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An Improved Procedure for the Preparation of Human Serum Albumin from Placental Extracts

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An improved procedure is described for the separation and purification of albumin from human placental extracts. Under controlled conditions of *p*H, ionic strength, ethanol and zinc concentration, and temperature, albumin has been separated from hemoglobin and other plasma proteins with increased yield and purity. The improvements have concerned an increased protein concentration, batch resin treatment, precipitation of lipoproteins at *p*H 5.2 and 18% ethanol and their subsequent removal at *p*H 5.8 and 40% ethanol followed by precipitation of the albumin at *p*H 4.8–4.9.

Introduction

We presented earlier a procedure[2] for the preparation of a stable, salt-poor, low heme content albumin from placental sources. That procedure employed the principles of the ethanol–water and metal cation methods of Cohn, *et al.*,[3,4] and Oncley, *et al.*[5] This paper presents significant modifications of the original method for the removal of hemoglobin and heme; this has resulted in an improved preliminary purification of albumin. The removal of zinc has been facilitated by employing batch resin treatment to replace the resin column. New, less critical, conditions for the removal of the α- and β-globulins during the final albumin purification yield a more reproducible albumin as a final product. Many of the improvements described have made the original method more adaptable to the large scale production of albumin from these sources.

Materials and Metheds

Placental Extract.—The placentas and postpartum blood were collected as described previously except that re-usable stainless steel boxes were employed, each with a capacity of about 30 placentas. The containers were stored at −30°, and thawed at +2–4° for 72–96 hours. The placentas were then chopped and each kg. of material added to a slurry of 1100 ml. of cold saline (0.8%) containing 22.4 g. of long fiber pulp.[6] This mixture contained considerable ice and was stirred for 1–2 hours at 2°. A major portion of the extract was obtained by straining the mixture through surgical gauze. Additional extract was recovered from the wet pulp by use of a Sweco vibrating screen separator.[7] This extract was clarified in a Sharples supercentrifuge and was found to contain approximately 19 g. of plasma and tissue proteins and 23 g. of hemoglobin per liter.

Resin Treatments.—The carboxylic exchange resin XE-64[8] was used to remove zinc by the batch method. The resin was prepared by treatment with 3 vol. of 1 *M* HCl for one hour. The acid was decanted and the resin washed with water and treated twice with 3 vol. of 1 *M* NaOH. The resin was stored under 1 *M* NaOH if not used immediately. Just before use the resin was washed free of excess NaOH with distilled water and titrated to *p*H 7.4–7.5 by addition of concentrated HCl. The regenerated resin was then separated from excess fluid by suction on a buchner funnel.

Duolite A-7-Resin.[9]—This anion-exchange resin was used to remove chloride, acetate, citrate and other anions from the purified albumin solution by exchange for OH^-. The resin was prepared by placing 1.8 kg. of dry resin in a 4 in. glass column and washing with 4 vol. of 0.75 *M* NaOH, using a flow rate of 200 ml./min. This was followed successively by a thorough flushing with water and then by 2 vol. of 0.75 *M* HCl at 200 ml./min. The final regeneration was accomplished by rinsing free of excess HCl and passing 3 vol. of 1.5 *M* NH_4OH through the column at 100 ml./min. and leaving in this solution at 0° until needed. Immediately before use the resin was washed with double-distilled water until neutral to litmus.

Other Analyses.—The methods for electrophoretic analysis and for the determinations of nitrogen, hemoglobin, heme, iron, zinc, sodium and caprylate were as described previously.[2]

System of Fractionation

Directions for the Preparation of Normal Serum Albumin (Human) from Placental Extracts.—Precipitate I is formed by the addition of 351 ml. of 95% ethanol (precooled to −10°) per kg. of extract at 0°, giving a final ethanol concentration of 25%. The alcohol is added with good stirring and at such a rate that the temperature is gradually lowered to −5° where it is held during the remainder of the addition. The *p*H is uncontrolled but lies in the range 6.6–6.9. The suspension is stirred for at least one-half hour after completion of the ethanol addition. Precipitate I is removed by centrifugation at −5° and stored at −10° for subsequent use in the preparation of immune serum globulin.

Precipitate A.—The supernatant from precipitate I is held at −5° while sufficient *p*H 4.0 buffer[10] is added with stirring to bring the *p*H to 4.95 ± 0.05. Each liter of supernatant I requires approximately 3.2 ml. of this buffer. Thirty ml. of 0.1 *M* zinc acetate in 25% ethanol (freshly prepared and precooled to −5°) per l. of original supernatant I is added slowly with stirring. The suspension is stirred for two hours at −5°, then precipitate A is removed by centrifugation at −5°.

