# Effect of Alkali Treatment on Viscosity and on the Sulfhydryl and Disulfide Content of Proteins[1]

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**ABSTRACT**

Changes in viscosity with time of various protein dopes following adjustment of pH to 12.0 with alkali were followed. Results indicated rapid increases in viscosity with bovine serum albumin and hemoglobin, moderate increases with immunoglobulin G and 12S rapeseed protein, and very slow change with 11S soybean protein. The sulfhydryl content decreased with time in 12S rapeseed and heat-denatured 11S soybean proteins only. The disulfide contents decreased in the first 15 min of treatment with all the sulfur-containing proteins. The rate of viscosity change is logarithmically related to the hydrophobicity value of the protein.

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Texturization of proteins by the spinning methods (Boyer 1954, Castaigne et al 1978) requires the use of concentrated protein solutions of high viscosity (spinning dope). Viscosity of soybean protein solutions increases rapidly to a maximum within 35 min from the addition of alkali and then decreases (Kelley and Pressey 1966). Therefore, the alkali treatment and spinning must be exactly synchronized (Westeen and Kuramoto 1964).

Very few research papers have been published on the effect of strong alkali on proteins at high concentrations. Ishino and Okamoto (1975) showed that viscosity of soybean protein isolate at pH 12.3–12.7 increased rapidly as the protein concentration was increased from about 5.0 to 10.5%. Their results also showed that the viscosity of the alkali-denatured protein at 10.4% concentration was lower at pH 11.0–13.0 than at pH 7.2–10.0. They attributed the high viscosity at these lower pHs to molecular aggregation and increased hydration not involving changes in structure, and at pH between 11.2 and 12.2 to molecular unfolding. They concluded that SS-SH interchange may play a large part in viscosity increase and gelation of soybean protein denatured by alkali.

The effect of alkaline treatment on protein SH and SS groups has been studied mainly with dilute solutions. The half-cystine content decreased following strong alkali treatment of soybean (de Groot and Slump 1969) and ovomucoid (Donovan and White 1971). Two

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types of reactions have been recognized with proteins as well as with small molecular weight compounds treated with alkali (Cecil and McPhee 1959): $OH^-$ catalyzed hydrolysis of SS to give SH and SOH groups, and $\beta$-elimination reaction giving as end product the highly reactive dehydroalanine residue. This residue may react with lysine residues to give lysinoalanine cross-linking in soybean proteins (de Groot and Slump 1969) and in wheat gluten and lactalbumin (Friedman 1979). SH-SS interchanges may also occur under conditions of high alkali treatment of soybean protein (Ishino and Okamoto 1975).

This work was undertaken to explain the increase in viscosity of protein dopes following strong alkali treatment. SH and SS groups and their reactions may be involved in the viscosity increase as well as noncovalent linkages, especially hydrophobic interactions. The proteins were selected because they offered a wide range of values for these characteristics.

