Serum Protein Monitoring and Analysis with Ion-Selective Electrodes

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The highly sensitive response of the silver sulfide membrane electrode to silver ions and sulfur containing functional groups is utilized, in a novel manner, to provide direct potentiometric measurement of individual proteins and protein mixtures in serum. A procedure is developed to minimize protein poisoning of the electrode and to permit its long term use for protein measurements in isotonic saline solution. The results of studies on native and denatured proteins, deproteination of serum samples, and denaturation kinetics are reported and discussed in relation to possible applications in diagnostic medicine.

Clinical applications of ion selective membrane electrodes are becoming increasingly significant as evidenced by the large number of recent publications (*1*). The main areas of interest so far have been in enzyme analysis (*2–4*), alkali and alkaline earth metal ion analysis (*5*), and amino acid analysis (*6*). However, no methods have yet been published on the direct potentiometric determination of serum proteins using ion selective electrodes. Indirect titrimetric methods have recently been reported for the determination of thiols and disulfide groups in proteins by potentiometric titrations using solid membrane electrodes (*7–9*), by a silver metal electrode (*10*), and by coulometric titration (*11*), while amperometric and spectrophotometric methods have been reviewed in detail (*12, 13*). An earlier potentiometric method for thiols using a silver "thiol" electrode (*14–16*) proved to be unsuitable for measurements in protein solutions because of protein poisoning (*17*).

We therefore investigated the possibility of directly monitoring changes in protein concentration or structure under various reaction conditions and of measuring the rates of such reactions by potentiometry. This paper reports the results of studies using a silver sulfide membrane electrode for the direct determination of changes in protein concentration in blood serum. The method is based on the measurement of the free silver ion activity after silver mercaptide formation with sulfur groups in proteins. Applications are shown to be possible in areas of interest to clinical analysis including changes in total protein levels and albumin/globulin ratios in serum, the kinetics of protein denaturation, and the analysis of serum filtrates.

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EXPERIMENTAL

Apparatus. A Radiometer pH meter Model No. 26 was used for all pH and mV measurements which were carried out in an Orion microdish. The indicator electrode was a silver sulfide membrane electrode, fabricated by a procedure previously reported (*18*), and used with a double junction reference electrode (Orion Model 90-02-00). The indicator electrode showed Nernstian response to standard silver ion solutions in the concentration range of 10^{-2}–$10^{-6}M$.

Reagents. Silver nitrate of analytical reagent grade was used for preparation of a stock 0.01M silver ion and was standardized by potentiometric titration with 0.01M sodium chloride. The silver-blank solutions, to which protein solutions were subsequently added, were prepared as follows where silver ion was added last in order to prevent precipitation of silver chloride: *Blank A.* Silver nitrate ($6 \times 10^{-6}M$) in isotonic saline (0.9%) and borax buffer (0.015M boric acid and 0.00375M sodium borate) at pH 8.4; *Blank B.* Silver nitrate ($6 \times 10^{-6}M$) in a solution of sodium hydroxide (1M) and isotonic saline (0.9%).

Proteins were obtained from the following sources: bovine serum albumin, Cohn Fraction V, from Sigma Chemical Co.; human serum albumin from Nutritional Biochemicals Corporation; human α-globulin, Cohn Fraction III, and human γ-globulin, Cohn Fraction II, purity >99% by electrophoresis from Calbiochem. A standard reference solution of human albumin was obtained from Miles Laboratories, Inc., lot No. 17/A8931 containing 10.3 gram % protein concentration by Kjeldahl and 10.9% by the biuret method, and chloride (8.4 mg/ml). Whole human serum was either Technicon reference serum, lot No. B2C061, containing 6.6 gram % total protein and 4.2 gram % albumin, or Calbiochem Caltrol-340 Mixpack, lot No. 13042, containing 6.4 gram % total protein by the biuret method.

