HUMAN PLASMA PROTEINS are known to vary both qualitatively and quantitatively from the normal in various acquired disease states. In addition, genetic alterations in plasma proteins of man are known; these have been summarized by Gitlin and Janeway.2

The present study resulted from the finding of a markedly abnormal serum protein pattern in a patient with Wegener’s granulomatosis. On paper electrophoresis, this was characterized by a large increase in a component with a mobility similar to α1-globulin and a concomitant decrease in the albumin fraction. This resembled the electrophoretic pattern described in the rare genetic defect, bisalbuminemia.3,6 In order to determine whether this finding represented a dysproteinemia characteristic of Wegener’s granulomatosis or was indeed another instance of bisalbuminemia, detailed studies of the patient’s serum and sera of his relatives were performed. The anomalous protein pattern in this subject and five members of his family was identified as bisalbuminemia; hence an additional family having this rare defect is available for study. In the course of attempts to characterize the two albumins in sera of affected members of the family, differences in their relative abilities to bind added 131-I-thyroxine were detected.

METHODS

Free electrophoresis of serum was carried out in a Perkin-Elmer instrument using barbital buffer pH 8.6, ionic strength 0.1.

Paper electrophoresis was carried out in a Duhrin-type cell (Spinco-procedure B) using barbital buffer pH 8.6, ionic strength 0.075.

Immunochemical studies were carried out by the agar-gel diffusion procedure of Ouchterlony.7 The serum proteins were separated by paper electrophoresis, using barbital buffer as described above. The protein areas of the air-dried unstained strips were located by cutting 0.8 cm. strips from each edge of the paper and staining with bromphenol blue. The unstained central segment was then aligned within the outer stained strips and the protein boundaries marked with pencil. The protein areas were cut from the paper, rolled on a curved forceps and placed into wells of agar plates containing 1 per cent agar in 0.05 M sodium phosphate buffer, pH 7.4, with Zepheran chloride 1:5000 as a preservative. The central well was filled with rabbit antiserum against human albumin; the peripheral wells with protein-containing paper strips were filled with 0.85 per cent saline. The plates were incubated at room temperature (22–24 C.) and were photographed after 48 hours of incubation.

Ultracentrifugal analysis was carried out in a Spinco ultracentrifuge (Model E) in 0.15 M saline at approximately 250,000 times gravity.

Measurement of the relative thyroxine binding abilities of the two albumins was carried

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A preliminary report of this work has been presented.1 Submitted Dec. 4, 1961; accepted for publication Apr. 17, 1962.
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Fig. 1.—Moving boundary electrophoretic patterns of double albumin containing serum. Protein concentration 1.5 per cent, barbital buffer pH 8.6, ionic strength 0.1, time 3800 sec., current 10 ma.

out as described by Robbins.8 Three different concentrations of $^{131}$-thyroxine (Abbott) were added to serum; the serum was incubated at room temperature for 15 minutes and then subjected to electrophoresis on paper in barbital buffer pH 8.6, ionic strength 0.075. Radioautographs of the air-dried unstained papers were prepared by placing the strips against Kodak “no screen” x-ray film for 48–72 hours prior to developing. The position of the paper with respect to the film was marked by punching holes through both paper and film so that a precise re-alignment of the subsequently stained strip and developed film could be achieved.

RESULTS

Free electrophoresis: The moving boundary electrophoretic pattern of serum from an affected individual (fig. 1) confirmed the previously observed paper electrophoretic pattern and demonstrates an albumin peak of reduced area but normal mobility, and a second large protein peak of approximately equal area with a mobility similar to normal human $\alpha_1$-globulin. This observation eliminates the possibility that the anomalous paper electrophoretic pattern was due to artefacts of the paper electrophoresis technic. The faster and slower moving albumin peaks have been designated A and B, respectively, following the nomenclature used by Earle et al.4

Agar-gel diffusion: Agar-gel diffusion of proteins A and B against rabbit antisera to human albumin (fig. 2, left) resulted in a single continuous line of precipitation of approximately equal density which did not cross throughout seven days of observation. In a second plate (fig. 2, right), rabbit antiserum gave a well defined single continuous precipitin line with albumins A and B from a different afflicted individual and with a normal human serum al-
Fig. 2.—Agar gel diffusion precipitin reactions of rabbit antihuman albumin serum (center well) against protein-containing paper strips (peripheral wells). Left: Albumins A and B (subject M. M.). Right: Albumins A and B (subject S. P.), serum albumin (N) and α₁-globulin area (α₁) from a normal subject.

bumin. A control paper strip of α₁-globulin from a normal serum, cut in the same manner as albumin B, did not reveal the presence of albumin. The purity of the rabbit antiserum to human albumin was determined by diffusing it against normal human serum and observing a single precipitin band. No evidence for albumin in protein fractions other than A and B could be detected by this technic.