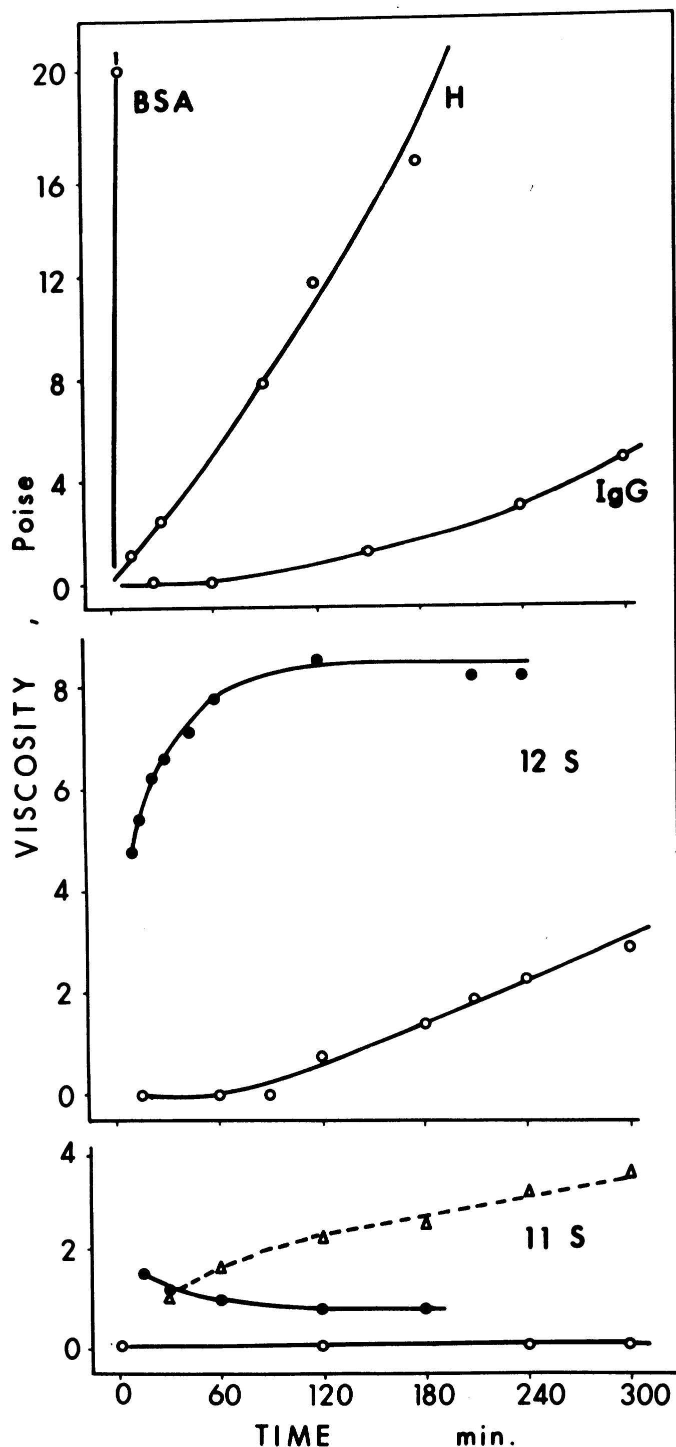


**Fig. 1.** Viscosity change with time of bovine serum albumin (BSA), hemoglobin (HG), immunoglobulin G (IgG), 12S rapeseed protein, and 11S soybean protein solutions after adjustment of pH to 12.0. ——— = 12% protein, --- = 16% protein, o or Δ = native protein, • = heat-denatured protein.

## MATERIALS AND METHODS

Protein fractions from soybean (11S) and rapeseed (12S) were prepared from hexane-defatted flours by fractional dissolution in ammonium sulfate solutions (Simard and Boulet 1978a). Immunoglobulin G (IgG) was prepared from 0–3-day bovine colostrum by fractional dissolution. The $(NH_4)_2SO_4$ solution used for dissolution of IgG was 35% saturation. Other proteins were obtained from commercial sources: bovine serum albumin (BSA) from Baker and hemoglobin (HG) from Nutritional Biochemical Corporation. Heat-treated proteins were prepared by submitting the native protein powder to water vapors at 120° C (103.4 kPa) in an autoclave for 1 hr.

Treatment with alkali was done at 23–24° C by adding 1*N* NaOH to the protein suspension to increase pH to the chosen value and then neutralizing to the initial pH of 7.0 after preselected exposure times.

Sulfhydryl groups (SH) were measured by a spectrophotometric method based on the reaction of proteins with 5,5′-dithiobis-2-nitrobenzoic acid in the presence of SDS. Disulfide groups (SS) were determined by difference between SH contents measured before and after reduction of SS groups with 0.25*M* $\beta$-mercaptoethanol in the presence of 6*N* guanidine-HCl. These methods were used as described by Simard and Boulet (1978b). Concentrations were expressed as mole per mole using the following molecular weights: 356,000 for 11S, 163,000 for 12S, 163,400 for IgG, 68,000 for HG, and 67,000 for BSA.

