[CONTRIBUTION FROM THE DIVISION OF LABORATORIES, MICHIGAN DEPARTMENT OF HEALTH]

The Preparation of γ-Globulin from Placental Blood by Ethanol Fractionation[1]

BY HAROLD L. TAYLOR, F. C. BLOOM, K. B. MCCALL, LEE A. HYNDMAN AND H. D. ANDERSON

RECEIVED OCTOBER 13, 1955

A procedure is described for the separation and purification of γ-globulin from human placental extracts and postpartum blood. Frozen placentas together with the postpartum blood are thawed, chopped and extracted with isotonic saline. The initial separation of a fraction containing γ-globulin is accomplished by the addition of ethanol in the cold. A large part of the α-globulins are separated from the γ-globulin fraction by the precipitation of the former at *p*H 4.8, and ethanol 8%. This is followed by the precipitation of a γ-globulin-rich (70%) fraction at *p*H 7.0–7.2 and ethanol 25%. A fraction rich (91%) in α- and β-globulins is separated at *p*H 5.1 and ethanol 17%, leaving a supernatant fluid from which the γ-globulin is again precipitated with a final purity of 96%.

Introduction

A procedure was presented earlier[2,3] applicable to the large scale preparation of albumin from placental extracts and postpartum blood. In the preparation of this albumin, a fraction (precipitate I) is separated which contains most of the γ-globulin, and this has served in our study as the starting material. The general principles of the ethanol fractionation procedure, as described by Cohn, *et al.*,[4] and as applied previously to the production of γ-globulin from venous blood by Deutsch, *et al.*,[5] and Oncley, *et al.*,[6] were followed in our study. Although the ethanol methods have been adapted for the production of γ-globulin from placental blood, no descriptions of methods have appeared in the literature.

Materials and Methods

The methods of collecting and handling placentas and postpartum blood were described previously,[2,3] as well as the methods of electrophoretic analysis, and the determinations of nitrogen and hemoglobin.[2]

Directions for the Preparation of γ-Globulin from Placentas (Scheme I)

The recovery of placental extracts and postpartum blood is accomplished by (a) thawing the placentas at 2–4° after removal from −30° storage; (b) chopping with a Hobart food cutter; (c) stirring each kg. of tissue plus blood into 1,100 ml. of saline (0.8%) containing 22.4 g. of paper pulp; (d) separating the extract at 0°; and (e) clarifying by Sharples centrifugation. This extract contains approximately 19 g. of plasma and tissue proteins and 23 g. of hemoglobin per liter.

Precipitate I.—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 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 frozen.

Precipitate II.—An initial purification is achieved by separating a fraction rich in α-globulins. Each kg. of precipitate I is suspended in 4.0 l. of water (0°) containing 1.75 moles of sodium chloride and then diluted with an additional 4.0 l. of water. After a uniform suspension has been obtained, the *p*H is lowered to 4.8 ± 0.05 by the slow addition of a mixed buffer[7,8] in an amount determined by an initial titration.[9] After 30 minutes stirring, the system is diluted with 12.9 l. of water (0°) per kg. of precipitate I. The mixture is stirred 2 hours and then 2.18 l. of 95% ethanol (−10°) per kg. of precipitate I is added through jets to bring the ethanol concentration to 8%. During the addition the temperature is lowered to and held at −2 to −3°. The suspension is stirred for one hour before removing precipitate II by centrifugation.[10] The supernatant fluid II is retained.

Precipitate III.—The supernatant fluid II is adjusted to *p*H 7.2–7.4 with a solution[11] of sodium bicarbonate and sodium chloride. Then 243 ml. of precooled (−10°) 95% ethanol, per kg. of solution, is added through jets as the temperature is gradually lowered to and held at −5 to −7°. The suspension is stirred for one hour before the γ-globulin fraction (precipitate III) is removed by centrifugation.

Precipitate III-1.—A fraction consisting principally of α- and β-globulins is precipitated from the γ-globulin by suspending each kg. of precipitate III in 10 liters of water (0°) and then adjusting the *p*H to 4.80 ± 0.05 by adding a mixture of 680 ml. of 0.05 *M* dibasic sodium phosphate and 4.17 l. of 0.05 *M* acetic acid. After stirring overnight, the *p*H is next raised to 5.10 ± 0.05 by the addition of about 390 ml. of 0.05 *M* sodium acetate per kg. of solution. This solution is then diluted with 10 l. of water (0°) per kg. of precipitate III, followed by raising the ethanol concentration to 17%. This requires adding 6.97 liters of precooled (−10°) 95% ethanol per kg. of precipitate III. During the addition the temperature is lowered to and held at −5 to −6°. The suspension is stirred for one hour before removing precipitate III-1 by centrifugation. The resulting fluid (supernatant III-1) is clarified by filtration through S-1 Seitz-type pads[12] to remove the remainder of Precipitate III-1.