Precipitate B.—The major portion of the hemoglobin in precipitate A is removed as precipitate B by suspending each kilogram in 12.5 l. of 0.15 *M* sodium chloride at 0°. The suspension is stirred without aeration for at least one hour; then 20 ml. of 0.1 *M* zinc acetate is added per l. of solution. The *p*H is adjusted to 7.2 ± 0.10 by the slow addition of approximately 85 ml. of 0.5 *M* sodium bicarbonate with stirring at 0°. The *p*H is rechecked and the suspension stirred for two hours prior to removal of precipitate B by centrifugation at 0°. Precipitate B contains the bulk of the hemoglobin along with lesser amount of α-globulin, β-globulin and albumin and is discarded.

Clarification.—The supernatant following the removal of precipitate B contains a fine suspension of insoluble protein (hemoglobin, α-globulin and β-globulin). This suspended protein is removed by filtration through Hercules C-3 pads

(1) Presented before the Biological Chemistry Division of the 126th National Meeting of the American Chemical Society in New York, September, 1954.

(2) F. H. Gordon, L. A. Hyndman, F. C. Bloom, H. D. Anderson, H. L. Taylor and K. B. McCall, THIS JOURNAL, **75**, 5859 (1953).

(3) E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin and H. L. Taylor, *ibid.*, **68**, 459 (1946).

(4) E. J. Cohn, F. R. N. Gurd, D. M. Surgenor, B. A. Barnes, R. K. Brown, G. Derouaux, J. M. Gillespie, F. W. Kahnt, W. F. Lever, C. H. Liu, D. Mittelman, R. F. Mouton, K. Schmid and E. Uroma, *ibid.*, **72**, 465 (1950).

(5) J. L. Oncley, M. Melin, D. A. Richert, J. W. Cameron and P. M. Gross, *ibid.*, **71**, 541 (1949).

(6) We have employed Schleicher and Schull filter pulp number 49.

(7) More recent studies have employed a basket-head centrifuge, with a 10–15% increase in fluid being obtained.

(8) XE-64 resin is finely ground I.R.C.-50 resin (100–200 mesh), available from Rohm and Haas Co., Resinous Products Division, Washington Square, Philadelphia 5, Pennsylvania.

(9) Duolite A-7 resin is available from the Chemical Process Co., 901 Spring Street, Redwood City, California.

(10) This *p*H 4.0 acetate buffer has a mole ratio of sodium acetate to acetic acid of 0.2 and is conveniently made by taking 200 ml. of 4 *M* sodium acetate and 400 ml. of 10 *M* acetic acid and water to make one liter. This buffer diluted with water 80 times has a *p*H of 4.0 ± 0.02 as measured with a glass electrode potentiometer at 25°.

and Hyflo filter aid (1% w./v.). The resulting solution, B-F-1, contains approximately 10 g. of protein per l., of which 6% is hemoglobin pigment.

Heme Removal.—To supernatant B-F-1 is added 1.828 g. of solid sodium caprylate per l. and the resulting solution stirred for one hour. The *p*H is lowered to 4.7–4.8 by the slow addition of approximately 150 ml. of *p*H 4.0 buffer[10] per l. After stirring for two hours at 0° the precipitate is removed by adding 1% Hyflo filter aid and filtering through Hercules C-7[11] pads laid over Hormann D-10-T pads.[12]

Precipitate C.—To each liter of supernatant solution (containing approximately 7 g. plasma protein and traces of hemoglobin) is added 1.536 g. of anhydrous citric acid. After stirring for one hour, the ethanol concentration is raised to 25% by the addition of 357 ml. 95% ethanol (precooled to −10°) per l. During the addition the temperature is lowered to and held at −8° to −10°. The suspension is stirred for four hours at −8° to −10° before removing precipitate C by centrifugation.

Zinc Removal.—Precipitate C (80% albumin) is suspended in 8.35 l. of water per kg. at 0° and stirred for one hour before adding 0.5% Hyflo. The suspended material is removed by centrifugation followed by filtration through Hercules C-3 pads (solution C). Zinc is removed by three batch treatments with XE-64 resin at 0–2°.[13] The first two treatments employ one part of wet resin per 19 parts of solution (w./w.), each for two hours, and the last employs one part resin to nine parts of solution for two hours. The resin is removed each time by centrifugation, decantation and filtration.

