Brom thymol blue was found to be a suitable indicator for the titrations in methanol. From the high autoprotolysis constant[29] of methanol it is clear that precise acid–base titrations should in principle be possible in this solvent. This indeed was found to be the case in the standardizations; however, in the kinetic experiments high precision was not obtained due to the dilute solutions (the infinite concentrations were about 0.02 M) and a rapidly fading end-piont.

The concentration of decalyl perbenzoate was determined from the weight of material (previously dried to constant weight and checked by melting point) made up to volume with absolute methanol. In runs conducted at 0, 20 and 30° the weighed sample of perester was placed in a volumetric flask and made up to volume with thermostated solvent. After rapid mixing an aliquot was withdrawn, and time was counted from the time of delivery of this first aliquot into a chilled flask. The standard procedure of analyzing aliquots as rapidly as possible after delivery was adopted and an attempt was made to treat all samples uniformly. Samples were titrated to the first blue color of a rapidly fading end-point. Except for the runs at 50° the reactions were carried out in the volumetric flask in which the solution was made up. In the runs at 50° solutions were prepared at room temperature, distributed in ampules containing a little over 5 ml. and placed in the thermostat. After a brief period for equilibration (about four minutes with constant agitation) an ampule was placed in ice-water and time was counted from this point. The aliquot was subsequently taken from the ampule and analyzed. In the runs at 0° the titrations were carried out at this same temperature and time was recorded at the end-point. In all cases aliquots were measured at the same temperature at which the solutions were made up or concentrations were corrected for thermal expansion of the solvent.

Figure 1 shows the number of points taken during a typical run and in a number of cases as many as 15 additional points were taken after 10 half-periods to study the increase in the infinity titer. The value of the infinity titer (A_∞) was determined directly from a point taken near 10 half-periods. If a point was not taken near 10 half-periods the value of (A_∞) was read from a smooth curve of a plot of A against time. From the rate of upward drift of the infinity titer it is clear that the amount of acid formed from the superimposed reaction is less than 1% of that formed by the primary process at 10 half-periods.

In a typical kinetic experiment (expt. 10) 4.069 g. (0.0148 mole) of perbenzoate was rapidly dissolved in methanol in a 100-ml. volumetric flask. The solution was made up to 100 ml. and thermostated at 20.11°. Five-ml. aliquots were delivered into a chilled flask at the time intervals indicated in Fig. 1 and analyzed. After obtaining a tentative value for A_∞, the specific rate constant k_1 was obtained from a plot of the type illustrated by Fig. 1. If the tentative A_∞ value corresponded to a time considerably different from 10 half-periods the A_∞ value was adjusted to correspond to 10 half-periods and the data recalculated.

Determination of the Ratio of Rearrangement to Solvolysis (k_2/k_3) for Decalyl Perbenzoate.—Each kinetic experiment provided the necessary data for calculating k_2/k_3 ratios by use of equation 7. The A_∞ values were taken at 10 half-periods. Additional experiments designed to determine this ratio are illustrated by the following example. *trans*-Decalyl perbenzoate (0.3669 g., 0.0132 mole) was added to 100 ml. of refluxing methanol. After 0.5 hour of reflux (estimated as 10 half-periods by extrapolation) the solution was cooled and analyzed for acid by titration. The total of 0.2956 millimole of acid produced corresponds to a k_2/k_3 ratio of 3.5.

Product Balance in the Rearrangement of Decalyl Perbenzoate in Methanol.—Refluxing 58.1 g. (0.212 mole) of pure decalyl perbenzoate (IIIa) in 600 ml. of methanol gave, after adding 200 ml. of water and cooling, 43.7 g. (0.160 mole) of rearranged benzoate (IVa). The mother liquors obtained after the crystalline ester had been collected were concentrated under reduced pressure to 400 ml. and then diluted with 200 ml. of cold water. This solution was extracted with ether several times and the combined ether extracts extracted with 200 ml. of 10% sodium hydroxide followed by water. After drying over magnesium sulfate and removing the ether 8.0 g. of a neutral oily material was obtained.

The basic extract above was acidified with concentrated hydrochloric acid and extracted with ether. Drying and removing the ether gave 4.8 g. (0.040 mole) of benzoic acid. Thus 95% of the starting material has been accounted for, 75.5% as rearranged ester and 18.9% as acid and solvolysis product based on the acid recovered.

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(29) L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., p. 256.

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

The Preparation and Properties of Human Serum Albumin Separated from Placental Extracts

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A procedure is described for the separation and purification of albumin from human placental extracts. Frozen placentas are thawed, chopped and extracted with isotonic saline. After the initial separation of a fraction containing γ-globulin, hemoglobin is separated from the albumin fraction by the addition of zinc and bicarbonate ions. Hematin, resulting from the breakdown of hemoglobin, is removed by filtration at *p*H 4.7 in the presence of sodium caprylate. The residual zinc concentration is reduced by an ion exchange resin and the placental albumin is concentrated and purified by the use of ethanol–water systems in the cold. The albumin is 95–97% pure by electrophoretic analysis and is low in salts and heme pigments.

