[CONTRIBUTION FROM THE UNIVERSITY OF TEXAS, M. D. ANDERSON HOSPITAL, RESEARCH AND TUMOR INSTITUTE]

Further Studies on Characterization of Human Serum Albumin by Means of Optical Rotation[1]

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Human serum albumin was isolated from fifty individual blood specimens by Cohn's method number ten, and the optical rotation of the albumin fractions was determined. Significant differences were found between the various albumin specimens in rotation values, as well as in the dependence of the specific rotation on *p*H. In many cases the albumins obtained from cancer patients had slightly lower optical activity than the albumins from normal subjects or from persons with non-malignant diseases. In several instances the albumin fractions which were obtained from the blood of tumor-bearing individuals possessed very low specific rotation values. Sedimentation diagrams of all the albumin samples including those with the lowest optical rotation showed only one peak. Electrophoresis on paper revealed small amounts of α- and β-globulins in the albumin fractions. Chemical tests showed that the low optical activity partly depends on the presence of glycoproteins, but it cannot be explained by this alone. The albumins themselves seem to be different in the various individual cases.

Introduction

The evidence is growing steadily that even the purest proteins are composed not of identical molecules but of closely related families of molecules.[2] Serum albumin, although homogeneous on the basis of sedimentation tests, is composed of a number of components,[3-6] and it is possible that both the ratio and constitution of these components may differ in various species, as well as in diseases.

The chief purpose of this study was to differentiate the albumins of normal subjects from those with cancer or other diseases. It was assumed that only slight differences in the constitution might be expected, and that the optical rotation method might be the most sensitive one in detecting such differences. This assumption was based on the finding that the optical activity changed in some cases after denaturation whereas viscosity and solubility remained the same.[7] There are also theoretical reasons to believe that the so-called "short range" differences in constitution will be reflected primarily in the optical activity.[8]

This study on optical rotation of blood proteins was started two years ago, and the first preliminary results showed that the optical activity of albumin and γ-globulin isolated from blood of cancer patients is somewhat lower than that of "normal" albumin and γ-globulin isolated by identical methods.[9] Differences were also found in viscosity of the albumin and γ-globulin obtained from various sources.[10] Interest in the possible diagnostic application of the findings was further stimulated by the fact that the albumins isolated from the blood of pregnant women behaved like the "normal" ones, whereas albumins from persons with multiple myeloma and Hodgkins disease also had low optical activity.[10] The possibility was investigated that the observed differences might be due to small variations in the isolation procedure which could conceivably lead to various amounts of impurities in the albumin fractions. Although it was found that the isolation procedure is reproducible,[10] some doubt about the role of the possible extraneous material prevailed. The albumin fractions were tested for purity by electrophoresis on paper. Initially, when a small amount of albumin was applied, no impurities could be found; however, the α- and β-globulin spots showed up when relatively large amounts (0.2 mg.) of albumin were put on paper. Quantitative estimates showed that the albumin fractions are 90–95% pure. These findings made it imperative to do more extensive studies on the extraneous material.

In this paper results obtained with fifty more albumin fractions are reported. The fractions were tested electrophoretically and by sedimentation in the ultracentrifuge. The chemical methods included tests for carbohydrates, lipids and phosphorus. Tests were also carried out for ash and some inorganic ions. As before, the dependency of the optical rotation on *p*H was determined, and significant differences were found between the albumin fractions. In a number of cases of albumin isolated from blood of cancer patients the optical activity was abnormally low. Some specimens with high optical activity also were encountered. Only individual (not pooled) plasmas were used in these experiments.

Experimental

Isolation of the Albumin.—Albumin was isolated from human plasma by the method of Cohn number ten, *i.e.*, fractionation with alcohol and heavy metal ions at low temperatures.[11] All the procedures of precipitation, washing and centrifugation were performed at −5°. The albumin solutions were treated with Nalcite HCR (cation-exchange resin obtained from the National Aluminate Corporation, Chicago) and dialyzed in the refrigerator at +4°. The completeness of dialysis was tested by means of conductivity measurements. Finally, the diluted albumin solutions were lyophilized.