(1) Presented before the Biological Chemistry Division of the 128th National Meeting of the American Chemical Society, Minneapolis, September, 1955.

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

(3) H. L. Taylor, F. C. Bloom, K. B. McCall, L. A. Hyndman, THIS JOURNAL, **78**, 1353 (1956).

(4) 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).

(5) H. F. Deutsch, L. J. Gosting, R. A. Alberty and J. W. Williams, *J. Biol. Chem.*, **164**, 109 (1946).

(6) J. L. Oncley, M. Melin, D. A. Richert, J. W. Cameron and P. M. Gross, Jr., THIS JOURNAL, **71**, 541 (1949).

(7) The *p*H 4.0 acetate buffer has a mole ratio of sodium acetate to acetic acid of 0.2 and is conveniently made up 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 4.00 ± 0.02, as measured with a glass electrode potentiometer at 25°.

(8) The *p*H 4.8 acetate buffer has a mole ratio of sodium acetate to acetic acid of 1.1 and is conveniently made by taking 250 ml. of 4 *M* sodium acetate and 90 ml. of 10 *M* acetic acid and water to make one liter. This buffer, diluted with water 100 times, has a *p*H of 4.80 ± 0.02 with a glass electrode potentiometer at 25°.

(9) The amount of *p*H 4.0 buffer is determined by a preliminary titration to *p*H 4.80. The amount of *p*H 4.8 buffer is calculated to make the sodium acetate contribution of the mixed buffers 0.25 *M* per kg. of precipitate I. The buffers are mixed and diluted with sufficient water (0°) to give an additional volume of 2 l. per kg. of precipitate I.

(10) We have employed a number 16 Sharples Supercentrifuge with a three-wing insert and a rate of 20–30 liters per hour.

(11) The amount of sodium bicarbonate is determined by a preliminary titration with care that all samples are aerated before the *p*H is determined. To the calculated amount of sodium bicarbonate is added an amount of sodium chloride such that the addition of these two substances will be in an amount of 0.023 mole per liter of supernatant II. Both substances are dissolved together in water at 0° and are added as a 0.5 *M* solution.

(12) Filter pads purchased from the Republic Seitz Filter Corporation, 17 Stone Street, Newark, New Jersey.

SCHEME I

Ppt. I redissolved

Ethanol	8%
*p*H	4.8 ± 0.05
Temp.	−3°
Protein	1%
Salt concn.	0.08 *M*
Sodium chloride	0.07 *M*
Sodium acetate	0.01 *M*

Ppt. II (discard) — Supernatant II

Ethanol	25%
*p*H	7.0–7.2
Temp.	−5°
Ionic strength	0.09–0.10
Protein	0.4%

Ppt. III (save) — Supernatant III (discard)

Ppt. III redissolved

Ethanol	17%
*p*H	5.1 ± 0.05
Temp.	−6°C.
Protein	0.7%
Ionic strength	0.01

Ppt. III-1 (discard) — Supernatant III-1

Filter

Ethanol	25%
*p*H	7.0 ± 0.01
Temp.	−5°C.
Protein	0.6%
Ionic strength	0.05

Ppt. III-2 — Supernatant III-2 (discard)

Precipitate III-2.—A final precipitation of the γ-globulin fraction from supernatant III-1 is accomplished by first raising the *p*H to 7.2–7.4 by adding a sufficient amount of a 0.5 *M* solution of sodium bicarbonate. The ethanol concentration is raised to 25% by the addition of 114 ml. of precooled (−10°) 95% ethanol per l. of solution. During the addition the temperature is lowered to and held at −10°. The suspension is centrifuged at −10° to remove precipitate III-2. This γ-globulin fraction is suspended uniformly in about 1.5 vol. of water–ice mixture (2:1) and, after the ice melts, the suspension is then frozen in suitable containers and dried from the frozen state.