Introduction

Placentas are a convenient, inexpensive and relatively unrecognized source for many human blood and tissue products, either as therapeutic biologicals or for investigative purposes. There is a mounting demand for immune serum globulin for prophylaxis against measles and poliomyelitis, and for albumin as a plasma substitute; placental sources for these products are large (3.6 million U. S. births annually) and merit consideration.

Placental plasma is approximately the equivalent of normal human plasma[1] in composition and placental extracts are essentially diluted placental plasma containing hemoglobin and tissue globulins. Placental extracts have been shown to contain diphtheria and scarlet fever antitoxins as well as poliomyelitis antibodies, and globulin from placental plasma can modify or prevent measles in the human.[2] Placental enzymes and hormones have

(1) R. M. Curtis and R. W. Worthington, Jr., *Am. J. Obstet. Gynecol.*, **42**, 428 (1941).

(2) Council on Pharmacy and Chemistry, *J. Am. Med. Assoc.*, **111**, 1764 (1938).

also been the subject of a number of investigations and postpartum plasma has been used for the treatment of rheumatoid arthritis.[3]

This paper describes a procedure applicable to large scale processing for the preparation from placental sources of a salt-poor, stable albumin, low in heme pigments, using the principles of the ethanol–water and metal cation methods of Cohn, *et al.*,[4,5] and Oncley, *et al.*[6]

Materials and Methods

Placental Extract.—The placentas were frozen and stored in polyethylene plastic bags at −30°, and thawed at 0–2° just before use. Plastic bags are ideal for collection and storage because they permit rapid heat transfer. The placentas were then chopped in a Hobart food cutter[7] with 15 g. of shredded filter paper per placenta. A meat chopper is preferable to a grinder, because it gives an even distribution of the filter pulp throughout the tissue thus providing channels for the extraction of fluid. The chopped placentas were then extracted with 0.75 l. of saline (0.8%) for one to two hours and the extracted protein was removed from the ground tissue with a Sweco separator.[8] Approximately 16 g. of plasma and tissue protein and 24 g. of hemoglobin were extracted per placenta by this method.

Ethanol Fractionation Techniques.—The general principles involved have been discussed elsewhere.[4,5]

Nitrogen Analysis.—Nitrogen was determined by a semi-micro Kjeldahl method similar to that of Ma and Zuazaga.[9]

Electrophoretic Analysis. The various fractions were analyzed with a Klett–Tiselius[10] apparatus. A diethylbarbiturate–citrate buffer of *p*H 8.6, ionic strength 0.1 was used on all but the final albumin preparations. These were done in diethylbarbiturate buffer as stipulated by the National Institutes of Health for normal serum albumin.

The methods for interpreting the photographs and the effects of the most important variables have been given by Armstrong.[11,12] When the solutions were highly colored, as were those containing hemoglobin, a composite of the descending and ascending limbs was used to give a complete pattern.

Hemoglobin.—Hemoglobin was determined by a method developed in this Laboratory by McCall[13] for plasma, in which the hemoglobin may be evaluated directly by the difference between the absorbency coefficients (optical density/cell thickness) of cyanmethemoglobin and of methemoglobin at the same wave length (540–550 mμ). This method is similar to that of Michel and Harris.[14]

Heme.—This method was kindly given us by Surgenor.[15] The final 25% solution of human serum albumin is diluted 1 to 25 with distilled water and read in the spectrophotometer in a 1-cm. cell at 403 mμ. The optical density is then multiplied by 25 to give $E_{1\text{ cm}}^{25\%}$ at 403 mμ. A light scattering blank, $E_{1\text{ cm}}^{25\%}$ of 0.25 is subtracted. The Commission on Plasma Fractionation[16] has established a maximum of 6.25 for the corrected $E_{1\text{ cm}}^{25\%}$. This corresponds to an approximate heme concentration of 4.80 mg./100 ml., calculated by multiplying the corrected $E_{1\text{ cm}}^{25\%}$ by 0.80. The factor of 0.8 is taken from the paper by Rosenfeld and Surgenor.[17]

Iron.—Iron was determined by the method of Kennedy.[18] Hydrogen peroxide rather than perchloric acid was used for the digestion of the sample.

Zinc.—Zinc was determined by a method developed in this Laboratory by Siders.[19] It is a modification of the method of Hoch and Vallee[20] and the alkaline titration procedure developed by Hughes.[21]

Sodium.—Sodium was determined in the final albumin preparations with a flame attachment for the model DU Beckman spectrophotometer.