Sedimentation in the Ultracentrifuge.—Twelve albumin specimens were spun in the Spinco analytical ultracentrifuge at 59,780 r.p.m. The *p*H of most of the solutions was 8.2–9.0, the concentration 0.4–0.9%, and the ionic strength of

(1) This study was supported in part by grants from the American Cancer Society and the National Cancer Institute, National Institutes of Health (C-1785). The results were presented in part at the American Chemical Society 126th National Meeting, New York, September 17, 1954.

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(3) T. L. McMeekin, THIS JOURNAL, **62**, 3393 (1940).

(3a) J. A. Luetscher, *ibid.*, **61**, 2888 (1939).

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(6) E. Brand, *Ann. N. Y. Acad. Sci.*, **47**, 187 (1946).

(7) B. Jirgensons, *Arch. Biochem. Biophys.*, **39**, 261 (1952).

(8) W. Kauzmann, Denaturation of Proteins and Enzymes in "The Mechanism of Enzyme Action," W. D. McElroy and B. Glass, editors, The J. Hopkins Press, Baltimore, 1954.

(9) B. Jirgensons and S. Sirotzky, THIS JOURNAL, **76**, 1367 (1954).

(10) B. Jirgensons and S. Sirotzky, *Arch. Biochem. Biophys.*, **52**, 400 (1954).

(11) E. J. Cohn, *et al.*, THIS JOURNAL, **72**, 465 (1950).

the glycine buffer was 0.05. The chief reason for these tests was to check the possible presence of any very fast or slow moving components. Some results are presented in Fig. 1. It is obvious that even the most abnormal albumins (with respect to optical rotation) show only one albumin peak.

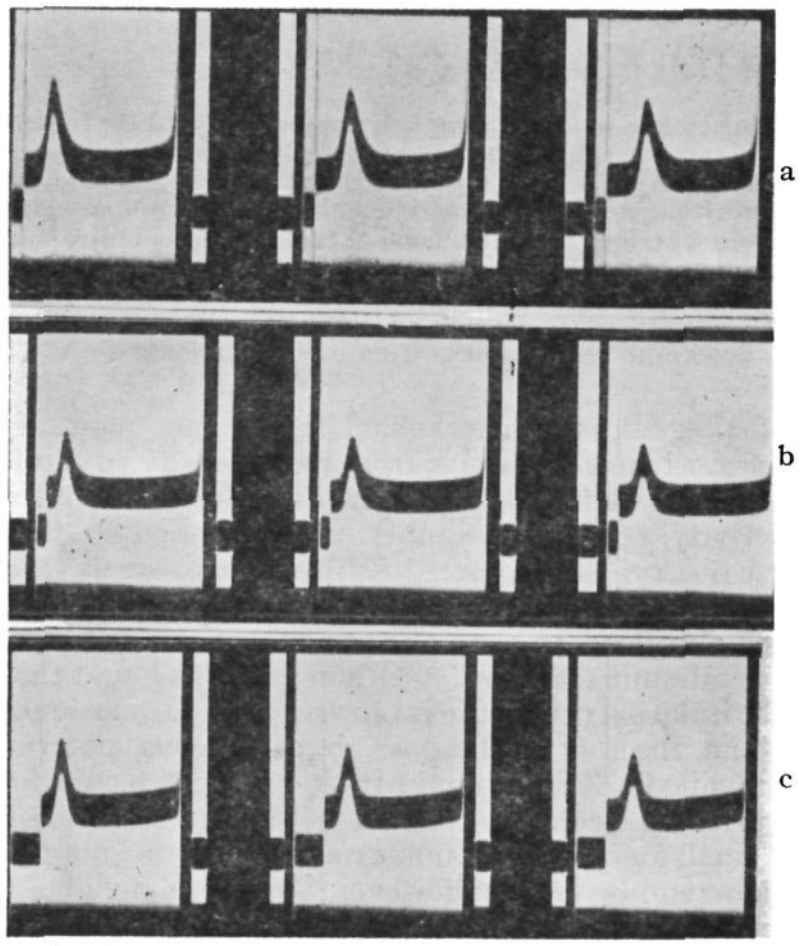

Fig. 1.—The sedimentation diagrams of albumin fractions (59,780 r.p.m., bar angle 60°): a, AC 31, 0.9%, in water, *p*H 5.0, 40, 48 and 56 min. after attaining full speed; b, AC 52, 0.45%, in water, *p*H 5.4; 32, 36 and 40 min. after attaining full speed; c, AC 60, 0.43%, in buffer, *p*H 8.2; 32, 36 and 40 min. after attaining full speed.