Electrode Conditioning. The silver ion response of the indicator electrode was found to be dependent on conditions of prior exposure to protein solutions. Immediately after polishing the electrode surface, the potential was very slow to reach a steady value for silver ion in protein solutions. However, by conditioning the electrode in a solution of Blank A containing 10 mg/ml albumin with efficient stirring for 48 hr, the response was improved significantly, as shown in the Result Section.

Procedures. For each protein, stock solutions (*ca.* 50 mg/ml) were freshly prepared before use by dissolving the appropriate protein in isotonic saline solution with gentle stirring. The solutions were standardized by the biuret method (*19*) using the standard 10 gram % human serum albumin as reference.

For the study of native protein solutions, all potentiometric measurements were made in isotonic saline solutions buffered at pH 8.4. To 1.0 ml of Blank A was added 1–100 μl of the appropriate protein solution, keeping the total volume constant at 1.50 ml by addition of isotonic saline. This gave a final total Ag^+ concentration of $4 \times 10^{-6}M$ in 0.01M borax buffer and isotonic saline. Potential readings were taken exactly 100 sec after the electrodes were immersed in the solution contained in the Orion microdish with continuous magnetic stirring using a small glass-encased metal bar of size approximately 100 mm × 1 mm.

Alkaline denaturation was carried out in the following way:

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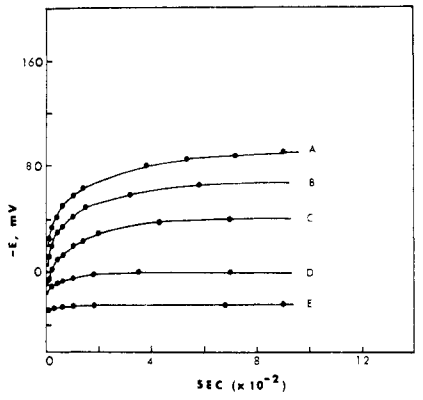

Figure 1. Electrode response times in protein solutions

Solutions of bovine serum albumin in Blank A for concentration: (*A*) 2.0, (*B*) 1.4, (*C*) 0.6, (*D*) 0.4, (*E*) 0.2 mg protein/ml

to 0.3 ml of a solution of NaOH (1.0*M*) and isotonic saline (0.9%) in a 3-ml centrifuge tube was added 1–100 μl of the appropriate protein solution, again maintaining constant total volume at 1.50 ml by addition of isotonic saline. The solutions were allowed to stand at room temperature for 2 hr and then refrigerated overnight for a total of 24 hr. A 1.0-ml quantity of Blank A was then added, giving a final pH 11.8. The solutions were finally transferred to the microdish and the potential was measured 100 sec after immersion of the electrodes.

The kinetics of the alkaline denaturation process were monitored immediately after the addition of protein solution (1–100 μl) to Blank B (1.0 ml), keeping total volume constant at 1.10 ml by addition of isotonic saline. Potentials were plotted as a function of time from the point of addition of the protein to the alkaline blank.

Analysis of serum filtrates was carried out in the following way: to 1.0 ml of Blank B in a 3-ml centrifuge tube serum was added in the range 10–100 μl, followed by isotonic saline to maintain constant volume at 1.10 ml. After standing for 45 min, 1.0 ml of a solution 20% in sulfosalicylic acid and 0.9% in saline was added and the solution was well stirred. The resulting precipitate was centrifuged and the potential of the clear supernatant was measured 100 sec after immersion of the electrodes in the test solution. Potentials were plotted as a function of total protein in the original test solution.

It should be noted that in no instance was any special precaution taken to maintain air-free conditions. This point is discussed in more detail in the Result Section. All measurements were made in the temperature range 25 ± 1 °C.

RESULTS

The simple method described for membrane conditioning has allowed the use of the silver electrode in a number of applications of direct potentiometry to the analysis of protein solutions under various reaction conditions, potentially of interest in both fundamental and clinical studies. However, reproducible potential measurements in protein solutions were obtained only by adhering to the definite procedure discussed below.

