Improved Isolation of Purified Siderophilin from Individual Sera.* (27910)

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Siderophilin(1), a major component of Fraction IV-4(2), has been crystallized in 95% purity, but the yield represented only a fraction of the starting material(3,4). In general, as the purity of siderophilin is increased, recovery decreases from 75% to 25%(3,4,5).

To study the kinetics of the iron binding and possible heterogeneity of individual siderophilins, maximal isolation of this β_1 globulin became necessary. The method to be described permits such an isolation from even small quantities of individual sera. The final preparation contains over 95% of the original iron binding globulin, contaminated with only 3% to 6% γ globulin.

Materials and methods. Sera. Healthy normal donors were phlebotomized into plastic bags† containing 25 ml of 1.5% disodium ethylenediamine tetra-acetate (EDTA). After centrifugation at low speed to obtain platelet rich plasma, clotting was effected by addition of an optimal quantity of 10% calcium chloride (0.45 ml/100 cc plasma) and the serum was recovered under sterile conditions after suitable defibrination. Such sera were stored frozen at –20°C if not used immediately.

The total iron binding capacity (TIBC) of the serum to be fractionated was determined (6). To achieve maximum binding of iron, 1.65 ml of 1% sodium bicarbonate per 100 ml of serum and 10% excess ferric ammonium citrate (50 μg Fe/ml)(6) containing 1 μc Fe^{59}/ml was added. The mixture was then incubated for 30 minutes and dialyzed until no radioactivity could be detected in the dialysate. Radioactivity was measured with a Picker well-type sodium iodide crystal, equipped with an automatic sample changer and recorder.

Serum proteins were precipitated at pH 9.4 using 3.5 volumes of 0.4% Rivanol‡ to each one volume of serum(7,8). The precipitate was removed by centrifugation and then washed twice with 0.05 M phosphate buffer, pH 8.0. The supernatant from the precipitation and the washings of the precipitate were dialyzed separately (to avoid formation of insoluble precipitates), against 7 changes of 0.02 molar phosphate buffer, pH 6.3, employing 100 volumes with each change. After dialysis of most of the Rivanol, the remaining pale yellow solutions were mixed and applied to carboxymethyl cellulose§ (CM) columns (1 g CM/1 ml serum) equilibrated with the same buffer. After application, the column was washed with 0.02 M phosphate buffer until the optical density of the effluent was less than 0.1. The CM bound proteins were then eluted with 1 M NaCl. The 0.02 M phosphate buffer eluate was then concentrated by lyophilization, dialyzed against 0.005 molar phosphate buffer, pH 8.0, and applied to diethyl aminoethyl cellulose (DEAE) column containing one g DEAE cellulose/2.5 ml of original serum. The column was washed with the initial buffer and then the siderophilin containing fraction was eluted with 0.04 molar phosphate buffer, pH 6.3. The proteins bound to the DEAE cellulose after elution of the siderophilin were recovered in 1 M sodium chloride. Optical density at 280 mμ served as a measure of protein concentration (Fig. 1a, b).

Each step of the isolation and purification procedure was examined by electrophoresis

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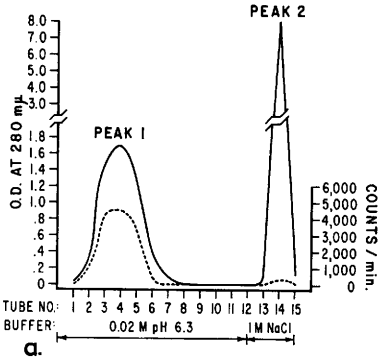
† JP-1 Platelet pack, Fenwal Laboratories, Inc., Framingham, Mass. 25 ml anticoagulant solution discarded immediately prior to phlebotomy.

‡ Ethodin Lot # N 480 TB distributed by Winthrop Laboratories, New York.

§ Selectacel reagent ion exchange cellulose #77 CM type 20, #70 DEAE type standard, Carl Schleicher and Schuell Co., Keene, N. H.



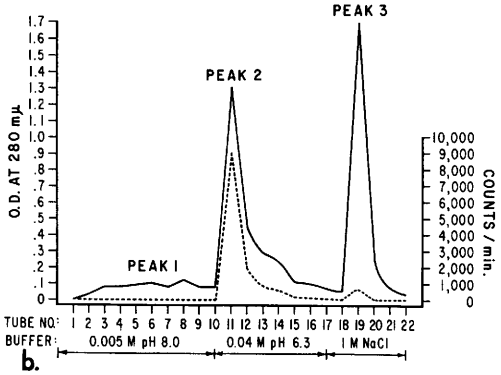


FIG. 1a. Chromatography of Rivanol supernatant. Rivanol supernatant of 2 ml serum equilibrated with 0.02 M phosphate buffer, pH 6.3 and applied to CM cellulose. 3 ml fractions collected at 6 ml/hr using the same buffer (peak 1), followed by 1 M NaCl (peak 2). Solid line represents absorbance at 280 mμ; broken line shows corresponding Fe^{59} radioactivity of the fractions. For protein distribution see Fig. 2 and 3.

