pable of generating variable repetition rate, 250-psec pulses with kilowatt peak powers. The high powers would allow very efficient frequency doubling while the narrow pulse widths would extend the range of measurable lifetimes down to ~15 psec.

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Automated Protein Determination with Ion-Selective Membrane Electrodes

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A newly developed flow system using a silver sulfide crystal membrane electrode makes possible the automated determination of protein in serum at sampling rates of 20–60 per hour. The automated method, which is based on measuring fixed-time denaturation kinetics of proteins, offers a high sensitivity in the μg/ml range and superior selectivity over conventional methods. This method can be employed to measure total sulfur-containing proteins or to distinguish between individual proteins on the basis of reactivity differences.

The application of ion-selective membrane electrodes in bioanalysis has been proceeding rapidly in recent years with the introduction of enzyme analysis (*1*) and related determinations of urea (*2*), glucose (*3*), and creatinine (*4*). The possibility of using the unique properties and advantages of ion-selective electrodes for determination of serum proteins has also recently been demonstrated (*5*) by showing that the silver sulfide membrane electrode is capable of sensing degradation products from the reactions of proteins in highly alkaline saline solutions containing silver(I).

Human protein analysis is of extensive interest in diverse fields and, as a result, a large number of analytical methods have been developed (*6*). However, widely used colorimetric methods such as the biuret (*7*) and Lowry methods (*8*) are known to be subject to interference by nonprotein substances (*9, 10*) and suggested improved methods continue to appear regularly (*11–13*).

We now describe an automated system, using ion selective electrodes in a flowing stream, which greatly improves the sensitivity and convenience of protein analyses. By carrying out the protein reactions in a flowing stream, it is possible to optimize both the required chemical steps and the conditions for best electrode response; this feature of the flow system represents a powerful advantage over our previous batch analysis method.

EXPERIMENTAL

The continuous-flow autoanalysis manifold for protein determinations is shown in Figure 1, using a Technicon Sampler II and proportioning pump. The sensing electrode assembly was comprised of Orion 94-16A and 90-02-00 electrodes with a flow-through cap fixed to the indicator electrode, as previously described (*1*). The potentiometric output of the electrodes was continuously recorded with a Beckman Model 1055 pH recorder.

Reagents. Silver nitrate, sodium hydroxide, and sodium chloride solutions were all prepared from analytical grade reagents, urea from Baker analyzed grade, lauryl sulfate from Sigma 95% grade, guanidine hydrochloride from Sigma Grade I, sodium sulfite from Baker analyzed grade, and all were used without further purification.

Bovine serum albumin (BSA) was obtained from Sigma, human serum albumin (HSA) from Nutritional Biochemicals Corporation and human γ-globulin (γ-G) from Miles Research Laboratories. Solutions of *ca.* 10 mg/ml were prepared in isotonic saline (0.9%) and standardized by the biuret method (*7*) *vs.* a standard solution of HSA (100 mg/ml) obtained from Miles Laboratories.

Procedures. For static solutions, rate data were obtained by immersion of the electrodes in the appropriate blank solution after conditioning of the indicator electrode as described previously (*5*). The blank potential was first established on the pH recorder and the required volume of protein was injected into the blank from a μl-syringe. Typically, a blank solution consisted of: Ag^+ ($6 \times 10^{-6} M$), OH^- ($1.0 M$), saline (0.9%), and denaturing or disulfide reagent at the chosen concentration. By changing the various reagent concentrations, a study was made to determine the optimum conditions for sensitive measurement of the protein in the automated continuous-flow system.

For measurements in a continuous-flowing stream, protein samples in isotonic saline of *ca.* 1.0-ml volume were prepared in

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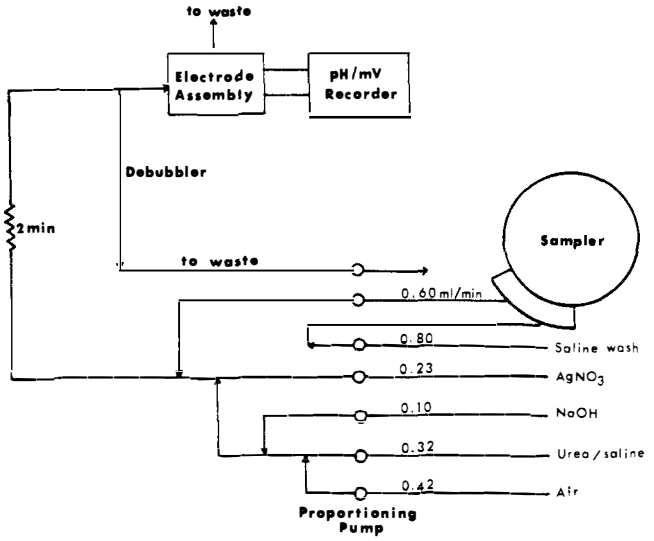

