[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY AND NUTRITION, UNIVERSITY OF SOUTHERN CALIFORNIA]

The Isolation of Rat Plasma Albumin[1-3]

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Method 10 of Cohn and co-workers has been modified for application to rat plasma, yielding a fraction V which is 90–92% albumin, and which contains about 87% of the total plasma albumin. A method is described for the refractionation of Fraction V which yields albumin containing less than 2% of other components as judged by electrophoresis at *p*H 8.6.

Despite the extensive use of the rat as a laboratory animal, relatively little attention has been devoted to the fractionation of rat plasma or the isolation of albumin from this source. The methods previously described[4] have failed to provide a product of the purity and yields desired for characterization studies, or for studies of the metabolism of rat plasma albumin. Consequently, an investigation of the applicability of Method 10 of Cohn and co-workers[5] was undertaken, with the object of finding conditions in which fraction V would contain most of the albumin of the plasma in a reasonably pure form, and of then investigating the refractionation of fraction V to obtain albumin of a high degree of purity.

Table I gives the electrophoretic composition and distribution of total plasma protein between the fractions obtained when Method 10 for human plasma is applied to rat plasma. Only about 68% of the albumin is recovered in fraction V, and the contamination with α- and β-globulin is considerable.

TABLE I

ELECTROPHORETIC COMPOSITION AND PROTEIN CONTENT OF FRACTIONS

Fraction	I + II + III, %	IV, %	V, %	VI, %	Unaccounted for, %
	Original Method 10				
Albumin + α_1	..	..	71	50	
α_2	5	..	13	50	
β	28	..	16	..	
γ	50	..	...	..	
0	6	..	...	..	
Total plasma protein, %	21	22	49	4	4
	Modified Method 10				
Albumin + α_1	..	8	90	40	
α_2	5	60	0–1	60	
β	28	32	9–10		
γ	50	..	...	..	
0	6	..	...	..	
Total plasma protein, %	21	23	51	1	4

The loss of albumin in fraction VI was decreased by increasing the alcohol concentration in solution 2. The extraction of precipitate IV + V presented the major problem, and no conditions were found which would eliminate both α- and β-globulins from fraction V. However, the inclusion of zinc and a reduction in *p*H of the extracting solution was found to largely eliminate the α-globulin without an undue reduction in the amount of albumin extracted. The composition of the fractions is given in Table I, and an electrophoretic pattern of fraction V in Fig. 1.

Fraction V was prepared for refractionation by the removal of salts, in order that conditions would not be limited by the salts originally present. A solubility curve for the fraction in alcohol at various *p*H values showed the anticipated minimum at about *p*H 4.8, but the precipitate obtained at this *p*H in the presence of buffer salts at a concentration of 0.005 *M* or more was still considerably contaminated. Attempts to remove contaminating globulin at higher *p*H values were also unsuccessful because of losses of albumin. The most satisfactory procedure which has been found so far involves the precipitation of albumin with alcohol at a very low ionic strength but with the addition of a small amount of zinc. The fraction obtained appears to be quite homogeneous as judged by electrophoresis at *p*H 8.5, as shown in Fig. 2, but like other plasma albumins it is heterogeneous at lower *p*H's. Only about 60% of the albumin in fraction V is recovered as purified albumin.

It is self-evident that, lacking pure fractions for study, it is impossible to avoid being rather empirical. For example, the addition of zinc in the refractionation of fraction V was more empirical than rational, but it turns out that the amount employed would be just slightly in excess of that required to saturate the histidine residues in the albumin if the composition is similar to that of bovine and human albumin. Doubtless the fractionation and refractionation can be greatly improved, but the present methods should provide a useful basis for further study.

Experimental

Plasma.—Plasma was obtained by cardiac puncture from male rats of the U.S.C. strain weighing about 300 g. Ten ml. of blood was withdrawn into a syringe containing 2 ml. of a solution consisting of 26.9 g. of trisodium citrate (5.5 H_2O), 8.0 g. of citric acid and 22.0 g. of glucose per liter (designated A.C.D. solution).[6] The formed elements of the blood were separated from the plasma by centrifugation, and the plasma was subjected to fractionation within two hours. If only albumin is desired, stored or frozen plasma may be fractionated successfully.

(1) Supported by a research grant from the United States Public Health Service (H-1620).

(2) Taken in part from a thesis submitted by Alan Keltz to the Faculty of the University of Southern California in partial fulfillment of the requirements for the degree of Master of Science.

(3) Presented in part before the forty-fourth annual meeting of the American Society of Biological Chemists, April, 1953.