Electrode Response Times. After conditioning the electrode, the response to changes in pAg on altering the total protein concentration became very much faster. Typical response times are shown in Figure 1 indicating an exponential approach to a steady potential at a given native protein concentration.

The procedure for measurement of silver ion potentials consisted of: first, measurement of the potential at exactly 100 sec after immersion of the electrode in the test solution and, second, measurement for Blank A after each test reading, prior to analysis of the next test solution. The second step was found to be necessary to reproduce the potential readings, otherwise the potentials for consecutive readings of a series of samples were dependent on the potential taken just prior to the solution being tested.

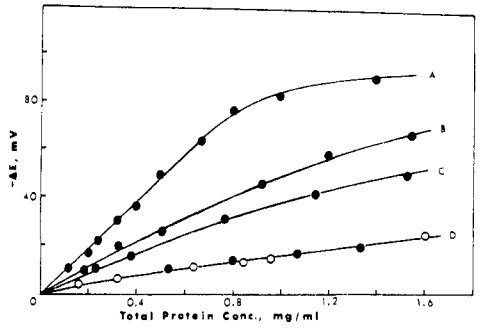

Figure 2. Calibration of native proteins in Blank A at pH 8.4

(*A*) Bovine albumin, (*B*) Human serum, (*C*) Human albumin, (*D*) Human globulins: α-globulin (○), γ-globulin (●)

Table I. Reproducibility of Ag-Potentials in BSA Solutions[a]

[BSA] = 4.0 mg/ml			[BSA] = 1.0 mg/ml		
$-E_{Ag}$	E_{blank}	$-\Delta E$	$-E_{Ag}$	E_{blank}	$-\Delta E$
48	61	109	26	58	84
48	60	108	25	58	83
50	60	110	19	58	77
49	59	108	21	58	79
50	54	104			

[a] $[Ag^+]_{Total} = 4 \times 10^{-6}M$, [Borax Buffer] = 0.01*M*, [NaCl] = 0.9%.

Table II. Effect of Silver Ion Concentration on Ag-Potential in Solutions of BSA[a]

$[Ag^+]_{Total}$	E_{Ag}
$0.1 \times 10^{-5}M$	−10
0.4	−51
0.6	−50
1.0	−27

[a] $[BSA]_{Total}$ = 2 mg/ml, [NaCl] = 0.9%, [Borax Buffer] = 0.01*M*.

This effect is typical of protein poisoning of electrode membranes and has been observed previously (5) for a calcium ion liquid membrane electrode.

Even after membrane conditioning, a very slow drift in blank potentials occurred over a period of 12 weeks, during which time the electrode was in continuous use with protein solutions without having been repolished. However, the potential differences between Blank A and test protein solutions remained constant and were reproducible. Typical data for reproducibility are given in Table I.

The effect of varying total silver ion concentration in buffered isotonic saline solutions of proteins was also investigated over the small range possible. At concentrations greater than $1 \times 10^{-5}M$, silver chloride precipitate was visible. At concentrations less than $1 \times 10^{-6}M$, it is well known (5) that the electrode response is no longer Nernstian. In the range $1 \times 10^{-6}M$ to $1 \times 10^{-5}M$ silver ion, no visible precipitation occurred if Ag^+ was added last to the buffered saline solution. When bovine serum albumin (BSA) was at a concentration of 2 mg/ml in the buffered silver ion solution, the largest change in potential was observed with the silver ion concentration in the range $4 \times 10^{-6}M$ to $6 \times 10^{-6}M$, as shown in Table II.