Caprylate.—Caprylate was determined by the method of Hink and Johnson.[22]

Resin Column.—The carboxylic cation exchange resin XE-64[23] was used for the removal of zinc. It was prepared and used as follows: The resin was stored under 10% sodium carbonate solution to remove pyrogens which may have been adsorbed. The sodium carbonate was removed by successive washings and decantations and the resin titrated to *p*H 7.3 ± 0.1 with concentrated hydrochloric acid. The resin was placed in a column over a layer of coarse sand and backwashed with pyrogen-free water, then washed down with 0° pyrogen-free water with approximately 10 lb./sq. in. in order to pack the resin. One-quarter inch of water was left covering the resin and the column was cooled to 0–2°. Six to ten vol./resin vol. of 3% protein solution (precipitate C-Red-F) from which the zinc was to be removed was put through the resin at 3–6 vol./resin vol./hr. The protein was displaced from the resin column with pyrogen-free water. The column was washed down with 0.15 *M* sodium chloride (2 vol./resin vol.), and was regenerated by backwashing with 1 *M* sodium hydroxide (3 vol./resin vol.) with care not to disturb the stratification of the resin. In this way the zinc was removed from the top of the resin column without passing it over the remaining resin. The sodium hydroxide was then replaced by 10% sodium carbonate and the resin was stored until needed again.

System of Fractionation

Directions for the Preparation of Normal Serum Albumin (Human Placental).—The placental extract is first taken to 25% ethanol with 351 ml. of 95% ethanol (precooled to −10°)/kg. of extract. The addition rate is controlled so that the temperature is maintained at the freezing point. 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 30 l./hr. The supernatant from precipitate I, containing the bulk of the α-globulins and the albumin, is held at −5° and sufficient *p*H 4.0 acetate buffer[24] is added to bring the *p*H to 4.95 ± 0.05. Each liter of supernatant I requires approximately 3.2 ml. of *p*H 4.0 acetate buffer. Thirty ml. of 0.1 *M* zinc acetate in 25% ethanol (freshly prepared and cooled to −5°) per l. of original supernatant I is then added

(3) L. W. Granirer, *J. Am. Med. Assoc.*, **146**, 995 (1951).

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

(5) 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).

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

(7) Hobart Manufacturing Co., Troy, Ohio, Model 84141.

(8) We have employed a Model G-ID-5 18-inch Sweco separator with a 50-mesh stainless steel wire screen, available from Southwestern Engineering Co., 4800 Santa Fe Ave., Los Angeles 53, California.

(9) T. S. Ma and G. Zuazaga, *Ind. Eng. Chem., Anal. Ed.*, **14**, 280 (1942).

(10) Klett Manufacturing Co., 179 East 87th St., New York 28, N. Y.

(11) S. H. Armstrong, Jr., "Conventions of Routine Electrophoretic Analysis used in the Control of Plasma Fractionation," Univ. Lab. of Phys. Chem., Harvard Medical School, Boston, Mass.

(12) S. H. Armstrong, Jr., M. J. E. Budka and K. C. Morrison, THIS JOURNAL, **69**, 416 (1947).

(13) K. B. McCall, unpublished method, Michigan Department of Health.

(14) H. O. Michel and J. S. Harris, *J. Lab. Clin. Med.*, **25**, 445 (1940).

(15) D. M. Surgenor, personal communication.

(16) Commission on Plasma Fractionation and Related Processes, 25 Shattuck St., Boston 15, Mass.

(17) M. Rosenfeld and D. M. Surgenor, *J. Biol. Chem*, **183**, 663 (1950).

(18) R. P. Kennedy, *ibid.*, **74**, 385 (1927).

(19) C. D. Siders, unpublished method, Michigan Department of Health.

(20) F. L. Hoch and B. L. Vallee, *J. Biol. Chem.*, **181**, 295 (1949).

(21) Described by F. R. N. Gurd and D. S. Goodman, THIS JOURNAL, **74**, 670 (1952).

(22) J. F. Hink, Jr., and F. F. Johnson, *J. Am. Pharm. Assoc.*, **40** 537 (1951).

(23) XE-64 is fine ground IRC-50 (100–200 mesh), available from Rohm & Haas Co., Resinous Products Division, Washington Sq., Philadelphia 5, Pa.

(24) This *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 should have a *p*H of 4.0 ± 0.02 as measured with a glass electrode at 25°.

slowly with stirring. After the addition is complete the suspension is stirred for two hours at −5°.

Precipitate A is removed by centrifugation with a Sharples supercentrifuge at −5° at 30 l./hr. This precipitate is approximately 75% plasma protein and 25% hemoglobin. Each kg. of precipitate A is resuspended with a Waring type blender in 25 l. of 0.15 *M* sodium chloride at 0°. Care is taken to avoid air bubbles in the body of the solution and the suspension is stirred 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 ± 0.1 by the slow addition of approximately 50 ml. of 0.5 *M* sodium bicarbonate with stirring at 0°. The *p*H is checked and the suspension stirred for two hours.

Precipitate B is removed by centrifugation at 0° and will contain the bulk of the hemoglobin as well as the α- and β-globulins. After the suspension of 0.5% Hyflow in supernatant B, it is filtered at 0° through C-3 pads.[25] The filtrate, supernatant B-F-1, contains approximately 5 g. of protein per l., of which about 2% is hemoglobin pigment. To supernatant B-F-1 is added 0.831 g. of solid sodium caprylate per l. and after this dissolves completely, the solution is stirred for one hour. Then the *p*H is lowered to 4.75 ± 0.05 by the slow addition of approximately 10 ml. of *p*H 4.0 acetate buffer. The suspension is stirred for two hours, 0.5% standard Super-cel is added, and the suspension filtered through D-5 pads.[26] Standard Super-cel is added again and the suspension filtered through D-10-T pads. The filtrate, supernatant B-F-3, contains about 4 g./l. of plasma protein, of which about 0.8% is hemoglobin. Supernatant B-F-3 is stirred at −5° to allow the formation of ice crystals. Then sufficient solid citric acid is added to make the solution 0.004 *M*. Precooled 95% ethanol is added with stirring to give a final ethanol concentration of 40%. The temperature is kept at the freezing point and finally adjusted to −8°. The suspension is stirred for 4 hours at −8°.