Genealogical data: The sera of 18 members of both sides of the patient’s family were subjected to electrophoresis on paper. The trait was found only in members of the family related to the patient’s mother. The patient’s son, two sisters, mother and mother’s sister were found to be positive (fig. 3). The patient’s father and maternal grandmother were negative. We were unable to obtain serum from three of the mother’s eight siblings. Since unaffected members of the family have not been shown to pass the anomaly onto their children, it was assumed that the anomaly was inherited from the grandmother’s deceased husband. However, it was subsequently disclosed by the maternal grandmother that her two children with bisalbuminemia (C. P. and M. M.) had a different father. Hence, the anomaly is presumed to be derived from an unidentified male ancestor.

Serum protein concentrations: Those individuals having bisalbuminemia, with the exception of subject S. L. P. (table 1), showed no evidence for an association of this trait with any change in total serum protein, total albumin, or individual proteins other than albumin. The consistently higher percentage of protein in the albumin B fraction, as compared to albumin A, was best explained by the similar electrophoretic mobilities of α₁-globulin and albumin B; thus, both proteins contributed to the total in this fraction. When a normal
Fig. 3.—Genealogical tree of family with bisalbuminemia. Solid areas represent individuals having the anomaly. The asterisk refers to the patient with Wegener's syndrome.

value of $\alpha_1$-globulin is deducted from the observed total protein in the “albumin B” fraction, then the ratio of albumins A:B becomes 1:1. Electrophoretic analysis of the serum of subject S. L. P., seriously ill with Wegener’s syndrome, revealed a decreased albumin fraction, a marked increase in $\alpha$- and $\beta$-globulins, and a less marked but distinct increase in $\gamma$-globulin, when compared to normal subjects.

**Ultracentrifugation**: Comparative ultracentrifugal analysis of bisalbumin sera and normal sera (table 2), for as long as 90 minutes, revealed little or no difference in the sedimentation constants of the A, G, and M fractions. Only a single A-fraction (which contains albumin) was evident. The percentage of the total protein found in the globulin containing G-fraction of the bisalbumin serum from subject S. L. P. was found to be elevated above normal values, confirming the globulin increases shown by electrophoresis. The so-called A-fraction of normal sera consists of albumin and part of the $\alpha_1$ and $\beta_2$-globulins, and not albumin alone. The G-fraction consists of the $\gamma$-globulins, part of the $\alpha_2$-globulins and about half of the $\beta$-globulins. The M-fraction refers to the macroglobulins and contains part of the $\alpha_2$- and $\gamma$-globulins of very high molecular weight.

**Thyroxine and Neohydrin binding**: When small amounts of $^{131}$-thyroxine (0.75 $\mu$g per cent) were added to sera, the radioactivity was found almost exclusively in the inter-alpha zone of both normal and bisalbumin sera (fig. 4). When the $^{131}$-thyroxine concentration was increased to 7.5 $\mu$g per cent, the radioactivity was found in both the inter-alpha and the albumin zones, as described by Robbins and Rall. However, in bisalbumin sera, although thyroxine binding in both the inter-alpha and albumin zones occurred, the radioactivity was bound to albumin B rather than to the expected albumin A, which
Table 1.—Serum Protein Concentrations in Members of Family with Double Albumin

<table>
<thead>
<tr>
<th>SUBJECT</th>
<th>TOTAL SERUM PROTEIN (Biuret reaction)</th>
<th>TOTAL ALBUMIN (Methyl orange reaction)</th>
<th>SERUM PROTEIN FRACIONS (Paper electrophoresis)</th>
<th>ALBUMIN RATIOS (corrected for oil)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>Per cent Total Protein</td>
<td>A  B  C  D  E  F  G  H  I  J</td>
</tr>
<tr>
<td>S.L.P.</td>
<td>7.45</td>
<td>2.7</td>
<td>13.1</td>
<td>21.6 15.2 18.8 31.2 1.0 1.2</td>
</tr>
<tr>
<td>C.P.</td>
<td>7.22</td>
<td>4.4</td>
<td>19.6</td>
<td>27.4 12.2 13.2 27.6 1.0 1.1</td>
</tr>
<tr>
<td>P.P.</td>
<td>8.02</td>
<td>4.1</td>
<td>25.8</td>
<td>29.7 9.3 10.5 25.1 1.0 0.9</td>
</tr>
<tr>
<td>S.P.</td>
<td>7.50</td>
<td>3.7</td>
<td>25.0</td>
<td>30.6 14.8 13.9 15.7 1.0 1.0</td>
</tr>
<tr>
<td>L.P.</td>
<td>7.84</td>
<td>4.1</td>
<td>23.4</td>
<td>25.4 11.6 13.8 28.8 1.0 0.9</td>
</tr>
<tr>
<td>M.M.</td>
<td>6.85</td>
<td>4.7</td>
<td>24.3</td>
<td>30.2 10.9 12.6 21.9 1.0 1.0</td>
</tr>
<tr>
<td>Mean ± S.E.</td>
<td>7.46 ± 0.00</td>
<td>4.1 ± 0.1</td>
<td>23.4</td>
<td>28.6 11.8 12.8 23.4 1.0 1.0</td>
</tr>
</tbody>
</table>