Native Protein Calibration. The extent of silver ion binding by proteins was investigated using a solution of silver ion at $4 \times 10^{-6}M$ in buffered saline at pH 8.4. Figure 2 shows potentials as a function of total protein concentration for various protein solutions, indicating appreciable changes in potential with increasing protein con-

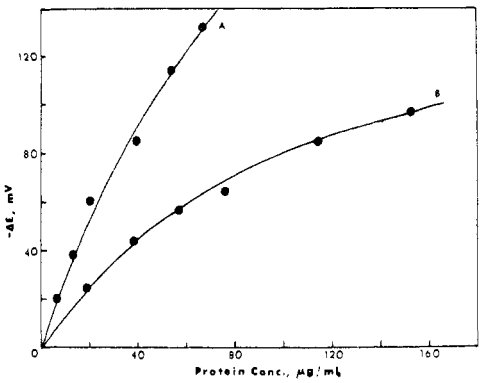

Figure 3. Calibration of proteins after alkaline denaturation for 24 hr

(*A*) Human albumin, (*B*) Human γ-globulin

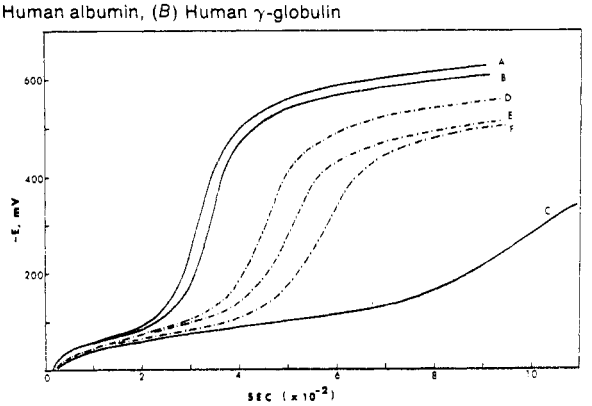

Figure 4. Rate of alkaline denaturation of human proteins

Concentrations of proteins in mg/ml in solutions of Blank B. (*A*) Total 6.0, Alb/Glob 5.0, (*B*) Albumin 5.0, (*C*) γ-globulin 1.0, (*D*) Total 6.0, Alb/Glob 0.2, (*E*) γ-Globulin 5.0, (*F*) Albumin 1.0

centration even in the presence of a large excess of chloride. For BSA, the potential change was approximately linear over a 10-fold change in protein concentration, but thereafter became independent of further changes in BSA concentration. Similar results were observed with the other proteins given in Figure 2 but the slopes were considerably lower, indicating a lesser extent of silver ion-binding than by BSA.

Human serum gave an effect similar to the individual proteins, the potential change being approximately as expected from the sum of the changes for each major protein constituent of the serum. The effect therefore was additive and must be attributed to total serum protein binding of silver ion and not to changes in [Cl^-] since constant chloride concentration was maintained in the test solution as the serum volume was altered.

Alkaline Denaturation of Proteins. The potential changes observed for the native proteins were quite small, particularly for the globulins. To obtain larger potential changes and, hence, a more sensitive method for determining variations in protein concentration, a possible approach was to denature the proteins, since it is well known (*17, 20*) that denaturation exposes active groups capable of strong binding of metal ions. Alkaline denaturation was chosen for investigation of this effect on pAg values.

Denaturation of HSA with alkaline saline for 24 hr followed by addition of the buffered Blank A gave very large changes in potential. The potential change was close to linear over a wide concentration of HSA from 1-100 μg/ml, as shown in Figure 3. A similar effect was observed for γ-globulin, but again the potential change was less marked than for albumin.

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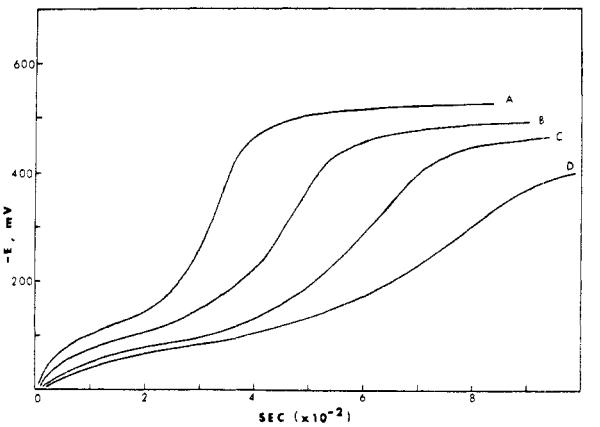

Figure 5. Rate of alkaline denaturation of total protein in human serum

Volumes of serum diluted to 1.10 ml in Blank B. (*A*) 50, (*B*) 30, (*C*) 20, (*D*) 10 μl

It was therefore possible to determine proteins in the range 1-100 μg/ml corresponding to potential changes up to 130 mV, provided that time was allowed for the denaturation process to approach completion.