FIG. 1b. Chromatography of peak 1 (tubes 1-8 Fig. 1a). Pooled fractions were lyophilized, equilibrated with 0.005 M phosphate buffer, pH 6.3, and applied to DEAE cellulose. 3 ml fractions collected at 6 ml/hr. Solid line represents absorbance at 280 mμ; broken line shows corresponding Fe^{59} radioactivity of the fractions. For protein distribution see Figs. 2 and 3.

on acrylamide(9), starch gel(10), and paper(11). Purity of the β_1 globulin was tested by immunoelectrophoresis(12).

Sedimentation analyses were carried out at 59,780 rpm in a Spinco model E ultracentrifuge equipped with phase plate and rotor temperature indicating and control unit. Sedimentation constants were calculated(13) and were corrected to 20°C with water as solvent.

γ globulin concentrations were estimated by double gel diffusion(14) employing anti-γ globulin serum obtained from rabbits that had been immunized with chromatographically (DEAE cellulose) isolated γ globulin.

TABLE I. Recovery of Protein Bound Fe^{59} after Precipitation with Rivanol.

No. of samples	ml serum precipitated	% loss (mean ± S.D.)
1	20	2.0
2	10	1.85 ± .21
7	2	2.85 ± 1.72

Results and discussion. Rivanol was found to precipitate about 25% of the added radioactive iron, but this was recovered by repeated washing of the precipitate with 0.05 M phosphate buffer, pH 8.0. Recoveries of protein bound Fe^{59} were found to be greater than 95% (Table I). The Rivanol supernatant combined with the washings of the precipitate was found to contain β and γ globulins and small quantities of α globulins and albumin (Fig. 2b, 3a). After separation on CM cellulose over 60% of the total protein applied (essentially γ globulin) was removed (Fig. 2d, 3c). Electrophoresis on acrylamide and immunoelectrophoresis now revealed only γ globulins having the electrophoretic mobility of β globulins, in addition to the previously described proteins (Fig. 2c, 3b). Subsequent chromatography on DEAE cellulose (see *Methods*) removed additional γ globulin (Fig. 3d and the α globulin and albumin contaminants (Fig. 2f, 3f). The β_1 globulin was now left contaminated only by γ globulin having an electrophoretic mobility of β globulin (Fig. 2e, 3e). The total pro-

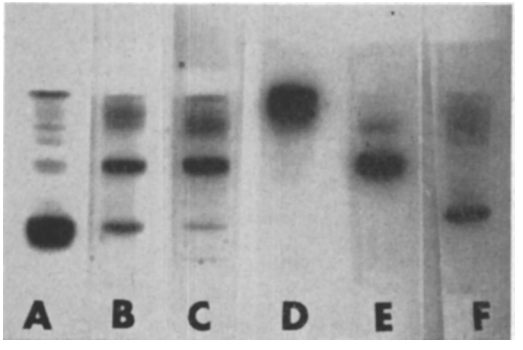


FIG. 2. Disc electrophoresis on acrylamide. A. Whole human serum. B. Rivanol supernatant. C-F. Chromatographic fractions pooled and concentrated by lyophilization: C. Peak 1, CM. D. Peak 2, CM. E. Peak 2, DEAE. F. Peak 3, DEAE.

TABLE II. Purification of Siderophilin.*

Sample No.	Serum, counts/min	Rivanol supernatant, counts/min	% loss	TIBC serum	TIBC after DEAE	% γ globulin	Ultracentrifugation mg protein/ml	Ultracentrifugation S^{w}_{20}
1	51,021	50,572	.9	276	265	3.3	2.48	5.26
2	32,901	32,726	.5	294	298	4.0	2.35	5.26
3	55,387	54,209	2.1	262	287	6.0	2.98	5.21
4	44,987	44,032	2.1	378	369	nd†	nd	nd
5	55,109	54,405	1.3	278	275	2.8	3.94	5.16
6‡							10.0	4.85

* 2 ml samples of serum used for each separation. † Not done. ‡ Pooled concentrate for ultracentrifugation.

tein in this fraction represented 15% of the starting material in the Rivanol supernatant. Data from representative experiments are given in Table II. The percent recovery of siderophilin in the Rivanol supernatant, as measured by Fe^{59} activity before and after Rivanol precipitation, was found to be in good agreement with the TIBC values obtained for whole serum and the isolated final product.

Ultracentrifugation of isolated transferrin revealed sedimentation coefficients in agreement with published values(5). Extrapolation to infinite dilution yielded an S value of 5.38 (Table II).