Precipitate C is removed by centrifugation at −8° and may be stored at this temperature. This contains approximately 75% albumin, 25% α- and β-globulins and 0.5% hemoglobin.

Each kg. of precipitate C is resuspended in 8.35 l. of pyrogen-free water at 0°, stirred for one hour and 0.5% Hyflo added. The suspension is filtered through C-3 pads and the precipitate C-Red-F is titrated to *p*H 7.2 with approximately 90 ml. of 0.5 *M* sodium bicarbonate per l.

The solution is then put through a column of XE-64[27] at a maximum flow rate of 6 vol./resin vol./hr.; the maximum amount put through should not exceed 10 vol./resin vol. The effluent from the column will be referred to as solution C-R. If pyrogenic substances are present, they may be removed by treatment with Decalso.[28] In some of our first lots of placental albumin, the placentas were thawed at room temperature and extracted at 5–10°. The albumin lots from these were pyrogenic; but if the placentas are processed carefully and thawed and extracted at 0–2°, this step probably can be eliminated.

The *p*H of solution C-R is adjusted to 5.2 ± 0.05 with *p*H 4.0 acetate buffer (approximately 12 ml./l.). Then to each liter is added 1.5 l. of a mixture containing 564 ml. of 53.3% ethanol and 436 ml. of water per l. precooled to −5°. This is added very slowly (50 ml./jet/min.) and the final ethanol concentration is 18% at −5°. The solution is stirred gently for two hours and allowed to stand for six additional hours without stirring to complete the formation of the precipitate.

Precipitate D is then removed by centrifugation and contains α- and β-globulins as well as heme pigment and a green euglobulin. If desired for experimental purposes, the paste may be diluted with water, frozen and dried *in vacuo*.

Standard Super-cel (0.25%) is suspended in supernatant D and the suspension filtered through D-10-T pads. The ethanol concentration of the resulting supernatant D-F is raised to 40% by the addition of 400 ml. of 95% ethanol/l. The temperature is held at −5° during the ethanol addition, cooled to −8°, and the suspension is allowed to stand for eight hours to complete the formation of the precipitate.

Placental albumin is removed by centrifugation, diluted with 1.5 volumes of water at 0°, frozen and dried *in vacuo*.

Discussion

Hemoglobin Removal.—Alhough it is possible to express blood from unfrozen placentas and to separate a serum low in hemoglobin, requirements for storage and the necessity for preventing pyrogenic contamination make this impractical for large-scale processing. The freezing and thawing of the placentas prior to extraction hemolyzes the red cells and about 50% of the total protein in the placental extracts is hemoglobin.

A modification of methods 6[4] and 9[6] had been successfully used for the separation of immune serum globulin from hemolyzed placental extracts, but method 6 is unsatisfactory for the separation of hemoglobin from albumin because of the high solubility of hemoglobin in alcohol–water mixtures. Salt fractionation with sodium sulfate, treatment with activated charcoal, and the mineral oil–water emulsion of Elkes, *et al.*,[29] were also found to be unsatisfactory for the separation of albumin and hemoglobin.

Rawlinson[30] has shown that oxyhemoglobin can be precipitated by zinc sulfate, and more recently Cohn, *et al.*,[5] have used zinc for the fractionation of plasma and have developed methods for its subsequent removal with ion exchange resins.[31]

The hemoglobin content of the placental extract can be reduced from 46% to approximately 3% by the addition of zinc acetate before adjusting the *p*H to 7.2 with sodium bicarbonate. The relative precipitating effect of zinc on the total protein, albumin, γ-globulin and hemoglobin of the placental extracts is shown in Fig. 1. It is evident that the hemoglobin may be precipitated with little relative loss of albumin. However, approximately two-thirds of the immune globulin is precipitated with the hemoglobin. The loss of immune globulin during the precipitation of hemoglobin is avoided by first removing the globulin as precipitate I. This separation is almost quantitative, and leaves the albumin and hemoglobin in supernatant I (Table I).

A study of the best conditions for purification of the albumin in supernatant I is shown in Fig. 2 and suggests two possible alternatives: (1) the albumin may be precipitated at *p*H 5.0, leaving the hemoglobin in the supernatant; or (2) the hemoglobin may be precipitated at *p*H 7.2, leaving

(25) Filter pads purchased from Hercules Filter Corporation, Paterson 3, N. J. Approximately 50 sq. in. pad/l. suspension were used. C-7 pads have also been used for this step and may be preferable.

