restoring to μ 0.15 and *p*H 7.5 in 0.055 *M* NaH_2PO_4 and 0.055 *M* tris-(hydroxymethyl)-aminomethane (Tris) with 0.01% thimerosal (sodium ethylmercurithiosalicylate).

I^{131}-BSA, I^{131}-CSA and I^{131}-Antisera.—These were prepared within two weeks of use by a procedure employing extraction of I_2^{131} in carbon tetrachloride.[10] The proteins were added in a buffer of 0.8 *M* glycine–NaOH, *p*H 9.4, to minimize denaturation. The products contained 10–100 μc. of I^{131} per mg. of protein, with an average of less than 1 iodine atom per molecule of protein. Less than 1% of the radioactivity was dialyzable, and all of the I^{131} albumin was readily precipitable with antiserum. The I^{131}-antisera did not appear to have lost potency on the basis of their precipitation with antigen, in agreement with the findings of others.[11]

Precipitations.—Five-tenths to 15 mg. of BSA, or CSA, was added to a previously-determined equivalent amount of the appropriate antiserum in the above described buffer of *p*H 7.5. Trace amounts of I^{131}-labeled proteins were included where desired. After 1 hr. at 40° the supernatants were removed by centrifugation at 1400 × *g* at 3° for 15 minutes. The precipitates were washed once with buffer and twice with 0.9% NaCl, then frozen and lyophilized. The remaining NaCl was found to be approximately 4 mg. per 100 mg. protein. Ratio by weight of antibody to albumin was 7/1.

Separations.—The dried precipitates were redissolved in 0.2–6 ml. of a glycine–HCl buffer, μ 0.1, *p*H 2.35,[4b] per mg. serum albumin and the *p*H adjusted to 2.40–2.44 by the addition of 5–8λ of 0.3 *N* HCl, or to other desired *p*H by suitable additions. All operations were conducted in an ice-bath at 0° except the determination of *p*H, which was performed at 23° in a Beckman one-drop glass electrode. Absolute ethanol was added slowly to the desired concentration with thorough mixing. The alcohol was made 0.005 *N* with respect to HCl if a *p*H below 2.7 was desired.

After 30 minutes the preparations were centrifuged at 15,000 × *g* (International Centrifuge PR-1, high-speed attachment) for 30 minutes. The use of Lusteroid tubes was avoided, as high alcohol concentrations were found to dissolve appreciable material from these tubes. The precipitates were gelatinous and tightly packed. They redissolved almost completely in the glycine–HCl buffer, but reprecipitation with alcohol to improve the recovery of serum albumin was not worthwhile because of additional contamination by the accompanying antibodies. When it was desired to recover the proteins from the supernatant and precipitate for further studies, the alcohol was removed by dialysis against water and the water removed by lyophilization.

Analytical Techniques.—I^{131} was determined in a well-type scintillation counter to 1% accuracy. Ultracentrifugal analyses were performed in a Spinco Model E instrument at room temperature on solutions which had been dialyzed against the *p*H 2.35 glycine–HCl buffer. The apparatus employed a Philpot–Svensson cylindrical lens-diagonal bar optical system.

Results and Discussion

Table I shows recoveries of I^{131}-BSA, I^{131}-CSA and their iodinated antibodies at *p*H 2.4 and alcohol concentrations of 71 and 80 vol. %. Protein concentration was 0.05% albumin or 0.4% specific precipitate. The optimum conditions were 71–75 vol. % alcohol, *p*H 2.4. At these conditions 88–92% of the albumin was recovered with only 0.4–2.8% of the total antibody. Since the initial ratio of antibody to albumin was 7/1, the recovered albumin was therefore 82–97% pure. Slower speed of centrifugation reduced the recovery of albumin, probably due to lesser packing of the precipitate. Use of 0.05 *M* H_3PO_4-NaH_2PO_4 buffer instead of the glycine–HCl buffer did not change the results appreciably.