Kinetics of Protein Denaturation. Since large changes in potentials occurred on denaturation for 24 hr, the possibility arose that the kinetics of the reaction over a much shorter time interval could also be monitored. In alkaline saline, results given in Figure 4 showed that a sharp transition in protein conformation occurred at the higher protein concentration range after relatively short reaction times. Figure 4 compares the rate profiles of solutions of HSA and γ-globulin, each showing a sharp transition at half-times depending on both the total protein concentration and albumin/globulin (A/G) ratio. In addition, Figure 4 shows a fast initial transition of relatively small potential change, preceding the sharp second transition of large potential change, and this was then followed by a slow continuous negative drift in potential over a 24-hr period.

The effect of alkaline denaturation of normal human serum was found to be similar to that of HSA, as shown in Figure 5. The half-time of the second transition was again dependent on the total serum protein concentration after dilution in the test solution, and an almost linear calibration curve was obtained for the range 10-50 μl of serum diluted to a final volume of 1.10 ml in the alkaline saline, as shown in Figure 6. The analogous calibration curve for HSA was compared to serum in Figure 6 in which potentials measured at 400 sec were plotted against protein concentration. The change in half-time with concentration indicated at least a second-order reaction with respect to protein; nevertheless, analysis for protein was possible within the limits given in Figure 6.

However, since the denaturation reaction was at least second order in protein, it would be expected that mixtures of proteins, *e.g.* in serum, would be denatured at rates quite different from the individual proteins. This is proved by the results given in Figure 4 and is further illustrated by Figure 6 where it is shown that there is not an exact correlation between the potentials for a given total protein content of serum and for the concentration of a single protein, HSA. It is therefore clear that the half-time of denaturation is closely related to both the A/G ratio and the total protein content of a mixture.

Serum Deproteination. After alkaline denaturation of serum in silver ion solution followed by acidification with sulfosalicylic acid, protein precipitation occurred. After centrifuging, sufficient soluble products were found to be

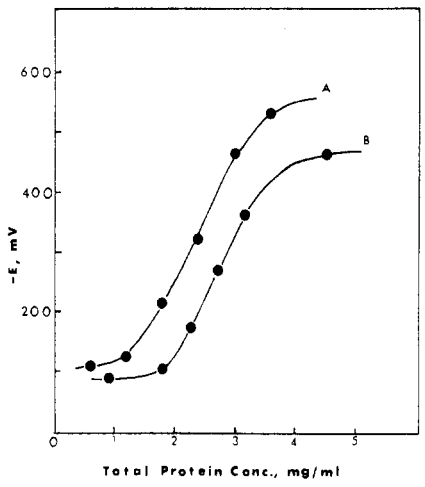

Figure 6. Calibration of proteins measured 400 sec after initiation of alkaline denaturation

(*A*) Human serum, (*B*) Human albumin

present in the supernatant to be monitored using the silver electrode. The potential of the supernatant was measured immediately after centrifuging and plotted as a function of total serum protein content of the serum volume originally sampled, as shown in Figure 7. The silver potential change was found to be linear with increasing volumes of serum sampled in the range 10–100 μl, *i.e.*, dependent on the total serum protein concentration initially denatured.

The potentials, however, were found to change after excessive exposure of the test solution to air. Figure 7 shows the potentials taken ½ hr after the initial readings. This effect would be expected (*17*) for thiols which are easily air oxidized in the presence of metal ions. This tendency was not observed in alkaline denatured protein solutions since there was a continuous negative change in potential even when measuring the solutions exposed to air. The increased stability toward oxidation in alkaline solution can possibly be attributed (*20*) to cross-linking between sulfhydryl groups of nonprecipitable species and disulfide groups in the protein.