Precipitate D.—The resulting solution is then adjusted to *p*H 5.2 ± 0.05, using *p*H 4.0 acetate buffer[10] diluted 1–10 with distilled water. The buffer is added through jet openings while maintaining the temperature at 0°. After the solution is stirred for one-half hour, precooled 95% ethanol (234 ml./l.) is added to give a final concentration of 18%. The solution is cooled to −5° during the addition and then stirred for one hour. After eight hours standing at −5° without stirring, the suspension is adjusted to *p*H 5.8–5.9 with solid sodium bicarbonate (the amount is determined by titration of aerated sample). Precooled 95% ethanol (400 ml./l.) is added to give a final concentration of 40%. The temperature is maintained at −8° during the addition, and after stirring for one hour the suspension is allowed to stand for 6–8 hours at −8°. Precipitate D thus formed is removed by centrifugation at −8° and is discarded.

Placental Albumin.—One-half of one per cent. of Standard Supercel is suspended in supernatant D before filtration through Hormann D-10-T pads. Following clarification, sufficient buffer[10] is added to lower the *p*H to 4.8–4.9. For this, the buffer is first diluted with an equal volume of cold ethanol (80%) and then added very slowly with adequate stirring, and the temperature is lowered to −10°. This slow addition of buffer and a standing period of eight hours or more are necessary to obtain albumin particles that are readily removed by centrifugation. The precipitate (albumin) is removed at −10° in the Sharples supercentrifuge equipped with a three-wing adapter.

Removal of Acid.—To minimize the amount of sodium bicarbonate that must be added to the albumin when prepared as a 25% solution at *p*H 7.0, Duolite A-7 resin is employed to remove acetic acid carried down with the albumin paste. The albumin precipitate is redissolved in two vol. of double distilled water at 0° and passed through a four in. glass column with a 1.8-kg. (dry) charge of resin at 100 ml./min. at 0°. These conditions are effective for raising the *p*H of 55–60 liters albumin solution to 6.0–6.3. The effluent is frozen in trays and dried *in vacuo*.

Discussion

The principles and general conditions involved in the separation of hemoglobin from albumin and other plasma proteins were discussed previously.[2] In the present method, we have doubled the protein concentration for the processing of precipitate A. This has materially reduced the fluid volumes involved and has in no way reduced the efficiency of the removal of hemoglobin. The same ratio of caprylate to protein was maintained but only one-half the amount of zinc acetate was required. Removal of precipitated hematin was greatly aided by simultaneous filtration through Hercules C-7 pads overlying Hormann D-10-T pads.

In the present method, precipitate C is removed at a final ethanol concentration of 25% instead of 40% as employed earlier. The percentage recovery of protein in precipitate C (Table I) is the same or greater than when the more dilute protein solution was precipitated with 40% ethanol (Table I, runs 9–10). The heme pigments are more completely removed by this change in procedure as shown by the data in Table II. The yields and purity of the albumins at this stage (precipitate C) are not materially increased over those of the previous method. The chief advantage of the changes in these steps is the reduction of processing volumes and consequent savings in processing times and reagents.

TABLE I

THE REMOVAL OF PRECIPITATE C AT SEVERAL ETHANOL CONCENTRATIONS

Ppt. A. run no.[a]	Protein in supernatant B-F-2 g./l.	Protein in supernatant B-F-2 Total g.	Protein in supernatant C g./l.	Protein in supernatant C Total g.	Ethanol, %	Protein in supernatant B-F-2 recovered in ppt. C, %
9	4.5	874	0.21	68	40	92.2
10	5.5	1149	0.30	105	40	90.9
11	4.0	876	.58	170	25	80.5
13	8.9	1898	.40	123	25	93.5
14	7.8	1962	.59	192	25	90.2
15	7.3	1764	.55	176	25	90.0
16	6.0	1560	.44	139	25	91.1
17	8.1	2395	.60	230	25	90.4

[a] 25 l. of saline per kg. precipitate A (original method) used in runs 9–10; 12.5 l. saline per kg. precipitate A (new method) used in runs 13–17.

TABLE II

HEME CONTENT OF SOLUTION C[a]

Ppt. A run no.	Solution C Protein, g./l.	Solution C Heme,[c] mg./g. protein
9–10[b]	36.3	1.96
13	34.0	0.42
14	33.7	0.76
15	37.9	0.56

[a] Solution C is obtained by the re-solution and clarification of precipitate C. [b] Original methods. [c] Calculated by ($E_{1\,cm.}$ 403 mμ)/mg. protein/ml. × 0.184/0.025.