(4) E. C. Gjessing, S. Ludewig and A. Chanutin, *J. Biol. Chem.*, **170**, 551 (1947).

(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. Lui, D. Mittleman, R. F. Mouton, K. Schmid and E. Uroma, THIS JOURNAL, **72**, 465 (1950).

(6) Reference 5.

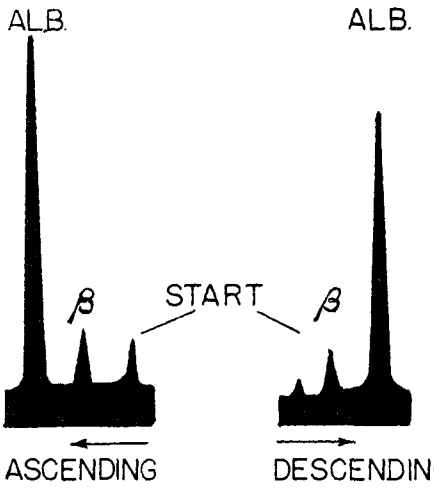

Fig. 1.—Electrophoretic patterns of fraction V from rat plasma; *p*H 8.5, ionic strength 0.1, Veronal buffer.

Some batches of plasma from female rats have been fractionated, and the fractions V were contaminated to a greater degree than those obtained from male rats, containing appreciable amounts of α- as well as β-globulins. Refractionation of fraction V obtained from this source appears to be equally effective, however.

Reagent Solutions.—Reagent solutions were freshly prepared for each fractionation from commercial 95% ethanol and C.P. reagents, and were cooled to −5° before use in the fractionation. The composition is given in Table II.

TABLE II

COMPOSITION OF FRACTIONATING SOLUTIONS

	95% EtOH, ml./l.	Buffer	Zn acetate, g./l.	Ba acetate, g./l.
Solution 1				
Original	250	2.5 ml. Na acetate acetic acid, *p*H 4.0, /2 = 0.8	..	..
Solution 2				
Original	200	....	54.8	..
Modified	270	....	54.8	..
Solution 3				
Original	160	20 ml. 1 *M* Na acetate 7.3 ml. 1 *M* acetic acid *p*H 5.5–5.6	..	2.6
Modified	270	20 ml. 1 *M* Na acetate 10*M* acetic acid to *p*H 4.7	2.0	3.8
Solution 4	230	CO_2 to *p*H 5.4		

Measurement of *p*H.—A Cambridge "Research Model" *p*H Meter, with a bulb-type glass electrode and saturated calomel half cell, was employed for all *p*H determinations. The instrument was standardized against phosphate reference buffer at *p*H 6.86 at 25° and all determinations were carried out on solutions at room temperature (25°). Solutions were *not* diluted before measurement, and the *p*H's are not strictly comparable with those specified by Cohn, *et al.*

Biuret Analysis.—Biuret determinations were standardized against Armour bovine serum albumin. Determinations were carried on aliquots of the original samples by adding an equal volume of a reagent containing 37.5 per cent. (v./v.) sodium hydroxide and 1.37% cupric chloride. It was necessary to substitute cupric chloride for the sulfate due to the presence of barium ion in some of the fractions. Optical densities of the reaction mixture were determined at 540 mμ with a Beckman Model B or DU Spectrophotometer.

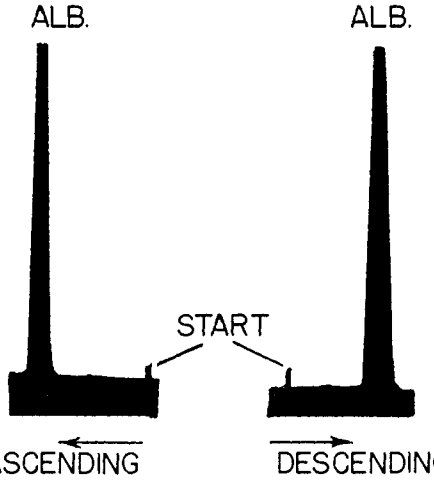

Fig. 2.—Electrophoretic patterns of refractionated fraction V from rat plasma; *p*H 8.5, ionic strength 0.1, Veronal buffer.

Electrophoresis.—Electrophoretic analysis of fractions has been carried out both by the moving boundary method of Tiselius and by zone electrophoresis on filter paper. The moving boundary measurements were carried out as described in previous papers from this Laboratory,[7] except that a 2.5-ml. cell was employed.