Electrophoresis on Paper.—The method of Kunkel and Tiselius[12] was used, with the following modifications. The paper strips were not squeezed between plates but they lay free on bakelite supports, and the top of the container was covered with a glass plate (the device of Bender & Hobein, Munich). The amount of albumin on the spots was 0.2 mg., the voltage 110 v., time 16–18 hours. The presence of α- and β-globulin spots was discernible on most of the strips. In order to determine the true values for the globulins, the amount of albumin adsorbed in the globulin sections of the strips was subtracted, and this amount was determined by making parallel runs with crystallized albumin. Mean values from many determinations were calculated, and they varied between seven to 10% of the total color for pure crystallized albumin. Because of these variations in the streak, and also because of the unequal adsorption of the brom phenol blue by albumin and the globulins, the method can be considered only as a semi-quantitative one. About thirty albumin fractions were analyzed in this way, and it was found that the albumins were 90–95% pure. However, in most cases the albumin fractions which possessed abnormally low optical activity had no more globulin impurities than the high-rotating fractions.

Chemical Tests of Extraneous Material.—Chemical tests for Zn and Ba were negative in all cases, and the ash content, which was tested in eight cases, was 0 to 1%. Fatty acids were extracted with methanol according to Cohn, *et al.*,[13] and cholesterol was determined after Bloor, *et al.*[14] The fatty acid content was found to be between 0.24–1.8%, being about 0.8% in most cases. The cholesterol content was 0.3–0.5%. There was no correlation between the optical activity and the variations in these impurities. The phosphorus content was determined after Gomori[15] in eight fractions, and only traces of P were found (0.02–0.05%). However, more significant variations were found in the amount of hexoses and hexosamine. Hexoses were determined by the orcinol reaction[16]; hexosamine was determined by the method of Elson and Morgan,[17] as adapted by Rimington.[18] A galactose–mannose standard was used for the hexoses, and a glucosamine standard was used for the hexosamine determinations. Most of the results are presented in Table I. It is obvious that the albumins isolated from "normal" blood contain less hexoses and less hexosamine than the albumins isolated from the plasmas of individuals with various diseases. The presence of carbohydrates indicates that the albumin fractions contain some glycoproteins and mucoproteins. Schmid isolated and crystallized a glycoprotein from human plasma,[19] and this protein contained 17.2% hexose and 11.5% hexosamine. However, the existence of various other glycoproteins in the albumin fractions is plausible. Examination of the table also shows that the abnormalities in optical rotation only partly depend on the carbohydrate content, as some specimens with high carbohydrate content show rather high optical activity.

Dependence of Optical Rotation on *p*H.—The optical activity was measured as before[9,10] with a precision polarimeter at 25 ± 1° using sodium light. Ten readings were made for each solution, and the variations are indicated by the vertical lines across each point in the graphs. The concentration of the albumin was determined gravimetrically after drying aliquots of aqueous solutions at 105–107°. The concentrations varied between 0.45 and 1.7%. The *p*H was varied by means of glycine buffers, the ionic strength being 0.05.

In Fig. 2 the two upper curves (circles) show the optical activity of albumins from the blood of tumor-bearing individuals, whereas the lower curve (crosses) represents the rotation *versus* *p*H dependence of crystallized human serum albumin obtained from Cutter Laboratories.[20] Noteworthy are two facts: first, the optical activity is not constant within the *p*H limits 4–10; second, the optical activity of the albumin fractions isolated from the plasma of cancer patients is lower than that of the normal albumin.