TABLE I

RECOVERIES OF ALBUMIN AND ANTIBODY AT *p*H 2.4

Prot.	EtOH, vol.%	Cent. force, × *g*	Buffer	% of albumin recovered	% of antibody recovered
BSA	71	15,000	Glycine	89.5	1.7
BSA	71	1,400	Glycine	79.0	0.7
BSA	80	15,000	Glycine	83.3	0.4
BSA	80	15,000	Phosphate	80.2	1.0
CSA	80	1,400	Glycine	83.6	1.2

Effect of Alcohol Concentration.—The effect of varying the alcohol concentration on the solubility of I^{131}-BSA and its iodinated antibody at *p*H 2.4 and 0.4% protein is shown in Fig. 1. Below 56 vol. % alcohol both BSA and antibody are soluble, but above this concentration the solubility of the antibody drops sharply to levels of 0.4–2.0% of that present initially. Recovery of BSA drops abruptly to about 90% when a precipitate of antibody appears but does not drop further until the alcohol concentration exceeds 80 vol. %.

Effect of *p*H.—The effect of *p*H on the solubility of I^{131}-BSA and its iodinated antibody at 71 vol. % alcohol and 0.4% protein is shown in Fig. 2. Less than 3% of the antibody initially present is soluble between *p*H 2.1 and 3.6. The gradual drop in solubility of BSA above *p*H 2.7 could have been anticipated from the work of Singer and Campbell.[4b] A plot of their data shows that dissociation of the "a" complex of BSA and antibody does not occur until a *p*H below 2.7 is reached.

The sharp drop in recovery of BSA above *p*H 3.4 probably is due to the reduced solubility of BSA as its isoelectric point is approached.

Ultracentrifugal Analysis.—A 15-mg. batch of BSA was separated from its antibody at *p*H 2.4, 80 vol. % alcohol and total volume 30 ml. Ultracentrifugal analysis of the mixture at *p*H 2.4 prior to addition of alcohol (Fig. 3a) showed that dissociation of the BSA and antibodies was complete.

Following separation, the supernatant contained BSA which appeared unaltered (3b) and showed only a small degree of asymmetry in the trailing edge of its peak due to remaining antibody. In this particular separation, 2.0% of the total antibody was unprecipitated. This means that the antibody admixture in the BSA might be as great as 14% by

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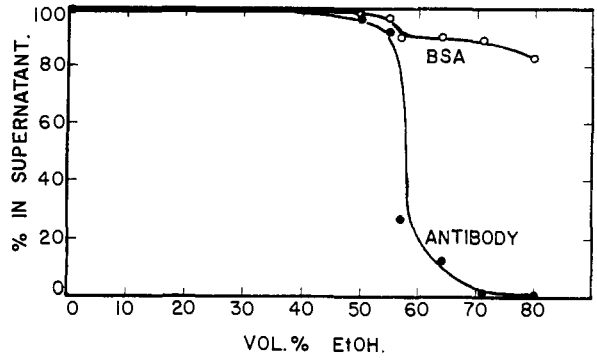

Fig. 1.—Solubility of I^{131}-BSA and iodinated antibody from specific precipitates as a function of alcohol concentration: 0.5-mg. samples of BSA were precipitated with antiserum, either the BSA or the antiserum being labeled with I^{131}. *p*H was 2.40–244 throughout; total volume 1.0 ml.

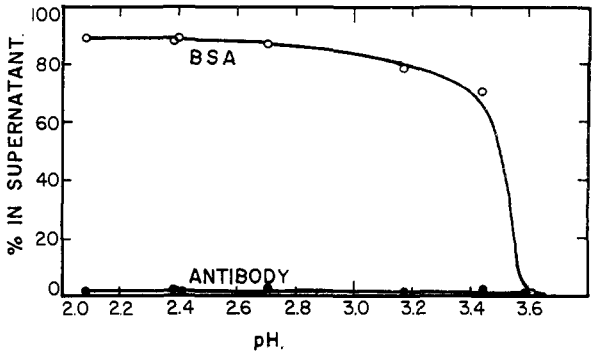

Fig. 2.—Solubility of I^{131}-BSA and iodinated antibody from specific precipitates as a function of *p*H: 0.5 mg. BSA; 71 vol. % alcohol; total volume 1.0 ml.

weight or 6% on a molar basis. Ultracentrifugation at *p*H 7.5 of BSA separated in another experiment, after removal of alcohol *in vacuo* and neutralization, showed a peak with no detectable asymmetry, suggesting that much of the antibody contaminant had been removed by such treatment.