Figure 1. Schematic diagram of the continuous flow apparatus

Concentrations used throughout were: Ag^+ ($1 \times 10^{-3}M$), urea ($10M$), NaOH ($4M$), saline (0.9%) with 1:1 sample to wash at 20 samples/hr unless otherwise stated in the text

Technicon sampling vials and automatically aspirated into the air-segmented reagent stream as shown in Figure 1. The wash solution was isotonic saline (Technicon), and data were recorded with a sample-to-wash ratio of 1:1 at various sampling rates from 20–60 samples per hour. The length of time allowed for reaction between reagent substrate and protein sample was determined by suitable choice of mixing coils but was found to have little effect on the potential, so that a 2-min delay was sufficient to allow formation of protein reaction products easily detectable by the indicator electrode. The reactions were carried out at room temperature 24–26 °C, as little effect on potentials was observed at the low protein concentrations even after passing the reaction mixture through a 30-min delay coil at 30 °C.

Essentially, this procedure is a fixed-time kinetic method of analysis, basically different from the quenched method used in enzyme systems (*1*). As in all automated continuous-flow systems (*14*), a series of peaks were recorded, one for each protein sample in sequence. In this case, the peak height is related through the Nernst equation to the free silver ion remaining after protein reaction. The peak height could therefore be related directly to the protein concentration via a calibration curve. Information regarding the stoichiometry of the reaction was evaluated, together with optimum conditions required for the highly sensitive automated analysis of proteins.

RESULTS

To increase the rate of the alkaline hydrolysis reaction of protein disulfide observed previously (*5*), an obvious possibility was to add either a denaturing agent or a disulfide splitting reagent to the protein solution. If a substantial increase in rate could be achieved, it might be expected that a rapid analytical method with sensitivity in the μg/ml range could be developed since this range had been successfully detected (*5*) but only after very long reaction times.

The disulfide splitting reagents chosen were cysteine and sodium sulfite, both commonly used for this purpose (*15*). Cysteine itself (or any thiol of course) was sensed by the Ag_2S electrode, and with the excess required to reduce the disulfide bonds, a large negative blank potential was observed, as shown in Table I. As observed previously (*16*), sulfite also gave a large negative blank potential, making these reagents unsuitable for electrode determination of low concentrations of protein disulfide groups.

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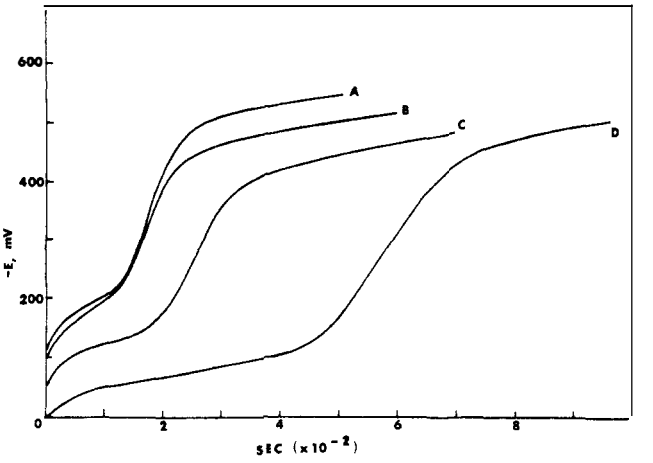

Figure 2. Rates of reaction of bovine serum albumin

Solution conditions: BSA (1 mg/ml), Ag^+ ($6 \times 10^{-6}M$), NaOH ($1M$), saline (0.9%) and urea at concentrations (molar) of: (*A*) 8.0, (*B*) 2.0, (*C*) 1.0, (*D*) 0

Table I. Blank Potentials of Reagents for Disulfide Splitting

Reagent[a]	Concentration, M	E, mV
None	. . .	+5
Cysteine	0.001	−320
Sodium sulfite	1.0	−210
Lauryl sulfate	1.0%	−40
Guanidine–HCl	4.0	−220
Urea	8.0	−110

[a] In solution of Ag^+ ($6 \times 10^{-6}M$), NaOH ($1M$), and saline (0.9%).