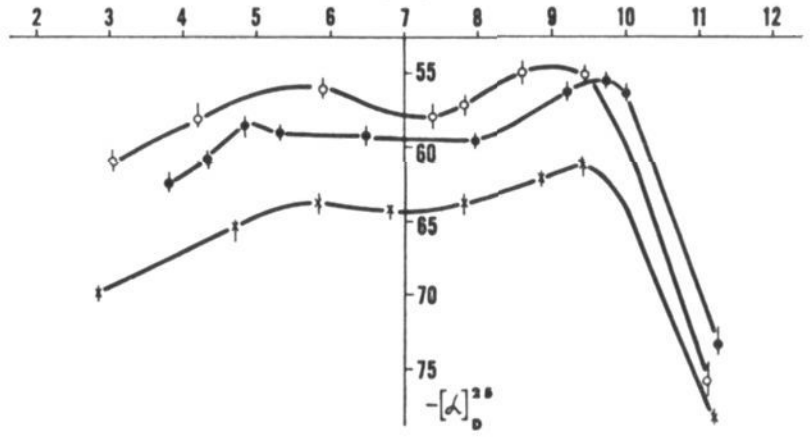

Fig. 2.—The dependence of the specific rotation on *p*H for various albumin specimens. Upper curve (open circles) represent the specimen AC40, 1.2% (cancer of uterus); the middle curve (discs) is that for AC42, 1.5% (cancer of cervix); the lower curve (crosses) shows the behavior of crystallized human serum albumin, 1.0%. The vertical lines through the appropriate points represent not the standard deviations but the maximum variations in the ten readings made in each case.

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(13) E. J. Cohn, W. L. Hughes and J. H. Weare, THIS JOURNAL, **69**, 1753 (1947).

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(19) K. Schmid, THIS JOURNAL, **75**, 60 (1953).

(20) The author is grateful to Dr. F. F. Johnson, associate director of research, Cutter Laboratories, Berkeley, California, for the protein specimens.

This is confirmed by the data of Fig. 3, which shows the optical activity of three "cancer albumin" specimens. The open circles represent the optical activity of the very abnormal specimen AC31 (cancer of the larynx); noteworthy is the surprisingly low optical activity of only 45–50°, and also the peak at *p*H 5. A similar case is that of AC52, an albumin isolated from the blood of a patient suffering from multiple myeloma.

Altogether eighty curves representing the dependence of the specific rotation on *p*H have been obtained, and great variety in the shapes of these rotation curves was observed. It seems that the curves can be classified in five or six general types. Three such types are represented by the three curves in Fig. 3. The common type showing constancy of optical activity in the *p*H limits 4–10, however, was observed only in about 10% of all cases, and both normal and "cancerous" albumins showed this type.

Optical Rotation and Carbohydrate Content of the Albumin Fractions.—The results are compiled in Table I.

TABLE I

OPTICAL ROTATION AND CARBOHYDRATE CONTENT OF ALBUMIN FRACTIONS

	Specimen	$-[\alpha]^{25}_D$ at *p*H 5	Hexose, %	Hexosamine, %	Glycoprot., %	$-[\alpha]_D$, calcd.
1	ANH97	62.3	0.37	0.24	2	59
2	ANW	60.2	.51	.30	3	59
3	AN15	59.7	.75	.26	3, 4	59
4	AN16	59.5	.69	.24	3	59
5	A54	59.6	.89	.54	5	58
6	A55	60.8	.99	.58	5	58
7	A62	58.8	1.35	.80	8	56
8	A68	59.6	0.82	.45	5	58
9	A70	58.4	1.12	.59	5, 6	57
10	A71	58.4	2.10	1.06	9, 12	55
11	A73	60.0	1.10	0.49	4, 6	57
12	A74	59.7	1.00	.53	5, 6	57
13	A75	59.8	1.00		6	57
14	AC32	54.4	0.92	.84	5, 7	57
15	AC33	54.2	1.72	.76	7, 10	56
16	AC35	58.4	.64	.52	4	58
17	AC36	56.5	1.57	.86	9	56
18	AC37	56.0	1.48	1.12	9	56
19	AC39	56.7	1.10	0.48	4, 6	57
20	AC40	55.8	0.93	.81	5, 7	57
21	AC42	58.2	1.22	.72	7	57
22	AC43	57.9	1.29	.58	5, 7	57
23	AC45	57.4	0.75	.48	4	58
24	AC46	58.6	.91	.66	5	58
25	AC48	58.8	.89	.72	5	58
26	AC51	58.0	.95	.66	5	58
27	AC53	57.9	1.10	.60	4, 6	58
28	AC57	58.2	0.69	.29	3	59
29	AC59	57.5	1.37	.47	4, 8	57
30	AC60	53.3	1.16	.72	5, 7	57
31	AC61	56.9	0.98	.50	5	58
32	AC63	55.8	1.22	.77	7	57
33	AC65	57.5	1.50	.70	6, 9	56
34	AC66	58.0	1.40	.72	6, 8	57
35	AC44	59.5	0.89	.78	5, 7	57
36	AC47	59.6	.64	.50	4	58
37	AC49	60.6	1.10	.75	6, 7	57
38	AC56	60.8	0.77	.62	4	58
39	AC58	60.4	.79	.41	4	58
40	AC69	59.3	1.58	.80	7, 9	56
41	AC31	45.5	1.11	.32	3, 6	58
42	AC50	45.8	0.77		4	58
43	AC52	47.4	1.16	1.20	7, 10	56