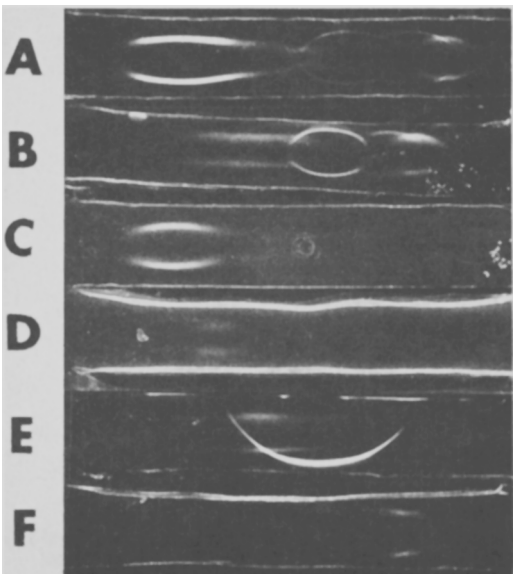


FIG. 3. Immunoelectrophoresis. A. Rivanol supernatant. B-F. Chromatographic fractions pooled and concentrated by lyophilization: B. Peak 1, CM. C. Peak 2, CM. D. Peak 1, DEAE. E. Peak 2, DEAE. F. Peak 3, DEAE.

Comparative analyses of serum, Cohn's Fraction IV-7-4, and Rivanol isolated siderophilin were identical immunologically, electrophoretically, and in iron binding properties.

Although the purity of the siderophilin is only slightly greater than that obtained by Koechlin(4), the yield has been increased to greater than 95%. Further purification of the siderophilin is in progress.

Summary. A method for isolation of over 95% of siderophilin from individual serum samples was described. The final product is contaminated with only small quantities of γ globulin of β mobility.

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Plasma Lactic Dehydrogenase-Elevating Agent of Mice: Effect on Levels of Additional Enzymes.* (27911)

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Studies by Riley *et al.*(1) on changes of lactic dehydrogenase (LDH) in the plasma of tumor-bearing mice uncovered a virus-like agent which is associated with a high proportion of transplantable tumors in mice. The LDH-agent is recognized by its ability to initiate a 5-10-fold increase in the plasma LDH of infected mice. The agent is filterable, nondialyzable and thermolabile(1). It is readily transmissible in mice and persists in the plasma indefinitely without causing overt disease(2). The term virus is used in this communication in an operational sense since information on the true nature of the LDH-agent is lacking. The relationship of the LDH-agent with most transplantable mouse tumors appears to be that of a common contaminant(3-7). The foregoing observations indicated the importance of ascertaining whether the LDH-agent is responsible for alterations in the levels of additional enzymes in the plasma of infected animals and in relating such changes to the effect of tumor development in the absence of virus.

Materials and methods. Female mice of strain CFW, a partially inbred line of Swiss Webster mice, were used. Animals 4-5 weeks of age were received at weekly intervals from a commercial source† to minimize transmission of the agent in the local animal quarters.

Sarcoma 180 (S180) cells freed from the LDH-agent by serial propagation *in vitro* were used(7). The LDH-agent used in this study was isolated originally from a mouse bearing a S180 and carried through 12 passages in primary mouse spleen cultures(7). Concentrations of virus were determined by bioassay(8).

Samples of plasma were obtained by decapitating 4-7 mice and collecting the blood in a tube containing 0.3 ml of heparin solution (100 units/ml). Tubes were placed in an ice bath until the erythrocytes were removed by centrifugation at 1200 $\times$ *g* for 10 minutes at 4°C. The plasma was stored at −17°C until tested. Plasma showing evidence of hemolysis was discarded.

When mouse tissues were assayed for enzyme activities the mice were killed by decapitation. Tissues were removed immediately, weighed and homogenized in 20 volumes (v/w) of cold 0.067 M phosphate buffer, pH 7.4 in a teflon pestle glass homogenizer. Homogenates were centrifuged at 1000 $\times$ *g* for 5 minutes and the supernatants were employed for enzymatic tests.

Various enzymes were assayed as follows. The activities of lactic dehydrogenase, malic dehydrogenase (MDH), glucose-6-phosphate dehydrogenase (G-6-PD), alpha-glycerophosphate dehydrogenase (GPD, isocitric dehydrogenase (ICD), and glutathione reductase (GR) were determined by measuring the rate of change of optical density at 340 mμ resulting from oxidation or reduction of the appropriate pyridine nucleotide coenzyme(9). The detailed conditions for individual enzyme assays are presented in Table I. The measurements were performed with a Zeiss M4 QII monochrometer attached to an automatic Gilford cuvette

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† Carworth Farms, New City, N. Y.