The alcohol precipitate, redissolved at *p*H 2.4, showed a peak corresponding to unaltered antibody plus appreciable faster-sedimenting material (3c). The small amount of BSA remaining was not detectable.

Properties of Recovered BSA and Antibody.—The BSA separated by any of the conditions described was completely soluble upon return to neutral aqueous solutions, although there was frequently a 10–20% loss on dialysis of the alcohol supernatants. The BSA so recovered was 98–100% precipitable with antiserum and showed a single peak, with the expected mobility, upon paper electrophoresis at *p*H 2.4 or 8.6.[12] Upon double diffusion in agar by the technique of Ouchterlony[13] it gave a single sharp band of precipitate which made a smoothly-rounded corner with the band produced by untreated control BSA, with no spurs

(12) Paper electrophoresis was performed at *p*H 2.4 in a horizontal suspended-strip apparatus using the glycine–HCl buffer at 3°. Paper electrophoresis at *p*H 8.6 was performed in a glass plate apparatus using 0.05 *M* veronal buffer at 24°. Proteins were detected by fixing in alcoholic $HgCl_2$ and staining with brom phenol blue (3,3',5,5'-tetrabromophenolsulfonaphthalein).

(13) J. Oudin, in A. C. Corcoran, "Methods in Medical Research," Vol. V, Year Book Publishers, Inc., Chicago, Ill., 1952, p. 335.

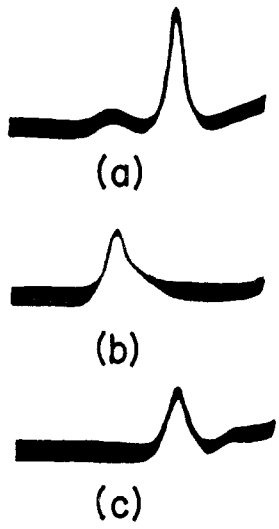

Fig. 3.—Tracings of ultracentrifuge patterns of (a) unseparated specific precipitate of BSA-antibody; (b) supernatant; (c) precipitate. *p*H 2.4; μ 0.1; speed 59,780 r.p.m.; concentration (a) 1.6% (b) 1.2% (c) 1.6%. Sedimentation proceeds toward the right.

or other evidence of immunological alteration.

Schwert[14] and Korner and Debro[15] have shown that serum albumin which had been exposed to trichloroacetic acid and 80 vol. % alcohol was apparently unaltered with regard to sedimentation constant, electrophoretic mobility, solubility, crystallizability and antigenic activity.

Most of the rabbit anti-albumin antibody was apparently irreversibly damaged by the alcohol and acid treatment. Only about 8% of the amount originally present could be recovered in solution upon neutralization. This amount, however, did precipitate in a normal fashion with BSA and gave a pattern on double diffusion in agar identical with that of untreated antiserum.

Other Proteins.—Attempts were made to effect a similar separation with ovalbumin instead of serum albumin. However, whereas the specific precipitates went into solution at *p*H 2.4, ovalbumin was found to precipitate at about 30 vol. % alcohol, so that the above technique could not be used. It might be possible to precipitate the dissociated ovalbumin while leaving the antibody in solution at some alcohol concentration between 30 and 56 vol. %.

Trials have not been conducted with insulin, the only other protein mentioned by Schwert as being soluble in 80 vol. % alcohol at low *p*H,[14] but it might be expected that the above method would prove satisfactory for the separation of insulin from its antibodies.

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