Zone electrophoresis was carried out by essentially the method of Tiselius and Kunkel,[8] except for the staining procedure. Strips were stained with Buffalo Black, as described by Grassman and Hannig[9] or by a phosphomolybdate method. In the latter procedure, the strips were immersed in phosphomolybdic acid, and the excess then removed by washing with 1:1 methanol–water acidified with acetic acid. The color was developed from the protein bound phosphomolybdate by reduction with a saturated aqueous solution of stannous chloride. The intensity of staining was determined on a strip photometer.

Fractionation.—The fractionation was carried out in 125-ml. lusteroid, centrifuge tubes, in a cold room at −5°. It was found necessary to place the tubes in an agitated bath of 20% ethylene glycol in order to adequately control the temperature of the tubes. Twenty-five ml. of A.C.D. plasma was placed in a centrifuge tube and allowed to cool to 0°, at which time 100 ml. of solution 1 was added at a rate not exceeding 20 ml. per minute, and with continuous stirring. Stirring was continued for 15 minutes, after which the precipitate was removed by centrifugation in an angle head at 4000 r.p.m. and at a temperature of −5° for 30 minutes. Precipitate I + II + III was found to contain essentially all the γ-globulin and fibrinogen and some β-globulin, and no modification of this step seemed necessary.

The supernatant, containing fractions IV + V + VI, was then transferred to a flask and 10 ml. of solution 2 was added with stirring. After 15 minutes this was centrifuged at 4000 r.p.m. for 30 minutes to remove the precipitate of fraction IV + V. It was found necessary to increase the alcohol concentration of solution 2 in order to prevent undue loss of albumin in supernatant VI.

Precipitate IV + V was suspended in 175 ml. of solution 3 and stirred for one hour before centrifuging at 4000 r.p.m. for 30 minutes. It will be noted that the composition of solution 3 has been modified by the inclusion of zinc and a reduction in *p*H in order to reduce the amount of α- and β-globulin in supernatant V to reasonable values without large losses of albumin in precipitate IV.

The original composition of solutions 1, 2 and 3 is given in Table I, together with the composition of these solutions in the present procedure.

Refractionation of Fraction V.—Fraction V was lyophilized after removal from the precipitate of fraction IV.

(7) J. W. Mehl, J. Humphrey and R. J. Winzler, *Proc. Soc. Expil. Biol. Med.*, **72**, 106 (1949).

(8) H. G. Kunkel and A. Tiselius, *J. Gen. Physiol.*, **35**, 89 (1951).

(9) W. Grassman and K. Hannig, *Hoppe-Seyler's Z. physiol. Chem.*, **290**, 1 (1952).

Our attention has been called to the danger of denaturation which accompanies the lyophilization of proteins in the presence of zinc and other metal ions,[10] and to the fact that the use of ion-exchange columns for the removal of zinc before lyophilization has been a standard practice in the Harvard laboratories for several years.[11,12] It is possible that some of the purification achieved in the refractionation is due to the denaturation of components other than albumin, but in general the elimination of any denaturation would be desirable, and advantages to be gained by such a modification of the procedure will be reported on in the future.

The lyophilized material was dissolved in a minimal volume of 0.2 *M* acetate buffer, *p*H 4.5, and dialyzed against 4 changes of the same buffer, followed by eight changes of distilled water. Since the zinc was not entirely removed by this procedure, the solution was then passed through a column of Dowex-50 in the hydrogen or sodium cycle. It is important for the success of refractionation that all of the zinc be removed, as indicated by a test with dithizone. The salt-free, zinc-free material was then lyophilized for storage.

Refractionation was carried out by dissolving 500 mg. of fraction V in 5 ml. of water, cooling to 0°, adding 25 ml. of a solution containing 230 ml. of 95% EtOH per liter, with stirring, and finally 0.5 ml. of solution 2. The additions were made in the −5° bath, and after one hour the precipitate was removed by centrifugation for 30 minutes at 4000 r.p.m. The alcohol solution must contain dissolved CO_2. When freshly distilled water is used, the *p*H of the 21.9% ethanol solution is adjusted to 5.4–5.5 at 25° by bubbling in CO_2.

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(10) Personal communication from Prof. John T. Edsall.

(11) W. L. Hughes, Jr., H. G. Psyras and W. C. Starr, in "The Separation of the Formed Elements, the Protein, Carbohydrate, Lipid, Steroid, Peptide and Other Components of Plasma," University Laboratory of Physical Chemistry, Harvard University, 1950.

(12) W. L. Hughes, *et al.*, in "Conference on New Mechanized Equipment for the Collection and Processing of Human Blood," University Laboratory of Physical Chemistry, Harvard University, 1951.