Of the denaturing agents investigated, lauryl sulfate had little effect on the rate of potential change during the protein reaction, and guanidine hydrochloride gave a blank potential too negative to be useful, as shown in Table I. Urea was the only denaturing agent for which the blank potential remained sufficiently positive (Table I) for the electrode to retain some sensitivity for detection of changes in free silver ion concentration in the protein test solution. In addition, urea at high concentration markedly increased the rate at which protein disulfide groups were ruptured in alkaline silver ion solution as shown in Figure 2. At the relatively high protein concentration of 1 mg/ml, the inflection in the rate profile was shifted to more rapid half-times as the rate of the second step in the protein reaction increased with increasing urea concentrations, as shown in Figure 2. As before (*5*), the inflection shifted to longer times as the protein concentration decreased and was not observed at all at low protein concentrations (<0.1 mg/ml) even in the presence of urea. However, there were easily measurable potential differences between protein concentrations only minutes after initiation of the reaction in the presence of urea.

Automated Analysis. A schematic diagram of the continuous-flow manifold developed for automated analysis of protein is shown in Figure 1, and a typical chart recording for protein calibration and random sampling is shown in Figure 3. The critical parameter that controlled peak resolution was the total silver ion concentration. At $1 \times 10^{-3}M$ as used for Figure 3, the final silver ion concentration in the flowing stream after allowing for dilution by sample and reagent streams is $1.8 \times 10^{-4}M$. Despite the large excess of chloride present, with the silver ion added last to the alkaline chloride urea solution, there was only a slight accumulation of silver chloride deposit at the

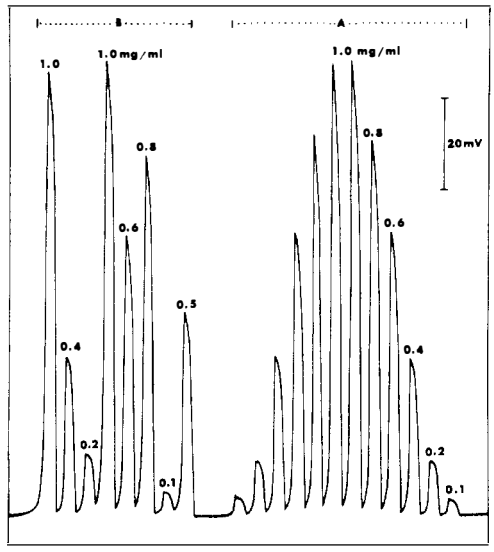

Figure 3. Continuous recording of BAS analysis

Rate: 20 samples/hr. (*A*) Calibrations in direction of increasing and decreasing concentrations; (*B*) Random samples

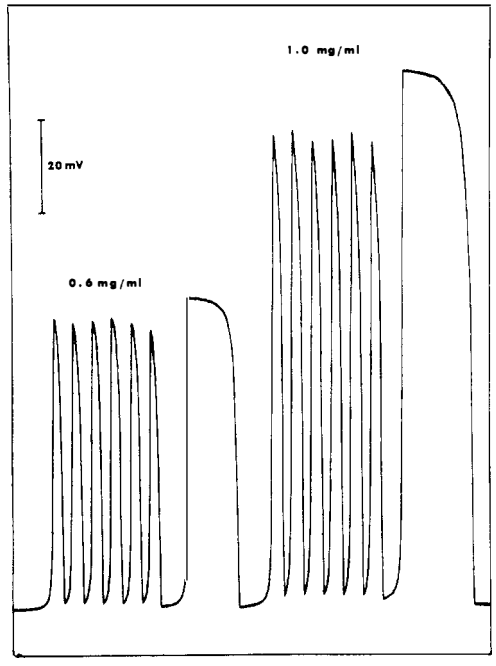

Figure 4. Steady state and replicates of BSA analysis

Rate: 20 samples/hr, using the manifold in Figure 1

point where the silver ion entered the flowing stream, and the stream remained perfectly clear in the mixing coil.