(26) Filter pads purchased from F. R. Horman and Co., Inc., 17 Stone St., Newark 4, N. J. D-10-T pads also have been used at this stage in conjunction with C-7 pads in the first stage, omitting the third filtration, and this combination may give equally efficient removal of heme pigments with a saving of filtration time.

(27) Small resin particles (100–200 mesh), a low flow rate (3–6 vol./resin vol./hr.), and the low concentration of residual sodium caprylate (ref. 15) all contribute to the removal of zinc. The final concentration of zinc for a 3% protein solution should be less than 4 μg./ml. This will give a level of about 20 μg. zinc/ml. in the final albumin preparation.

(28) W. E. Smith and R. B. Pennell (*J. Bact.*, **54**, 715 (1947)) have shown that the pyrogenicity of a variety of protein solutions may be reduced with a synthetic zeolite cation exchanger, Decalso (manufactured by the Permutit Co., 330 West 42nd St., New York 36, N. Y.). The resin was used as described in their paper (30 g. of washed Decalso/l. of solution C-R).

(29) J. J. Elkes, A. C. Frazer, J. H. Schulman and H. C. Stewart, *Proc. Roy. Soc.* (*London*), **A184**, 102 (1945).

(30) W. A. Rawlinson, *Australian J. Exptl. Biol. Med. Sci.*, **16**, 303 (1938).

(31) Minutes of the Formed Elements Meeting, Feb. 15, 1951, May 4, 1951, Univ. Lab. Phys. Chem., Harvard Med. School, Boston, Mass.

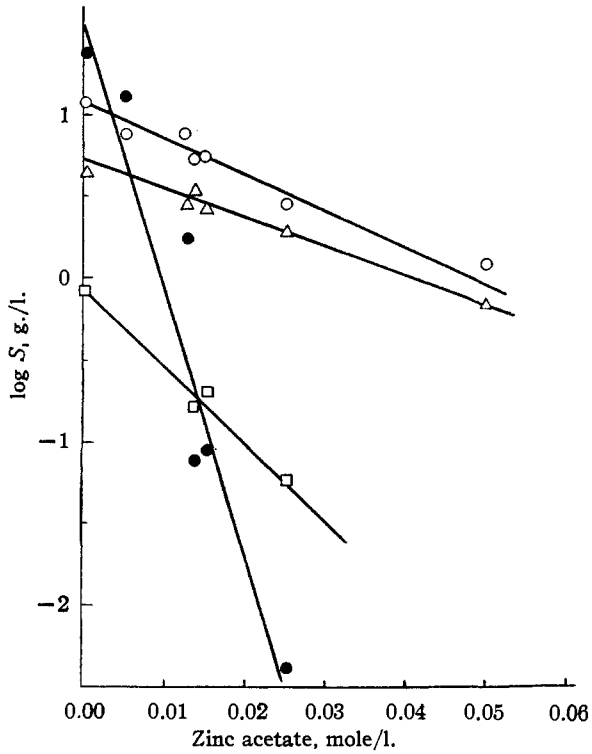

Fig. 1.—Effect of zinc acetate on the solubility of the proteins in placental extract, ionic strength 0.15, *p*H 7.2–7.5, temp. 5°: O, total plasma protein; ●, hemoglobin; Δ, albumin; □, γ-globulin.

TABLE I

DISTRIBUTION OF PLACENTAL PROTEINS IN FRACTIONS DURING PROCESSING

Fraction	Albumin	α	β+ Fibrinogen	Hb[c]	γ	Total
	Grams per 10 placentas recovered in fractions					
Extract	77	49	61	194	21	405
Supernatant I	75	49	16	187	0	327
Precipitate I	0	0	44	5.7	22	72
Supernatant A	7.9	23	14	148[a,b]	0	208
Precipitate A	54	18	4.1	26	1	104
Supernatant B	41	5.7	7.4	2.9	0	57
Precipitate B	3.3	9.8	6.1	28	0	47
Supernatant B-F-3[d]	32	3.6	4.4	0.3	0	40
Supernatant C	3.2	2.2	1.2	0	0	6.6
Precipitate C	23	3	4.5	0.15	0	30
Precipitate D	1	1.9	0.8	1.3	0.2	5.2
Supernatant albumin	1	0.02	0.03	0	0	1
Albumin	22	0.4	0.7	0.04	0	23

[a] Calculated from iron content. [b] Estimated from precipitate A values. [c] By cyanmethemoglobin method. [d] The difference between supernatant B and supernatant B-F-3 is the protein removed by filtration through D-5 and D-10-T pads.

the albumin in the supernatant. The albumin was separated at *p*H 4.95, as precipitate A since this precipitate could then be brought to conditions similar to those shown by Fig. 1 to be effective for the separation of hemoglobin from the original extract. Bicarbonate ion appears to be an essential factor in the action of zinc. In experiments with oxyhemoglobin, if bicarbonate is replaced by chloride, phosphate or tartrate, the hemoglobin is approximately ten times as soluble. Zinc carbonate probably selectively adsorbs the hemoglobin. The point of attachment of the zinc to the hemoglobin may be either with the imidazole