Further purification of the albumin in precipitate C is necessary, but the excess zinc is removed first. The present method results in much lower concentration of zinc in solution C (precipitate C redissolved; Table III). In the earlier method the zinc concentration was reduced to acceptable levels by raising the *p*H of solution C to 7.2 and employing a column of XE-64 resin. When the improvements of the present method were followed as described, batch treatment with resin was found effective (Table III) when the *p*H of solution C

(11) Filter pads purchased from Hercules Filter Corporation, Paterson 3, New Jersey. Approximately 14 sq. in. pad per l. were used.

(12) Filter pads purchased from F. R. Hormann and Company, Inc., 17 Stone Street, Newark, New Jersey.

(13) Our most recent studies indicate that only two batch treatments are necessary.

TABLE III

REMOVAL OF ZINC FROM SOLUTION C AND ASSOCIATED LOSS OF PROTEIN

Ppt. C run no.	Solution C			Solution C-R-1			Solution C-R-2			Solution C-R-3		
	Vol., l.	N × 6.25, g./l.	Zinc, μg./ml.	Vol., l.	N × 6.25, g./l.	Zinc, μg./ml.	Vol., l.	N × 6.25, g./l.	Zinc, μg./ml.	Vol., l.	N × 6.25, g./l.	Zinc, μg./ml.
10[a]	43.8	36.3	69	48.5	30.9	2						
11[a]	33.5	38.8	53	38.0	31.2	3						
12[b]	43.8	34.0	73	47.5	..	33	47.2	28.8	12	44.8	27.8	6
13[c]	45.2	33.7	26	44.8	32.9	8	44.2	29.9	3	43.2	30.1	1
14[c]	39.8	37.9	33	39.8	34.4	8	39.8	36.2	4	33.8	35.9	3
15[c]	85.5	35.0	40	84.5	34.0	4	84.3	33.9	2	80.5	33.1	1

[a] Zinc removal in column; solution C at *p*H 7.2. [b] Zinc removal by batch method; solution C at *p*H 7.2. [c] Zinc removal by batch method; solution C unadjusted (*p*H 4.5–4.6).

was unadjusted (*p*H 4.5–4.6). The XE-64 resin column, although more efficient for zinc removal, cannot compete with the batch method for large-scale application. The slow flow rates and column capacities seriously limit the processing volume. Batch treatment allows for almost unlimited processing volume with very short process times.

In the earlier method the removal of globulins from solution C (after zinc removal) was accomplished by raising the ethanol concentration to 18% and lowering the *p*H to 5.2 ± 0.05. We have found that the previous system at this point was too sensitive to changes in *p*H to use in a routine production method. When this precipitate was formed at *p*H 5.8 and 40% ethanol, the subsequent yield and purity of the albumin were satisfactory although the albumin was found to contain material that was unstable to heating at 60°. This material was largely removed by (a) incorporating the changes cited above for the removal of hemoglobin and heme pigments and (b) adjusting solution C, after removal of the zinc, to *p*H 5.2 and 18% ethanol and allowing to stand for 8–12 hours (−5°) before proceeding to the conditions of *p*H 5.8 and 40% ethanol. These conditions are quite similar to those employed in method 6 of Cohn, *et al.*, for the preparation of fractions IV-1 and IV-4. In this case, no attempt is made to remove separately the precipitate formed at *p*H 5.2 and 18% ethanol.

With the supernatant from precipitate D already at 40% ethanol, it becomes necessary only to lower the *p*H to 4.8–4.9 to precipitate the albumin. Lowering the *p*H only to 5.2, as in the original method, results in the formation of a colloidal albumin which is difficult to separate. Considerable acetic acid is occluded in the albumin paste at *p*H 4.8. As a consequence, we have employed a hydroxyl exchange resin, Duolite A-7, to remove acetate and other anions rather than to neutralize them.

The final albumin has been produced in satisfactory yield and purity (Table IV) and has the same general characteristics as cited previously. By all accepted standards, the final albumin solutions are identical to those prepared from fresh venous blood.

TABLE IV

DISTRIBUTION OF ALBUMIN AND HEMOGLOBIN IN FRACTIONS DURING PROCESSING

	Yield per placenta				Recovery of extract albumin, %
	Protein (N × 6.25), g.	Hemoglobin g.	Hemoglobin %	Albumin, g.	
Extract	42.3	23.0	18	7.6	(100)
Precipitate I	5.5	0.5	7	0.49	5
Precipitate A	13.7	2.1	48	6.67	87
Supernatant A	21.9	..	..	(0.6)	(8)
Precipitate C	4.0	0	82	3.3	43.5
Albumin	2.3	0	98	2.2	29.5

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