Response Characteristics. With the reagent conditions given in Figure 3 and data recorded at 20 samples per hour and 1:1 sample to wash ratio, Figure 3 shows excellent response characteristics—*viz.*, little sample interation or peak tailing, good precision between calibrations measured in the directions of increasing and decreasing protein concentrations, and good accuracy for random samples. Figure 4 illustrates steady state and replicate measurements at two different protein levels. The steady state readings show the slow approach of the electrode to its equilibrium potential, accounting for the fact that the continuously recorded replicate peaks reached only *ca.* 90% of the steady state potential at a sampling rate of 20 per hour. Despite this, excellent precision was obtained

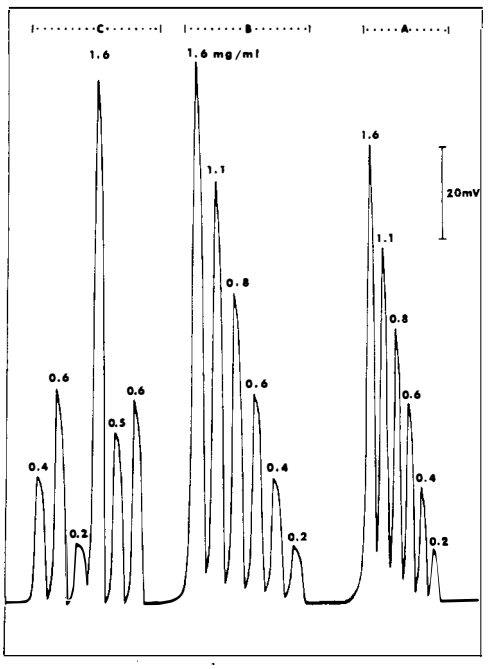

Figure 5. Continuous recording of BSA analysis at increased sampling rates

(*A*) 60/hr, (*B*) 40/hr, (*C*) Random sampling at 40/hr

Table II. Precision of Ag_2S Electrode Response to Albumin at High Total Silver Ion Concentration

$[Ag]_T = 1.8 \times 10^{-4} M$[a] [BSA], mg/ml	Mean peak heights, mV[b]		
	20 S/hr	40 S/hr	60 S/hr
0.2	12.5 ± 1.0	12.2 ± 1.0	12.1 ± 1.0
0.4	36.2 ± 1.5	33.4 ± 1.3	25.7 ± 1.3
0.6	61.3 ± 1.0	47.1 ± 1.3	43.9 ± 1.2
0.8	82.7 ± 0.9	74.4 ± 1.8	62.8 ± 1.3

[a] Concentration of silver ion in flowing stream. [b] Average of at least 6 determinations. Range is standard deviation.

for replicates in the concentration range 0.1–1.0 mg/ml as shown in Figure 4 and Table II.

Effect of Sampling Rate. Good response characteristics were also observed at increased sampling rates of 40 and 60 samples per hour as shown in Figure 5. The data indicated greater sample interaction and decreased peak heights as sampling rate was increased. The major factor controlling peak height was the slow electrode response characteristic at a fixed set of reagent conditions. Shorter exposure of the electrode to the test and wash solutions at the high sampling rates resulted in the altered characteristics shown in Figure 5. Table II gives the precision for replicates at various sampling rates and Table III, the accuracy of random samples, showing the resulting poorer precision and accuracy. It should be noted that the slow electrode response caused lower peak heights than the mean when suddenly exposing the electrode to a high protein concentration. However, as shown in Tables II and III, the accuracy and precision are still acceptable at the high sampling rates, allowing protein determinations in the range 0.1–2.0 mg/ml at 60 samples per hour with a relative error of ±5%.

Effect of Variation of Silver Ion Concentrations. As a consequence of using the relatively high total silver ion concentration of Figure 3, the peak heights were not as

Table III. Relative Error in Random BSA Samples under Continuous Flow Conditions at Various Values of $[Ag]_T$