DISCUSSION

The silver sulfide electrode is known to possess unique properties regarding Nernstian response over a very wide concentration range of free silver ion (*5*) and satisfactory function at high pH (*21*). These properties have permitted the development of direct potentiometric methods of analysis of interest in both fundamental and clinical aspects of protein chemistry.

The applications of protein analytical methods in medical diagnosis are of course, well known (*22, 23*) and include analysis for total protein, specific methods for albumin and globulin, and A/G ratios as well as chromatographic and immunochemical methods of separation. The electrode method reported here represents a single technique for measuring a number of different protein values: changes in total protein concentration, A/G ratio, and serum filtrate thiol content. The proposed procedures are easily carried out and are reproducible providing that the slow response of the electrode is taken into account.

One point of interest arising from these results is the identity of the species responsible for the potential changes. In native protein solutions, the reaction of Ag^+

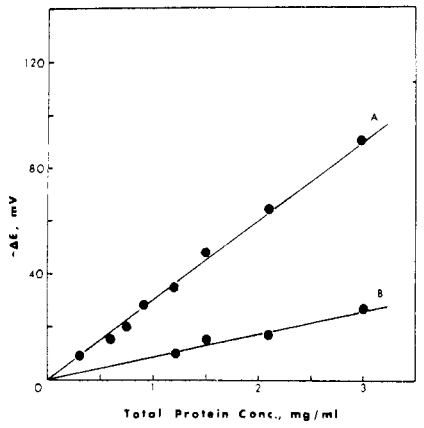

Figure 7. Calibration of protein degradation products in serum centrifugates after deproteination of denatured serum

(*A*) Immediately after centrifuging, (*B*) 30 min after exposure to air

with protein–thiol groups to give silver mercaptides would be expected to change pAg values and hence the potential, although some doubt still exists on the stoichiometry of this reaction (*7, 9*). This was confirmed by the nonlinear shape of the calibration curves in Figure 2, which reached a limiting value due to effectively complete silver mercaptide formation in the presence of excess protein. It is emphasized that potential changes were observable even in the presence of excess chloride which markedly decreased the free silver ion concentration available for mercaptide formation.

In alkaline denatured protein solutions, disulfide cross-links are known (*17, 20*) to be ruptured, producing thiol groups in excess of the silver ion concentration. Thiols affect the potential of silver sulfide membrane electrodes whether added silver ion is present or not (*7, 9*) and, hence, the calibration curve in Figure 2 was linear over a much wider range of denatured protein concentrations than in the case of native proteins. The main problem in solutions of high thiol concentration is the very slow response of the electrode when it is re-immersed in the blank solution, taking up to 20 min to return to the base potential before the next test solution can be measured.

Figures 2 and 3 also show that total protein analysis is possible in a biological fluid such as blood serum with little pretreatment either in the native or denatured state. The method, like most other protein analytical methods (*9*), suffers from the fact that the response to individual native or denatured proteins varies. However, the electrode method is more sensitive than the biuret method and is less liable to interference from turbidity or ammonium salts.

Of even greater interest is the capability of the electrode to monitor the kinetics of protein denaturation at high pH, and to analyze serum filtrates. It has been established in Figures 4 and 5 that sharp transitions in protein conformation occurred in alkaline silver ion solutions. Rate data at extremely high pH have not been reported previously using the common techniques for monitoring denaturation reactions such as optical rotatory dispersion, intrinsic viscosity, and difference spectroscopy (*20, 24*). Alkaline denaturation is not well understood (*20*), but because of the specificity of the silver electrode for thiol groups, it seems certain that the transition observed in Figures 4 and 5 can be attributed at least partially to random-coil formation on rupture of the disulfide cross-links of the serum proteins. The method therefore appears promising for use in fundamental studies on protein conformation.