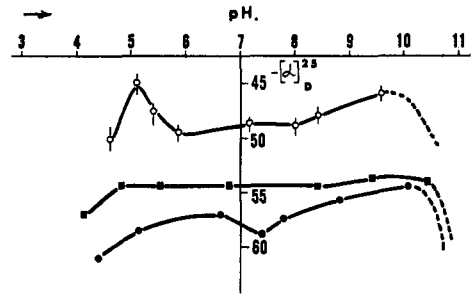

Fig. 3.—The optical rotation of albumin AC31, 1.2% (open circles), AC33, 0.60% (squares, gastro-intestinal cancer) and AC35, 0.95% (discs, cancer of pharynx).

There are five groups of albumins. Numbers one to four comprise normal albumins isolated by the method of Cohn. The specimen designated ANH 97 is an albumin obtained from the Harvard University Medical School and it is supposed to be a 97% pure albumin. The second group of albumins (no. 5–13) consists of albumin fractions isolated by the method of Cohn from the blood of patients with nonmalignant diseases. For example, A 71, which has a very high carbohydrate content, was isolated from the plasma of an individual with cirrhosis of the liver. The next largest group (no. 14–34) represents albumins from cancer patients. They all have somewhat lower specific rotation values than the normal albumins, and it is obvious that the lowering in the optical activity in this group can be explained by the presence of extraneous carbohydrate-containing proteins. The following group (no. 35–40) contains "cancerous" albumins which possess relatively high rotation values, although the carbohydrate content is higher than in the normals. The last small group (no. 41–44) comprises the very abnormal specimens. AC50 and AC52 here are albumin fractions from two patients with multiple myeloma. It is noteworthy that neither electrophoresis nor sedimentation in the ultracentrifuge revealed any particular abnormalities of these specimens (Fig. 1). The semi-quantitative determination of α- and β-globulins by means of paper electrophoresis gave 5% α- + β-globulins for AC31, 4% for AC50, and 9% for AC52. The high-rotating AC47 had 6% α- + β-globulins and AC49 9%. The glycoprotein content was calculated from the percentage of hexose and hexosamine, assuming that the glycoprotein contains 17% hexose and 11.5% hexosamine. When two numbers are given, the glycoprotein content differs depending upon whether it is calculated from the hexosamine or the hexose content. These latter cases indicate that there are indeed different glycoproteins in the albumins.

The last column contains specific rotation values which were calculated in the following way. It was assumed that the specific rotation of pure albumin at *p*H 5 is −62°, and that all of the fractions contain 3% of some optically inactive material; in addition to this the rotation contributed by the glycoprotein is then taken into account, assuming that its specific rotation is −20°. Such a computation, of course, can give only approximate results, because the composition and optical rotation of the glycoproteins contaminating the albumins is unknown.