[BSA], mg/ml	$-\Delta E$, mV	[BSA], found	Relative error, %
$[Ag^+]_T = 1.8 \times 10^{-4}M$, 20 S/hr, 1:1 S/W			
0.50	44	0.49	−2.0
0.10	5	0.105	+5.0
0.80	78	0.77	−3.8
0.60	60	0.59	−1.6
1.00	98	0.98	+2.0
0.20	13	0.21	+5.0
0.40	34	0.40	0
1.00	96	0.96	−4.0
$[Ag^+]_T = 1.8 \times 10^{-4}M$, 40 S/hr, 1:1 S/W			
0.60	44	0.59	−1.6
0.50	37	0.51	+2.0
1.60	113	1.57	−1.9
0.20	12	0.21	+5.0
0.60	47	0.62	+3.4
0.40	27	0.41	+2.5
$[Ag^+]_T = 1.8 \times 10^{-5}M$, 20 S/hr, 1:1 S/W			
0.030	14	0.029	−3.3
0.100	49	0.105	+5.0
0.300	86	0.290	−3.3
0.080	37	0.078	−2.5
0.040	20	0.041	+2.5
0.200	69	0.186	−7.0

Table IV. Precision of Ag_2S Electrode Response to Albumin at Low Total Silver Ion Concentration

$[Ag^-]_T = 1.8 \times 10^{-5}M$ [BSA], mg/ml	Mean peak heights, mV	
	20 S/hr	60 S/hr
0.08	31.6 ± 1.5	26.8 ± 2.6
0.10	48.1 ± 1.4	34.5 ± 3.1
0.14	61.0 ± 1.7	55.8 ± 3.9
0.40	102.1 ± 1.2	82.0 ± 3.3

sensitive to protein as for the static system, described earlier (*5*), in which the silver ion was $6 \times 10^{-6}M$. The effect of decreasing $[Ag]_T$ was therefore examined in more detail by recording protein calibration of various $[Ag]_T$ values in the range 10^{-3} to $10^{-4}M$. Figure 6 shows the recorder output for calibration of BSA at various sampling rates with $[Ag]_T$ initially $1.0 \times 10^{-4}M$, diluted in the flowing stream to $1.8 \times 10^{-5}M$. Figure 6 indicates clearly the much greater sample interaction than for the calibration using $1.0 \times 10^{-3}M$ Ag^+ in Figure 3, with resulting poorer precision and accuracy as shown in Tables III and IV. However, sensitivity of the protein determination was improved by a factor of approximately 10 by decreasing the silver ion concentration, as shown in the calibration curves given in Figure 7. From Tables III and IV, the precision and relative error of measurements made with low total silver ion at 20 samples per hour are comparable to the 40 sample per hour data at the high total silver ion concentration. High sensitivity can, therefore, be achieved at the expense of high precision.

The slopes of the calibration curves in Figure 7 should depend on the number of silver ions bound per mg of protein, the equilibrium constant for the reaction between the silver ion and protein, and on the rate of the reaction. The peak heights increased with decreasing silver ion, confirming that a greater percentage of total silver was bound by protein at the lower silver ion concentrations. In addition, the plots of potential against $[Ag]_T$ were con-

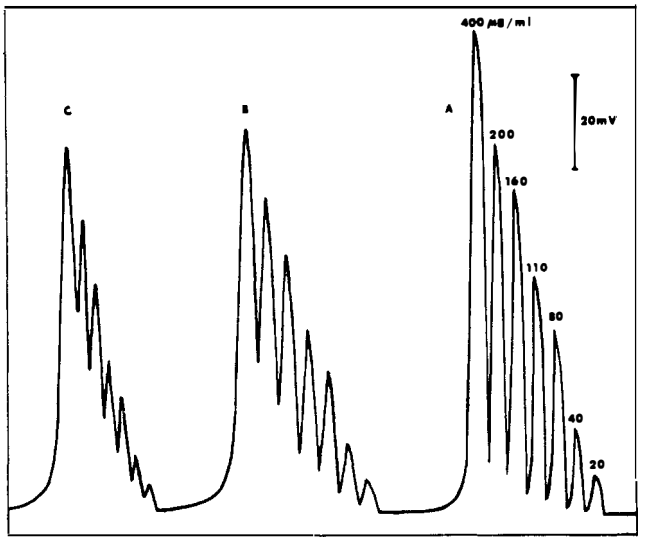

Figure 6. Continuous recording of BSA analysis at low-total silver ion concentration

$[Ag^+]_T = 1.0 \times 10^{-4}M$. Sampling rates: (*A*) 20/hr, (*B*) 40/hr, (*C*) 60/hr