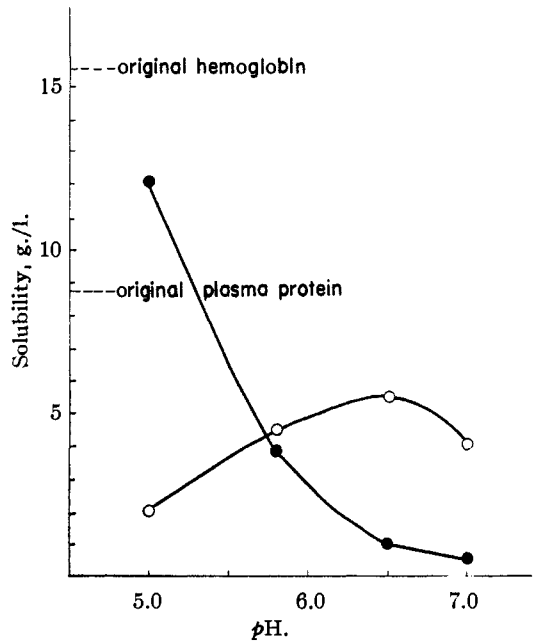

Fig. 2.—Solubility of total plasma proteins and hemoglobin in supernatant I, ionic strength 0.10, ethanol 25%, zinc acetate 0.005 *M*: O, plasma protein; ●, hemoglobin.

group[21] or with the carbamino groups formed by the combination of carbon dioxide and hemoglobin.[32]

Removal of Heme Pigments.—When the protein in supernatant B-F-1 was precipitated and recycled through the zinc step, or treated with resins, filter-pulp and charcoal, the pigment measured as hemoglobin was not reduced further. Most of the pigment at this stage is probably in the form of methemalbumin formed from the hematin produced at the low *p*H used in the removal of precipitate A. It has been shown by Bloch and Oelsner[33] that the rate of hematin production from hemoglobin is accelerated markedly at low *p*H.

Rosenfeld and Surgenor[17] have reported that spectrophotometric titrations at the 403 mμ absorption band of methemalbumin indicate that human serum albumin reacts with ferriprotoporphyrin IX to form protein–heme complexes in the molar ratio of 1:1 and 1:2. Between *p*H 7.2 and 10.0 the reaction was not affected by *p*H, specific ion effects, or moderate changes in ionic strength. Reversible dissociation of the complex occurs between *p*H 7.0 and 4.6; at *p*H 4.6 the complex is almost completely dissociated. Lemberg and Legge[34] state that methemalbumin is probably the only compound of biological importance in which the linkage is from the carboxylic side chains of the hematin to basic groups in the protein molecule. Most of the evidence for this is based on spectroscopic observations. The binding of caprylate to albumin has been studied by Boyer, *et al.*[35] The extent of this binding

(32) J. K. W. Ferguson, *J. Physiol.*, **88**, 40 (1936).

(33) E. Bloch and E. Oelsner, *Klin. Wochschr.*, **2**, 141 (1923).

(34) R. Lemberg and L. W. Legge, "Hematin Compounds and Bile Pigments," Interscience Publishers, Inc., New York, N. Y., 1949, p. 243.

(35) P. D. Boyer, G. A. Ballou and J. M. Luck, *J. Biol. Chem.*, **167**, 407 (1947).

increases as the *p*H is lowered over the range studied, with the solubility of caprylic acid as a limiting factor. From these observations, it appears that a low *p*H and the introduction of caprylate as a competing ion will dissociate the heme albumin complex and make it possible to filter out the freed heme aggregates.

When sodium caprylate (0.005 *M*) was added to supernatant B-F-1 and the *p*H reduced to 4.75, the solution turned black, probably because of the free hematin released. If this was followed by filtration through Horman D-5 and D-10-T pads[26] in succession it was possible to lower the heme pigment to 0.8% (measured as hemoglobin) as shown in Table I. The additional filtration with the very retentive D-10-T pads was necessary in order to accomplish the most effective removal of pigment, because of the colloidal nature of the hematin.[36] The effectiveness of this step may be seen in Fig. 3 by the marked change in the absorption spectrum before and after treatment.

The albumin is precipitated (precipitate C) to reduce the total volumes required and also the level of zinc. Originally this was done by adding zinc acetate to 0.005 *M* at 25% ethanol. However, insoluble zinc caprylate coprecipitated with the albumin. This made resolution difficult and resulted in high levels of zinc in the redissolved paste. In the present procedure, therefore, the ethanol concentration is raised to 40%, the *p*H remains constant, and citric acid is added to bind the zinc. This results in a marked reduction of zinc in precipitate C[37] and makes easier the subsequent removal of zinc by an ion exchange column.

Rework of Solution C-R.—After the zinc and the pyrogens, if any, have been removed, a further purification of the albumin is desirable. Solution C-R is about 90% albumin, 10% globulin (including globin) and about 0.3% hemoglobin.