Unaffected Members of Family (6) 7.20 ± 0.06 4.4 ± 0.3
Mean ± S.E. 7.44 ± 0.06 4.1 ± 0.1

migrates similarly to normal albumin. There is some evidence, based on somewhat less density in the radioautograph, that the relative lack of thyroxine binding by albumin A persisted even when the I\(^{131}\)-thyroxine concentration was raised to 75.0 \(\mu\)g per cent; however, this is equivocal. At this concentration of thyroxine, radioactivity was found associated with nearly all of the serum proteins in a nonspecific manner. The pre-albumin zone of both normal and bisalbumin sera appears to bind a small but comparable amount of radioactivity at this concentration of thyroxine. This marked selective affinity of albumin B for I\(^{131}\)-thyroxine relative to albumin A was observed in each of the six anomalous sera tested. When, on the other hand, a tracer amount of Hg\(^{203}\)-labeled Neohydrin* was added to the sera, this compound was found to bind to both albums A and B. Based on the relative densities of the film, however, there appeared to be slightly less binding of the Hg-labeled compound by albumin A than by albumin B.

**DISCUSSION**

The anomalous protein observed in this subject and in five of his relatives both by paper and moving boundary electrophoresis has been identified as albumin by immunochemical methods. The Ouchterlony agar diffusion tests with rabbit antisera against normal human serum albumin revealed that both albumins A and B reacted in a manner indistinguishable from normal human serum albumin. These observations prove the presence of albumin in protein fractions A and B and indicate their identity or very close antigenic relation to normal serum albumin. In control studies with normal human serum, no evidence for the presence of a second albumin with a mobility similar to \(\alpha_1\)-globulin was obtained. Thus an additional family having bisalbuminemia first

*3-chloromercuri-2-methoxypropyl urea.
Table 2.—Ultracentrifugal Analysis of Normal and Bisalbumin Sera*  
(Protein Diluted to 1.7 per cent with 0.9 per cent NaCl)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>A</th>
<th>G</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
| M. H. (normal) | Sedimentation constant ($S_{20}$) | 3.67S | 5.75S | 13.9S  
|          | Per cent total protein | 85.0 | 8.0 | 7.0  |
| S. L. P. (bisalbuminemia) | Sedimentation constant ($S_{20}$) | 3.42S | 5.63S | 13.9S  
|          | Per cent total protein | 81.0 | 15.0 | 4.0  |

* Determined by F. Wissler, Roswell Park Memorial Institute, Buffalo, N. Y.  
† Not corrected for radial dilution.

The genealogical data confirm the genetic transmission of bisalbuminemia during three generations. Since both the mother of the subject S. L. P. and her sister were carriers of the anomaly while the father was negative, the maternal grandparents must have transmitted the defect. The finding that the grandmother did not have the anomaly indicates that the source must have been the maternal grandfather, who, unfortunately, remains unidentified. Both the grandmother and her husband were Italian immigrants from the Naples area; however, no information regarding the origin of the grandfather of the affected children is available. These observations emphasize the potential pitfalls involved in all genealogical studies.

Of interest is the apparent 100 per cent incidence of bisalbuminemia in those exposed to the genetic risk in this family. Wieme has observed a similar high incidence.

With the exception of subject S. L. P., who was seriously ill with Wegener's granulomatosis, the presence of bisalbuminemia was not associated with any significant change in total serum proteins, total albumin, or serum components other than albumin. The ratios of albumins A:B were found to be 1:1 after the subtraction of a normal value for $\alpha_1$-globulin from the albumin B-fraction. These ratios were similar in each of the afflicted individuals of different ages, from three generations in this family. No evidence was obtained in other members of this family for an association of bisalbuminemia with any disease. The decreased albumin and increased $\alpha_1$, $\beta$-, and $\gamma$-globulin fractions revealed in sera of subject S. L. P. by serum electrophoresis confirms the findings of Budzilovich and Wilens and Linderholm and Nelsson for patients with Wegener's syndrome.

The ultracentrifugal data indicates that no differences in molecular weight exist between albumins A and B and normal serum albumin. Hence, the former are not split molecular products of normally occurring serum albumin. The elevated G-fraction observed in the sera of subject S. L. P. is a reflection of the increased globulin observed by serum electrophoretic analysis.