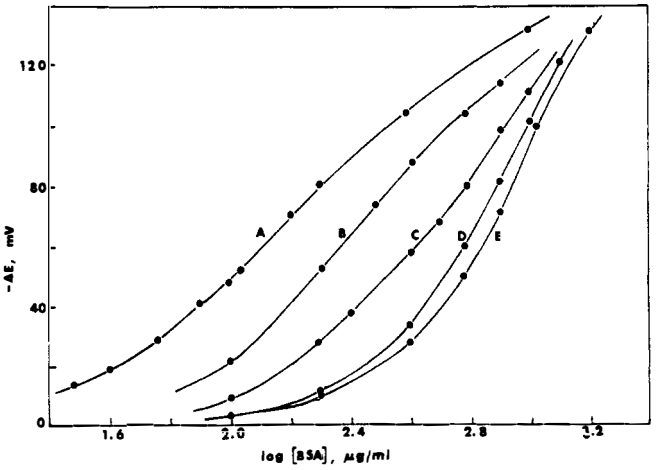

Figure 7. Calibrations for BSA analysis

Using the manifold in Figure 1 (20 samples/hr) with silver ion concentrations: (*A*) 0.1, (*B*) 0.2, (*C*) 0.5, (*D*) 1.0, (*E*) $1.2 \times 10^{-3}M$

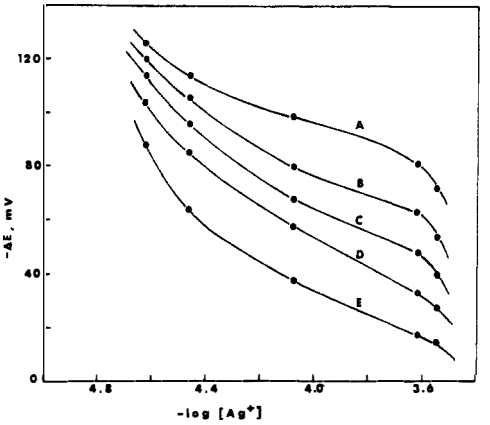

Figure 8. Dependence of peak heights on $[Ag^+]_T$

BSA concentrations (mg/ml): (*A*) 0.80, (*B*) 0.63, (*C*) 0.50, (*D*) 0.40, (*E*) 0.25. Sampling rate 20/hr

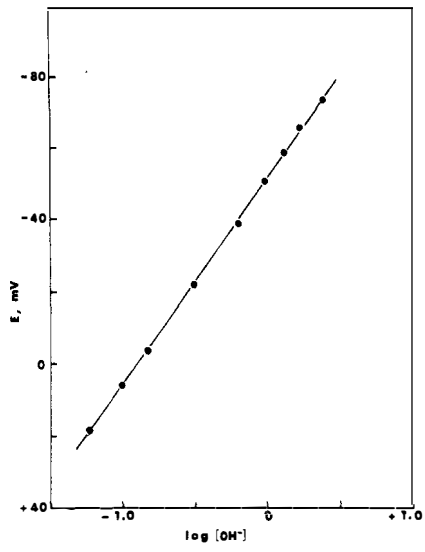

Figure 9. Effect of hydroxide ion on electrode response to BSA

Conditions described in text. [BSA] 2 mg/ml. Sampling rate 20/hr

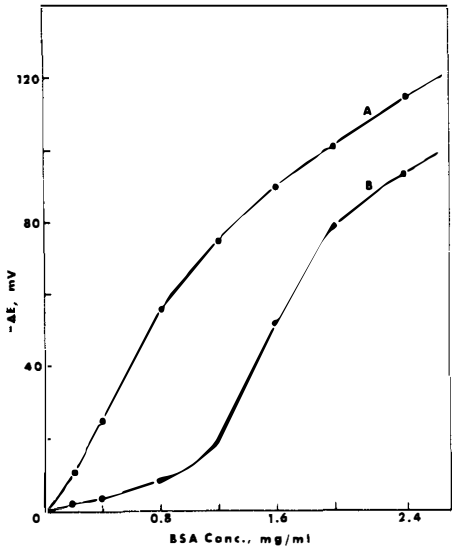

Figure 10. Effect of urea on BSA calibration

Flow conditions as given in Figure 1 but with NaOH (1 5*M*) and urea: (*A*) 10.0*M*, (*B*) 0

structed in Figure 8, further illustrating the marked effect of silver ion on potential. The slopes of these plots gave data in relation to the stoichiometry of the protein reaction, as given in the Discussion Section.