TABLE II

ANALYTICAL DATA FOR SEVERAL PREPARATIONS OF NORMAL PLACENTAL ALBUMIN (HUMAN)

Rwk. no.	Prot., g./l.	Sodium, mg. %	Hb,[a] mg. %	Albumin, %	Heme, mg. %	Zinc, μg./ml.
1	248	275	52	95	6.68	20
2	306	300	46	95	7.20	23
3	271	270	56	95	8.60	21
4	250	305	12	97	2.92	12
5	248	252	73	95	19.0	12
6	247	209	29	88	5.08	23
7	246	198	12	98	1.76	13
NIH[b]	250m	330M	[c]	97m	4.80[d]	[e]

[a] By cyanmethemoglobin method. [b] Established maxima (M) and minima (m) of the National Institutes of Health for Normal Serum Albumin (Human). [c] No limits established. [d] An approximate limit of 4.80 mg. % heme for a 25% final solution of normal serum albumin has been established (see Materials and Methods). [e] No limits have been established; however, normal serum albumin prepared by method 6,[4] currently distributed by the Michigan Department of Health, has a mean value of 18 μg./ml. and a range of 9–34 μg./ml.

(36) J. Shack and W. M. Clark (*J. Biol. Chem.*, **171**, 143 (1947)) have shown from diffusion and ultracentrifuge data that heme does not form a true solution even in alkali, and that it is always present in the form of aggregates with a molecular weight as high as 50,000.

(37) The zinc is reduced from 23.2 mg./g. protein in supernatant B-F-3 to 3.25 mg./g. protein in precipitate C.

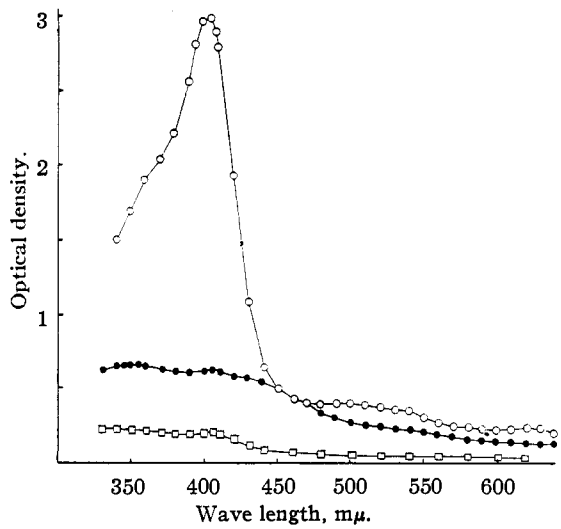

Fig. 3.—Spectrophotometric absorption curves for supernatants B-F-1, B-F-2 and B-F-3: O, supernatant B-F-1 (4.6 g. protein/l.); ●, supernatant B-F-2 (4.2 g. protein/l.); □, supernatant B-F-3 (3.9 g. protein/l.).

For this rework step a *p*H of 5.2 and an ethanol concentration of 15% was first used. After the removal of precipitate D (some of the heme pigment remains in this fraction, along with a green protein) by centrifugation and filtration through a D-10-T pad, a final albumin (rework no. 1, Table II) was obtained. The method was subsequently modified to give the procedure as included herein, by raising the ethanol concentration to 18%, which gave a more stable final albumin. After filtration through a D-10-T pad, the albumin was precipitated at 40% ethanol and *p*H 4.7. This resulted in the albumins of precipitate C rework No. 2 through 7 (Table II).

Placental Albumin

General Characteristics.—The protein recoveries in various fractions per 10 placentas are shown in Table I. The over-all recovery of the albumin is 2.2 g. per placenta or 29%. A summary of the conditions for the separation of the various fractions is presented in Table III.

TABLE III

CONDITIONS FOR THE SEPARATION OF VARIOUS FRACTIONS FROM PLACENTAL EXTRACTS

Fraction	*p*H	Temp., °C.	Zinc, mM.	Ethanol, %	Protein concn., %[a]
Supernatant I	6.6–6.9[b]	−5°	..	25	3.2.
Precipitate A	4.95	−5°	3	25	2.4
Supernatant B	7.2	0°	2	0	1.1
Supernatant B-F-3	4.75	0°	..[c]	0	0.5
Precipitate C	4.75	−8°	..[d]	40	0.2
Supernatant D	5.2	−5°	0	18	1.2
Albumin	5.2	−8°	0	40	0.6

[a] As observed. [b] Uncontrolled. [c] Sodium caprylate step 0.005 *M*. [d] Sodium citrate used at 0.004 *M*.

The zinc concentration, salt content, heme pigment (measured both as hemoglobin by the cyanmethemoglobin method[13] and as heme pigment by the Surgenor method[15]) and the electrophoretic purity of the placental albumin are given in Table

II. The National Institutes of Health has established criteria of purity for some of these properties and this information is included in the table.

Stability.—The final product meets National Institutes of Health requirements for stability when heated as a 25% solution and stabilized with either 0.04 *M* acetyl DL-tryptophanate or 0.02 *M* acetyl DL-tryptophanate and 0.02 *M* sodium caprylate. A study of the effect of added zinc on the stability of solutions of final albumin in 0.02 *M* tryptophanate plus 0.02 *M* caprylate showed no effect for amounts of zinc as high as 65 μg./ml. in tests lasting 144 hours at 57°.