The electrophoretic, immunochemical, genealogical and ultracentrifugal evidence obtained in the present study is in agreement with that reported by other investigators and appears consistent with the conclusion that albumin A is identical to normal serum albumin and that albumin B is an anomalous
protein. However, the unexpected observations regarding the failure of albumin A to bind added $^{131}$-thyroxine leads to speculation that neither albumin A nor B are identical to normal human serum albumin. The lack of thyroxine binding by albumin A relative to albumin B could be ascribed to modifications in the amino acid sequence leading to the introduction or deletion of charged side-chain groups, similar to those reported by Gitlin et al.\textsuperscript{13} for albumin B. On the other hand, such charge differences and, hence, binding ability could reflect the manner of folding of the polypeptide chains of the albumin molecule to expose or mask charged groups in response to a configurational change.

It is equally conceivable that the amino acid sequence of albumin A is identical to normal human serum albumin and that other factors influence the thyroxine binding of albumin A in a specific or nonspecific manner. Klotz and Urquhart\textsuperscript{14} have reported that veronal buffer may seriously interfere with the capacities of various proteins to bind low-molecular weight organic compounds. Christensen\textsuperscript{15} has presented evidence that barbiturate is bound to serum proteins and can displace thyroxine from some of its binding sites. Since barbital buffer was used in the present investigation, it may be argued that this can explain the lack of binding of $^{131}$-thyroxine by albumin A. However,
STUDIES IN BISALBUMINEMIA

the observation that the albumin of control normal sera, studied simultaneously with bisalbumin sera under identical conditions, always bound added $^{131}$-thyroxine would rule out this explanation. If barbital does combine with thyroxine binding sites or displaces thyroxine from such sites, then albumin A must contain thyroxine binding sites which are more sensitive to barbital than either normal albumin or albumin B and, hence, differs from normal serum albumin.

The total serum iodine and protein bound iodine of the subjects L. P. and P. P were found to be 5.7; 5.2 μg. per cent and 5.2; 4.2 μg. per cent respectively. These data, which are within the normal range of 4–8 μg. per cent, does not support the possible explanation that the thyroxine binding sites of albumin A were previously saturated by an excessively high level of circulating unlabeled thyroxine in these subjects.

The difference between the binding affinities for thyroxine of these two albumins is a relatively specific one, since both albumins A and B bound added Hg$^{2+}$-Neohydrin, a compound known to bind to serum albumin.16

The presence of an inhibitor in bisalbumin sera which prevents the binding of added thyroxine to albumin A must also be considered. These questions are under current investigation. Whatever the explanation, our studies show that in this particular family, decreased thyroxine binding by albumin A in veronal buffer pH 8.6 is a hereditary trait associated with an abnormally slow migrating albumin B. At present this genetically linked defect cannot be attributed to a specific defect in thyroxine binding sites on the albumin A molecule. It is evident, however, that such information would have an important bearing on the question of whether lack of thyroxine binding by albumin A represents partial suppression of a normal allele by the presence of a single abnormal gene or whether the faster moving albumin represents the expression of a mutation at a second different gene site.

It would be of considerable interest to know if the thyroxine binding defect of albumin A is found associated with all cases of bisalbuminemia, or is restricted to this particular family.

Summary

1. An abnormal serum protein pattern in a patient with Wegener's granulomatosis and five of his relatives was identified as bisalbuminemia by electrophoretic and immunochemical methods.

2. With the exception of the patient with Wegener's syndrome, the presence of bisalbuminemia was not associated with a significant change in total serum proteins, total albumin, serum components other than albumin, or any disease.

3. Addition of $^{131}$-thyroxine to bisalbumin sera resulted in thyroxine binding by albumin B but not by albumin A. The failure of albumin A to bind added $^{131}$-thyroxine leads to speculation that, in this family, neither albumin A nor B are identical to normal human serum albumin.

Summary in Interlingua

1. Un anormal tableau de proteina seral in un paciente con granulomatose de Wegener e in cinque consangüineos de ille esseva identificate como bisalbuminemia per medio de methodos electrophoretic e immunochimic.
2. Con l'exception del patiente con le syndrome de Wegener, le presentia de bisalbuminemia non esseva associate con ulle alteration significative del total proteinas seral, del total albumina, del componentes del sero altere que albumina, o de ulle morbo.

3. Le addition de thryoxima marcate per $^{131}I$ a seros bisalbuminic resultava in le ligation de thyroxina per albumina B sed non per albumina A. Le facto que albumina A non ligava addite thyroxina a $^{131}I$ induce le speculation que in iste familia ni albumina A ni albumina B es identic con normal albumina de sero human.

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Studies in Bisalbuminemia: Binding Properties of the Two Albumins

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