[CONTRIBUTION FROM THE RESEARCH DEPARTMENT, CALCO CHEMICAL DIVISION, AMERICAN CYANAMID COMPANY]

Analogs of Pteroylglutamic Acid. X. N^1-(Pteridyl-2)-sulfanilamides[1]

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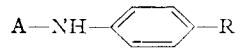
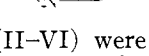
A number of N^4-acetyl-N^1-(substituted-pteridyl-2)-sulfanilamides have been prepared from N^4-acetyl-N^1-(4,5-diamino-6-hydroxypyrimidyl-2)-sulfanilamide by reaction with 1,2-diketones, diethyl oxalate and dichloroacetic acid. The N^4-acetyl group was hydrolyzed to yield the N^1-(substituted-pteridyl-2)-sulfanilamides.

It has been established that the sulfonamide drugs are antimetabolites for *p*-aminobenzoic acid (PABA) for many bacteria, and owe their therapeutic effectiveness to this fact.[3] The *p*-aminobenzoic acid moiety occurs in pteroylglutamic acid (I)[4] and it was logical to expect that the introduction of a *p*-aminobenzenesulfonamide grouping into I in the place of PABA might give interesting substances for biochemical studies, and possibly useful as antimetabolites of I. The first compound of this type to be reported was 4-(2-amino-4-hydroxypteridyl-6-methylamino)-benzenesulfonylglutamic acid (II), which was synthesized by Viscontini and Meier[5] by methods analogous to those used for I. Forrest and Walker subsequently prepared II also.[6] A somewhat similar compound, N^4-(2-amino-4-hydroxypteridyl-6-methyl)-sulfanilamide (III) was reported by Martin and Avakian,[7] and also by Sato, *et al.*[8] The same compound in pure form also was described by Forrest and Walker,[6] who were concerned with a theory of sulfonamide bacteriostasis which involved incorporation of sulfanilamide groups into biologically inert analogs of pteroic acid and pteroylglutamic acid. By the reaction of reductone with sulfanilamide, *p*-methylsulfonylaniline, sulfanilylglycine, diethyl sulfanilylglutamate and sulfadiazine, they obtained "reductone-anils" which were subsequently condensed with 2,4,5-triamino-6-hydroxypyrimidine to give pteridines of the general structures II–VI.



A = 2-amino-4-hydroxypteridyl-6-methyl-
I, R = $-CONHCH(COOH)CH_2CH_2COOH$
II, R = $-SO_2NHCH(COOH)CH_2CH_2COOH$
III, R = $-SO_2NH_2$
IV, R = $-SO_2CH_3$
V, R = $-SO_2NHCH_2COOH$
VI, R = $-SO_2NH$



These compounds (II–VI) were tested as antimetabolites of I for a strain of *Streptococcus lactis;* II and V inhibited growth and were reversed by I. All (II–VI) were inactive for the Walker rat carcinoma.

Three other related substances have been reported, namely, N^4-(2,4-diaminopteridyl-6-methyl)-sulfanilamide,[8] 4-(2,4-dihydroxypteridyl-6-methylamino)-benzenesulfonic acid[9] and sodium 4-(4-hydroxypteridyl-6-methylamino)-benzenesulfonate.[10] A number of derivatives of I bearing benzenesulfonyl groups on the 10-nitrogen have been synthesized by Weisblat and co-workers.[11]

Our work was undertaken with the purpose of preparing N^1-pteridylsulfanilamides analogous to Sulfadiazine and other chemotherapeutically important N^1-heterocyclic sulfanilamides. Compounds of the general structure VIII have been prepared.

(1) For the preceding paper in this series, see THIS JOURNAL, **75**, 4675 (1953).

(2) To whom inquiries regarding this paper should be addressed.

(3) For a review, see R. O. Roblin, Jr., *Chem. Revs.*, **38**, 263 (1946).

(4) R. B. Angier, *et al.*, *Science*, **103**, 667 (1946).

(5) M. Viscontini and J. Meier, *Helv. Chim. Acta*, **32**, 877 (1949).

(6) H. Forrest and J. Walker, *J. Chem. Soc.*, 2002 (1949).

(7) G. J. Martin and S. Avakian, U. S. Patent 2,476,557, July 19, 1949.

(8) Hideo Sato, *et al.*, *J. Chem. Soc. Japan*, **72**, 866 (1951); *C. A.*, **47**, 5946 (1953).

(9) H. Urist and G. J. Martin, U. S. Patent 2,504,471, April 8, 1950.

(10) D. J. Brown, *J. Chem. Soc.*, 1644 (1953).

(11) D. Weisblat, *et al.*, THIS JOURNAL, **75**, 3625 (1953).