Safety.—Safety tests[38] showed that the albumin had no demonstrable toxicity in the guinea pig or mouse, and in the pyrogen tests with rabbits, there were no toxic symptoms in any case other than a temperature rise with pyrogenic samples.

Zinc.—The data given in Table II indicate that the zinc levels in the placental albumin are well within the range of those occurring in the albumin now being distributed for human use. It appears from the review of Hegsted[39] that there is no danger of chronic toxicity resulting from these concentrations of zinc, since it can be absorbed and excreted in large quantities with no evidence of tissue damage.

Acknowledgment.—We wish to acknowledge the assistance of the staff of the Michigan Department of Health Plasma Fractionation Laboratory which included, besides the authors, F. C. Coryell, W. Gay, P. Jordan, A. Kennedy, G. Nonhof, Dr. O. Olson, L. Rasmussen, J. Regan and J. Sgouris. For assistance in the many analytical determinations and biological testing we are indebted to A. Burger, E. Carr, J. Collins, A. Forell, V. Ku, J. Mapes, T. Misenheimer, S. Mizuba, L. Roberts, H. Samaras and C. D. Siders.

(38) These tests were carried out in the manner prescribed by the National Institutes of Health for Normal Serum Albumin.

(39) D. M. Hegsted, J. M. McKibbin and C. K. Drinker, U. S. Pub. Health Repts., Suppl. No. 179 (1945).

LANSING, MICHIGAN

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE UPJOHN COMPANY]

The Isolation and Purification of Amicetin[1]

BY J. W. HINMAN, E. LOUIS CARON AND C. DEBOER

RECEIVED JULY 27, 1953

Amicetin, a new antitubercular antibiotic, is produced in deep culture fermentation by *Streptomyces vinaceus-drappus* on a soya meal-cerelose-yeast medium and extracted by a simple butanol process using a Podbielniak extractor. The antibiotic has been obtained in crystalline form as the free base and various salts. It has been characterized on the basis of its physical, chemical and biological properties.

Amicetin[2] is produced by a previously unidentified microörganism which has now been characterized and given the name *Streptomyces vinaceus-drappus*.[3,4] Representative yields of amicetin produced by *S. vinaceus-drappus* on a soya meal-cerelose-yeast medium in different sized fermenters are given in Table I. These yields are expressed in streptomycin units as measured by a disc-plate assay procedure using *Mycobacterium avium* as the test organism.[5] By this method pure streptomycin sulfate assays at 800 units per mg., and crystalline amicetin free base, at about 1000 units per mg.

TABLE I

Fermenter (capacity)	Vol. medium	Yield, *M. avium* units/ml.
Erlenmeyer flask (500 ml.)	100 ml.	350
Glass fermenter (5 gal.)	8 l.	300
Stainless steel tank (100 gal.)	75 gal.	250
Stainless steel tank (2000 gal.)	1500 gal.	250

The clarified fermentation liquor from the larger fermenters was conveniently extracted with about one-quarter its volume of 1-butanol at *p*H 8.5–9.5 using a Podbielniak extractor. The butanol solution was extracted with approximately one-quarter its volume of dilute sulfuric acid of such strength to give a final aqueous extract of *p*H 2. These operations were repeated until the aqueous volume was sufficiently small that when the solution was brought to *p*H 8.5 and seeded, amicetin crystallized as the hydrated free base.

The crude crystals so obtained were tan to white in color, had the appearance of matted needles, and melted at about 160–165°. Material of this quality assayed 850–950 *M. avium* units per mg. When examined by the countercurrent distribution method of Craig[6] in an all-glass machine using 60 to 65 transfers with a system of 1-butanol, di-*n*-butyl ether and water (2:1:3 by volume), material at this stage of purification was found to contain 70–90% of one component, depending somewhat on the quality of the starting fermentation liquor.

Further purification was accomplished by dissolving the antibiotic in dilute hydrochloric acid, stirring with activated carbon, filtering and reprecipitating the free base by adjusting the filtrate to *p*H 8–8.5. The slurry of hydrated needle crystals was heated with constant stirring to 60–65° to convert the antibiotic to the granular anhydrous crystal form which melted at 244–245°. The low-melting needle crystals could also be converted to the granular form by warming a methanol suspen-

(1) Presented before the Division of Medicinal Chemistry at the National Meeting of the American Chemical Society at Los Angeles, California, March 15–19, 1953.

(2) C. DeBoer, E. L. Caron and J. W. Hinman, THIS JOURNAL, **75**, 499 (1953).

(3) C. DeBoer, manuscript in preparation.

(4) In the Lilly Research Laboratories, amicetin is produced by an organism known as *Streptomyces fasciculatis*.

(5) Y. H. Loo, P. S. Skell, H. H. Thornberry, J. Ehrlich, J. M. McGuire, G. M. Savage and J. C. Sylvester, *J. Bact.*, **50**, 701 (1945).

(6) L. C. Craig and H. O. Post, *Anal. Chem.*, **21**, 500 (1949).