Discussion

It was confirmed again that the curves representing the dependence of the specific rotation on *p*H differ considerably, depending on the specimen. The specific rotation in most cases is not constant in the *p*H limits 4–10 as assumed formerly, and found even in some recent work of other authors.[21] First, the levorotation begins to increase at *p*H 5 rather than *p*H 4. Second, the values at *p*H 6.5–8 are often more negative than at *p*H 5. Finally, in about 75% of all cases the lowest values are observed at the weakly alkaline *p*H range of 8.5–10. This pertains

(21) M. A. Golub and E. E. Pickett, *J. Polymer Sci.*, **13**, 427 (1954).

not only to the albumin fractions which are 90–95% pure but also to the purest crystallized serum albumin (Fig. 2). The particular curves are reproducible. It is impossible to explain all these differences, although it is plausible that three factors may be decisive: the presence of extraneous material, the variations in ionization on the surface of the albumin molecules, and differences in the constitution of the various specimens. At *p*H values below four (and above ten), a reversible expansion of the molecule takes place.[22]

The differences observed between the various specimens are remarkable. In about 70% of all cases the albumins from cancer patients showed small but significantly lower optical activity than normal albumin. However, in three instances the optical rotation of the "cancer albumins" was below −50°, and in five other cases it was just above −50°, which is surprisingly low in comparison with the ordinary values of −60 to −62° (Na light). However, a number of albumin specimens isolated from the blood of tumor-bearing individuals showed the same optical rotation as normal albumin.

The presence of extraneous material was the first factor suspected as being the cause for the abnormalities. As the amount of inorganic contaminants, phosphorus and lipids was too low to have a decisive influence the carbohydrate containing α- and β-globulins were considered. Table I shows that the albumin fractions from patients with cancer or other diseases contain more carbohydrates than the normal ones. These findings indicate that one factor which might be responsible for the abnormally low rotation is a relatively larger content of the low rotating glycoproteins in the albumin fractions. For example, the acid glycoprotein of Schmid[19] has a specific rotation value of only −20° (for Na light). If one assumes that a similar glycoprotein is the only extraneous carbohydrate-containing material in the albumin fractions, the content of it can be calculated from the percentage of hexose and hexosamine. It was found in this way (Table I) that the fractions might contain about 3–12% glycoprotein.

The important question now can be raised: can the optical rotation abnormalities be explained by the presence of low-rotating and optically inactive extraneous material, or are the albumins themselves different? There are two approaches to a solution of this problem. The first is a direct one: to determine the optical rotation of the plasma fractions very rich in glycoproteins and of mixtures of crystallized albumin with these fractions. It was found that the specific rotation of fraction IV-4, which is very rich in glycoproteins,[23] is −50°, and that mixtures with crystallized albumin containing 20% of this fraction had a rotation value of −60°.[10] Consequently, the carbohydrate-rich proteins alone seem to be unable to depress the optical activity to −50°. Another way to substantiate this is a simple calculation as presented in Table I. Let us assume that the specific rotation of pure albumin is −62°, that the fractions contain 3% of optically inactive material, and in addition a glycoprotein with the specific rotation of only −20°. If the glycoprotein accounts for 9% of the total fraction, the specific rotation should be −56°. Since this value is much higher than the values obtained for several of the fractions studied, it seems likely that the albumins *per se* are different. This is substantiated also by the other exceptions observed in the opposite direction. In seven cases the optical rotation of the albumin fractions at *p*H 5 was around −60°, although chemical determinations showed that they contained practically the same amount of carbohydrate and of inactive material as the low rotating specimens. Accordingly, the specific rotation should be about three degrees less than observed. Moreover, considerable differences were found even in pure, crystallized specimens of albumin, the limits so far observed in this Laboratory being −59.5 up to −64.5 at *p*H 5. If we assume the highest value of −64.5 for the "truly pure" albumin, the calculated values in the last column of the table then would be higher, and most of the "cancer albumins" then appear even more abnormal in respect to optical rotation.

Another possibility to consider would be that in the various individual cases there are different glycoprotein contaminants in the albumin fractions; one has to assume then that these various glycoproteins possess various optical rotation values, *i.e.*, in the cases of very low optical activity of the albumin fraction they should be dextrorotatory. Such an assumption, however, does not seem to be very plausible.

The views and data outlined support the conclusion that the chief reason for the optical activity differences of the various fractions are differences in the constitution and configuration of the albumins *per se*.

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(22) (a) J. F. Foster and J. T. Yang, THIS JOURNAL, **76**, 1015 (1954); (b) J. T. Yang and J. F. Foster, *ibid.*, **76**, 1588 (1954).

(23) The author is grateful to Dr. Oncley and the staff of the Harvard Medical School Laboratories for the donation of this and other protein specimens.