Discussion

Preliminary Purification.—We have employed a saline extract of the placentas as a starting material for the preparation of both albumin and γ-globulin. The presence of large amounts of hemoglobin in this extract rendered electrophoresis useless in the early steps of purification. However, by using infrared film and a tungsten lamp,[13] it was possible to determine the γ-globulin electrophoretically in supernatant II and the succeeding fractions. A fair estimate of the amount of γ-globulin in the extract and in precipitate I was possible by superimposing ascending and descending electrophoretic patterns. The clarified extract was estimated to contain 1.2 to 1.3 g. of γ-globulin per liter (or per placenta) by this method.

The removal of precipitate I from the extract was accomplished under the same conditions as employed by Cohn, *et al.*,[4] for the preparation of a similar fraction (II + III) from human plasma. Attempts to further purify the γ-globulin by the method employed for fraction II + III from plasma resulted in a satisfactory product, but a very poor yield. Instead, conditions for the extraction of γ-globulin from precipitate I were selected on the basis of data presented in Table I.

(13) G. L. Miller, *Science*, **116**, 687 (1952).

TABLE I

EFFECT OF *p*H AND IONIC STRENGTH ON THE EXTRACTION OF γ-GLOBULIN FROM PLACENTAL PRECIPITATE I[a]

Exp. no.	Extraction conditions[b] *p*H	Extraction conditions[b] Ionic strength	Extracted material Total protein, g.	Extracted material γ-Globulin, g.	Extracted material γ-Globulin, % of that in ppt. I
(Starting material for Experiments 1–4)			(38.9)	(5.44)	(100)
1	4.85	0.08	11.4	3.82	70.1
2	5.38	.08	14.0	3.18	58.5
3	5.70	.08	11.8	2.94	54.1
4	5.40	.04	4.8	0.24	4.4
37	5.38	.08	843	328	65
38	4.85	.08	981	520	100

[a] Experiments 1–4 represent 150 g. precipitate I; Experiments 37 and 38 represent 9.8 kg. precipitate I. [b] All extractions performed at 8% ethanol, −2°.

These small scale experiments, 1 through 4, indicated that the extraction of γ-globulin was more nearly complete at the lower *p*H (4.85). In exp. 4, a lower ionic strength reduced the solubility of all proteins in precipitate I. When large-scale runs (37–38) verified the findings of the small-scale work (experiment 1), we adapted the conditions of *p*H 4.8 for this step. It was also determined that less hemoglobin was extracted at this *p*H.

The conditions for the precipitation of the bulk of the γ-globulin from supernatant II are essentially those employed by Oncley, *et al.*,[6] for the removal of a total γ-globulin fraction. They differ only in ionic strength and protein concentration. Since Oncley, *et al.*,[6] had reported that γ-globulin was more soluble in the presence of sodium acetate than sodium chloride, our original experiments on the extraction of γ-globulin from precipitate I were performed with sodium acetate. However, the quantity of acetic acid required in the *p*H 4.8 acetate buffer used earlier for the extraction of precipitate I nearly doubled the ionic strength at the *p*H of 7.2–7.4 employed for the subsequent precipitation of γ-globulin (as precipitate III). We then replaced $^7/_8$ of the sodium acetate with sodium chloride to determine whether or not the extraction of γ-globulin from precipitate I was as complete as sodium acetate alone and would still increase the recovery of γ-globulin in precipitate III (Table II).

TABLE II

THE EXTRACTION AND PRECIPITATION OF γ-GLOBULIN[a]

	Extraction solution: 0.08 *M* sodium acetate[b] Protein, g./kg. ppt. I	γ-Globulin %	γ-Globulin G.	Extraction solution: 0.01 *M* sodium acetate[c] and 0.07 *M* sodium chloride Protein, g./kg. ppt. I	γ-Globulin %	γ-Globulin G.
Precipitate I	226	16	43.4	211	16	40.6
Supernatant II	106	..	..	97	59	
Supernatant III	40	..	..	39	9	
Precipitate III	64	53	33.9 (78%)	55	70	38.5 (95%)

[a] All extractions were performed at *p*H 4.80 ± 0.05; 8% ethanol, and −2°. [b] Average values from three runs. [c] Average values from ten runs.

The data indicate that although the sodium acetate–sodium chloride solution yielded a lesser quantity of precipitate III, the total amount of γ-globulin recovered was significantly greater (13%).