Effect of Hydroxide Ion. Using the manifold in Figure 1, the effect of varying hydroxide concentration, while maintaining all other reagent concentrations constant, was determined by replacing the hydroxide with a standard BSA solution (2 mg/ml) and sampling hydroxide from the Sampler II. Blank corrections for changing [OH^-] were made by replacing the BSA solution with isotonic saline. The results shown in Figure 9 with peak height plotted as a function of log [OH^-] gave a slope of 58.5 mV, indicating a decade increase in bound silver for a decade increase in total hydroxide concentration.

Effect of Urea on Calibrations. Calibration curves for BSA were measured with urea as shown in the manifold in Figure 1, and for comparison in the absence of urea by replacing it with isotonic saline. As expected from the effects of urea on the reaction rate in Figure 2, the calibration without urea was in the form of an s-shaped titration curve as shown in Figure 10, where the potential changes were very small at the low concentrations of BSA. As the protein concentration was increased, a sharp increase in ΔE was observed near 1.0 mg/ml. In the presence of urea, however, the calibration was almost linear at the low BSA concentrations <1.0 mg/ml. This was attributed to the faster exposure of SS groups to attack by hydroxide.

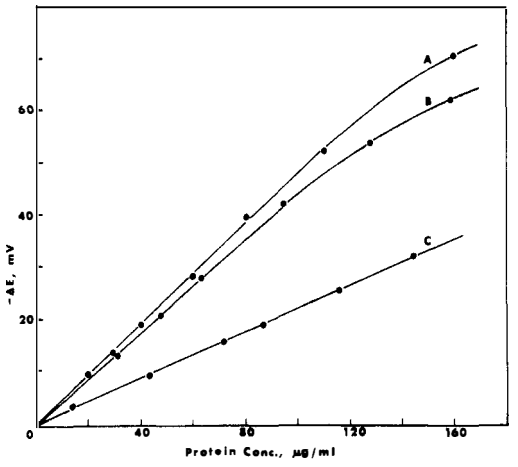

Figure 11. Calibration curves for proteins using low total silver ion concentration

$[Ag^+]_T = 1.0 \times 10^{-4}M$; (*A*) Bovine albumin, (*B*) Human albumin, (*C*) Human γ-Globulin. Sampling rate 20/hr

Table V. Interference Effects on the Ag_2S Electrode under Continuous Flow Conditions

Interference	Concentration, *M*			
Ammonia[a]	1.0	0.1	0.01	0.001
Peak height, mV	123	38	3	1
Urea[a]	10.0	1.0	0.1	0.01
Peak height, mV	63	6	1	0
Urea[b]	10.0	1.0	0.1	0.01
Peak height, mV	58	6	0.5	0

[a] Peak heights measured for $[Ag^-]_T$ $1.8 \times 10^{-4}M$, 20 S/hr, 1:1 S/W. [b] Peak heights measured for $[Ag^-]_T$ $1.8 \times 10^{-5}M$, 20 S/hr, 1:1 S/W.

Albumin and Globulin Response. The effect of the reagent system on human serum albumin (HSA) and human γ-globulin (γ-G) was also studied in comparison to BSA and calibration curves are given in Figure 11, measured at $[Ag]_T$ of $1.0 \times 10^{-4}M$ in the continuous flow system. The calibrations were linear in the protein range form 10-100 μg/ml and there was little difference between BSA and HSA response, as expected from the number of disulfide groups in each molecule (*17*). The response for γ-G was less than half that of the albumins for equivalent concentrations, in agreement with the much slower rate of reaction of γ-G, than HSA observed previously (*5*) and also agreeing with the fact that γ-G contains fewer disulfide groups per mole than albumin (*18*).

Interferences. The effect of nonsulfur containing compounds on the electrode response was also investigated under the chosen continuous-flow conditions. Any donor group that complexes silver ion may be likely to interfere with the protein response, and particularly nitrogen-containing groups which complex silver strongly (*19*). Ammonia and urea, known to interfere with the biuret reaction (*19*), were chosen as likely interferences, and concentrations varying by a factor of 1000 were aspirated into the reagent stream. Table V shows the results observed at two

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$[Ag]_T$ values, and indicates no interference except at very high concentrations. The analysis system, therefore, is not completely specific for sulfur groups but is highly selective because of the nature of the electrode and reagent system used.