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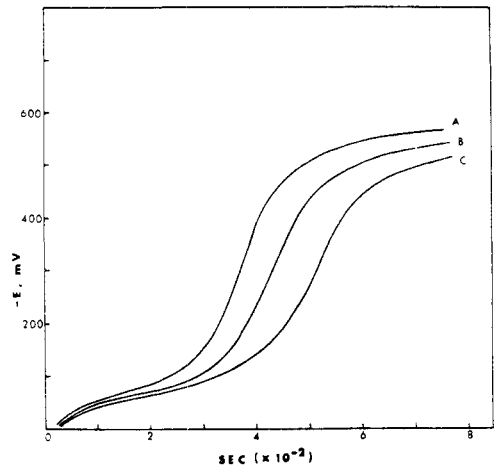

Figure 8. Comparison of rate of alkaline denaturation of protein mixtures approximating to normal and abnormal serum samples

Albumin/γ-Globulin ratios in mg/ml after dilution by a factor of 12.5. Normal: (*A*) 3.6/2.2; Abnormals: (*B*) 2.2/2.5, (*C*) 1.6/3.0

Furthermore, in Figure 4 it has been shown that the rates of these reactions depend first on protein structure and second, on the total protein composition of a mixture. Serum albumin thus reacts at a faster rate than γ-globulin at the same concentration. Keeping the total concentration constant in a mixture of these two proteins, but changing A/G ratio from 5.0 to 0.2, gave quite different half-times. Therefore, for conditions where albumin is in excess, the half-time of the reaction approaches the value for albumin itself. In abnormal serum samples, in which globulin is in excess (hyperglobulinemia), the half-time would be expected to approach the value for globulin. Measurement of the half-times of normal controls as compared to unknowns can therefore be used to distinguish this condition, *in conjunction with* the total protein analysis.

As an example, synthetic solutions were prepared containing A/G ratios published for abnormal and normal cases (*5*). Figure 8 shows that the rates of denaturation of the samples were quite different. As expected from Figures 4–6, the rate of denaturation of the normal sample was considerably faster than the abnormals and measurement of potentials at 400 sec gave remarkably large potential differences.

However, hyperglobulinemia is certainly not diagnostic of any one disease (*22, 23*) and is characteristic, for example, of most inflammatory conditions. Better specificity of diagnosis might be expected (*25*) if the above determinations were used in conjunction with analysis of protein degradation products after deproteination of the denatured serum, as shown in Figure 7. Analogous determinations have previously been carried out by polarography using the Brdicka serological filtrate test, recently reviewed by Homolka (*25*). In conjunction with this test, Müller and Davis (*26*) proposed the calculation of the so-called protein index, *viz.* the ratio of serum filtrate sulfur components to total serum denatured protein sulfur levels determined by polarography. Such data are of particular interest in cancer studies and, although not specific for cancer, gave some correlation with malignant conditions (*25*).

It is now clear that similar studies can be carried out using ion electrode potentiometry, with the well known advantages of simplicity of potentiometry over polarography. It is emphasized that the most important property of the electrode kinetic method is the extremely sensitive change in potential with small changes in serum volume as shown in Figures 5 and 6. The potential difference between 20- and 30-μl samples in Figure 5 measured at 500 sec was approximately 200 mV, equivalent to a change in free Ag^+ concentration of a factor of over 1000 for a change in total protein concentration of a factor of only 1.5. This high sensitivity is ideally suited to clinical analysis for differentiating between normal and abnormal protein levels in serum.

On the other hand, it is clear from these kinetic studies that if the denaturation is allowed to proceed too long, as shown in Figure 8, then the potential differences between abnormals and normals become very small relative to the overall change. This is possibly the reason for the relatively small differences between normal and abnormal samples observed by Brdicka (*27*) using polarography of alkaline denatured serum. The electrode methods described herein therefore offer interesting possibilities in clinical analysis, particularly with regard to determination of the protein index in cancer studies.

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