Final Purification.—Analyses of precipitate III have shown a γ-globulin content of approximately 70% (Table III). For further purification we have employed the extraction procedure of Deutsch, *et al.*,[5] for our precipitate III, with slight modifications. The procedure of Deutsch, *et al.*,[5] for fraction II + III of plasma employs a mixture of dibasic sodium phosphate and acetic acid to adjust the *p*H to 4.80 ± 0.05. After stirring overnight, that system is raised to 5.10 ± 0.05 by the use of additional dibasic sodium phosphate and acetic acid. We have modified this method by employing sodium acetate for the *p*H adjustment from 4.8 to 5.1. Our preliminary experiments with acetate indicated that the γ-globulin extracted from precipitate III was equal or slightly greater in amount than that obtained with the phosphate. Except for this change, all conditions of the method of Deutsch, *et al.*,[5] have been applied without modification.

Distribution of γ-Globulin.—The results of electrophoretic analysis of the several precipitates and supernatant fluids are presented in Table III. The data for the several precipitates are considered more reliable than those for the supernatant fluids, since all the supernatant fluids were concentrated before electrophoretic analysis by drying from the frozen state. In our experience, this often leads to error because of altered solubilities of many of the proteins.

TABLE III

ELECTROPHORETIC ANALYSIS OF PLACENTAL FRACTIONS

	Albumin, %	Globulins, % α	β_1	γ	$\phi + \beta_2$
Precipitate I[b]	7	47	22[a]	16	8
Precipitate II[b]	8	70	14[a]	3	5
Supernatant II[b]	3	12	23[a]	59	3
Precipitate III[b]	1	13	10	70	6
Supernatant III[b]	6	25	55[a]	9	5
Precipitate III-1[c]	2	47	24	7	20
Supernatant III-1-F[c]	0	0	4	95	1
Precipitate III-2[c]	0	0	4	96	0
Supernatant III-2[c]	0	0	6	90	0

[a] Hemoglobin included in β_1 1-globulin peak. [b] Data are means of ten analyses; batch size—527 placentas. [c] Data are means of five analyses; batch size—2,464 placentas.

The distributions of total protein and γ-globulin are presented in Table IV. The most serious discrepancy would appear to be that the sum of the γ-globulin recovered in precipitate II, precipitate III and supernatant III is about 8% greater than that in precipitate I. This is probably due to a low figure for the γ-globulin content of precipitate I, since we feel confident that the electrophoretic data on the original extracts of precipitate I are less reliable. The data do indicate that a good separation of the γ-globulins and the α- and β-globulins was accomplished by the removal of precipitate II. The greatest losses of γ-globulin apparently occur during the removal of precipitate III and the filtration of supernatant III-1; these losses may be due to occlusion of γ-globulin by the filter pads and filter medium. Recently we have employed an alcohol–buffer wash which tends to minimize this loss.

TABLE IV

DISTRIBUTION OF TOTAL PROTEIN AND γ-GLOBULIN IN PLACENTAL FRACTIONS

	N × 6.25, g./plac.	Nitrogen, factor	Protein, g./plac.	γ-Globulin %	γ-Globulin g./plac.
Extract	42.3	7.19	48.6	2.5	1.22
Precipitate I	5.50	7.53	6.66	16	1.06
Supernatant I	36.8	..	..	1	
Precipitate II	2.76	8.30	3.66	3	0.11
Supernatant II	2.36	..	..	59	
Precipitate III	1.34	6.60	1.41	70	0.99
Supernatant III	0.91	(6.60)	0.96	9	.086
Precipitate III-1	.42	7.49	.50	7	.035
Supernatant III-1-F	.69	6.25	.69	95	.655
Precipitate III-2	.63	6.25	.63	96	.605
Supernatant III-2	.06	(6.25)	.063	90	.05

The yield of γ-globulin is 0.8 g. per kg. of placental tissue and postpartum blood. This represents an over-all recovery of approximately 50%. The product is 96–98% pure; the other major component is reported as β-globulin. The purified γ-globulin has been prepared in final solution containing 16.5 g. per 100 ml. and this solution has a light amber color. Although this globulin has not been tested clinically, it has been subjected to all the appropriate laboratory tests for safety, pyrogenicity, stability and content of diphtheria antitoxin, Influenzae A and poliomyelitis virus neutralizing antibodies. In all these tests this product was found to be indistinguishable from the γ-globulin prepared from fresh human plasma.

Acknowledgments.—We wish to acknowledge the assistance of the staff of the Michigan Department of Health fractionation laboratory which included, besides the authors, F. C. Coryell, William Gay, R. Murray, A. Kennedy, G. Nonhof, R. Johnson, J. Sgouris and F. Gordon. For assistance in the many analytical determinations and biological testing we are indebted to L. Rasmussen, E. Hagenbuch, R. Yasin, J. Collins, A. Forell and V. Ku.

LANSING, MICHIGAN