DISCUSSION

The continuous-flow system described has allowed the development of a simple, rapid method for determination of protein concentrations, apparently dependent on the disulfide content of the protein. The proposed method is a considerable improvement over the manual method described previously (*5*).

Some information about the stoichiometry of the reaction can be deduced from the data given in Figures 7–11. Because of the high selectivity of the electrode for thiol groups (*20*), the reaction must certainly involve rupture of disulfides giving a thiol product capable of strongly complexing silver ion. The slope of the plot in Figure 9 showing the effect of hydroxide ion on potential indicated a 1:1 stoichimetry between Ag^+ and OH^- as the extent of disulfide splitting was increased. The reaction can therefore be written similarly to the stoichiometry determined by Çecil and McPhee (*21*) for low molecular weight disulfides:

$$\text{P-(SS)}_x\text{-P} + x\text{Ag}^+ + x\text{OH}^- \rightarrow \text{P(SAg)}_x + \text{P(SOH)}_x \quad (1)$$

where X is the number of disulfide groups in the protein. The slopes of the calibrations in Figure 7, 8, 10 and 11 should then depend on the number of x, but will also be influenced by the factors mentioned in the Result Section, the equilibrium constants for complexing of silver by the reagent substrate, and by the presence of free SH groups in the protein. This is an extremely complicated situation and direct information about x cannot be easily obtained from these data.

However, the electrode response is at least semiquantitatively related to the number of disulfide groups in the protein, as shown in Figure 11. BSA and HSA both contain 17 SS groups per molecule (*15*) with molecular weights of 66,000 and 69,000, respectively, while γ-G contains only 12 SS bonds/molecule for a molecular weight of 160,000 (*18*). In principle, it should be possible to use the Ag_2S electrode directly to monitor protein breakdown without the addition of silver ion. However, in practice it is found that electrode response is too sluggish to permit effective monitoring at low protein concentration levels unless silver ion is added to the solution.

Interference effects have been shown to be minimal in Table V. Expected interferences from ammonia and urea are negligible at respective concentrations of $10^{-3}M$ and $10^{-1}M$. For 0.1 mg/ml BSA, it would be expected that one could tolerate ratios of *ca.* 10^3 and 10^5 of ammonia and urea in excess before interference occurred. These high selectivities can be attributed to the very high stability constant of silver thiol complexes in comparison to nitrogen donor complexes (*19*).

The method suffers from the fact that different proteins give a different response depending on protein structure, as in some colorimetric methods (*6*), but possesses a number of advantages over these methods because of the use of the electrode sensing system. The instrument cost is low; the method is virtually specific for sulfur-groups and interferences common in the Lowry and biuret methods cannot occur. Sensitivity in the range 5–100 μg/ml compares favorably to the Lowry method (*8, 13*), and the sensitivity of response can be varied simply by changing total silver ion concentration. Linear response is obtained over a wider concentration range than Hartree's (*13*) modified Lowry method and the method requires only 2-minute mixing time as compared to 30 minutes for the biuret and Lowry methods.

Despite the variation in response for different proteins, these advantages should allow wide application in biological studies, including sensing for column chromatography and immunochemical separations, and determination of protein-sulfur content of biological fluids. Presumably, the sensitivity could be still further improved by miniaturization of the manifold, improved electrode flow-through design and increasing the length of time for reaction.

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Digital Simulation of a Rotating Photoelectrode

Photolysis of Benzophenone in Aklaline Media

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A program is described for the digital simulation of convective–diffusional mass transport, photolytic and homogeneous chemical processes, and electrochemical reaction of the photoproducts at a rotating photoelectrode. The simulation required use of expanded equations for axial and radial convection. A rotating photoelectrode and results of the simulation were applied in a kinetic study of the photodimerization of benzophenone in alkaline alcohol–water solutions. The rate constant determined is in agreement with literature values determined by independent methods.

Rotating ring-disk electrodes (RRDE) have been demonstrated to be useful for the study of mechanisms of electrochemical processes and the kinetics of homogeneous reactions involving the products of electrochemical reactions. The applicability results because the rate of mass transport at the electrode can be easily controlled, reactive intermediates with a large range of half-lives can be

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