Protein concentrations were determined by a spectrophotometric method using the wavelength 280 nm and extinction coefficients (1%, 1 cm) 16.1 for BSA, 13.1 for IgG, 15.2 for HG, 9.6 for 11S, and 10.3 for 12S.

Viscosity of the protein solutions was determined at 25° C with the Epprecht-Rheomat 15 at a fixed rotation speed (setting 15 corresponding to a shear stress of 137.1 $sec^{-1}$). The limit of sensitivity under the conditions used was 0.2 poise.

## RESULTS AND DISCUSSION

Changes in viscosity after adjustment to pH 12.0 as a function of time for the various proteins are shown in Fig. 1. The addition of alkali caused progressive increases in viscosity of the different native (untreated) protein solutions with the exception of 11S soybean protein. The concentration of the latter had to be increased from 12 to 16% before the increase of viscosity became measurable. Ishino and Okamoto (1975) showed that the viscosity of a concentrated soybean protein isolate depended on the concentration and pH of the solution. They obtained rapidly increasing viscosities with solutions containing 10.4% proteins at pH between 11.0 and 12.7. The mixed protein fractions as they existed in their isolate appeared to develop larger viscosity values than the isolated fractions we used under similar conditions (Fig. 1). The 12S rapeseed protein and the IgG solutions gave about the same rates of increase in viscosity, whereas HG gave a more rapid increase. BSA gave the largest rate of increase even at the lower concentration of 8%. Higher concentrations gave rapidly setting gels.

Treatment of the 11S and 12S proteins by heat caused an increase in viscosity of the solution, which could be observed immediately after the addition of alkali, but as with the native proteins, the rate of change thereafter was nil with the 11S and relatively rapid for the 12S protein. Heat treatment of the 12S protein appeared to modify the protein in such a way that the rate of increase in viscosity was markedly accelerated (Fig. 1).

Separate measurements were made with solutions of the 11S and 12S proteins, and hemoglobin adjusted to pH 10. Results show that at pH 10 the viscosity did not increase above 0.2 poise within the

5-hr period following the addition of alkali.

Viscosity of diluted protein solutions is related to molecular qualities such as molecular size, degree of hydration, molecular shape, and molecule-to-molecule interactions (Cragg and Bigelow 1955). In concentrated protein solutions, however, the effect of molecular interactions are presumed to be predominant. This is illustrated by the exponential increase in apparent viscosity ($\eta_a$) when the concentration (C in %) of soybean protein is increased in the dispersion. A similar relation determined in our laboratory with a soybean protein isolate was

$$\log \eta_a = 0.26\ C - 3.347.$$

Deviation of protein solutions from Neutonian behavior at high concentrations supports the assumption (Shen 1981).

Treatment of protein solutions with alkali causes structural changes in the protein molecules. Diep and Boulet (1977a, 1977b) have shown with diluted soybean 7S and 11S proteins that the proteins react to extreme pHs (2.0 and 11.0) by unfolding their tertiary structure; electrostatic charge repulsion is responsible for this effect.

Increases in viscosity in concentrated solutions would thus be influenced by molecular unfolding and be largely determined by the exposure of hydrophobic regions to environments that favor protein-to-protein interactions.

The number of SH groups was determined with the proteins as a function of time of exposure to pH 12.0. Results are shown in Fig. 2. The initial values (15 min) at pH 12.0 for the 12S and 11S proteins were 4.0 and 6.7 groups per mole values, which are 1.4 and 1.1 mole per mole, respectively, higher than the values reported by Simard and Boulet (1978b) at pH 7.0. The alkali treatment appeared to increase the number of SH groups by about one mole per mole of protein. Draper and Catsimpoolas (1978) observed an increase of 9.2 groups per mole of glycine when pH changed from 7.0 to 12.0; however, the protein concentration used was less than 0.1%.

The stability of SH groups under conditions of high concentration and pH differs markedly among proteins. The SH increased with time in IgG, remained constant in native 11S, and decreased in native 12S. The changes in SH content and in viscosity (Fig. 1) of the treated protein solutions apparently were not related. The heat treatment caused a decrease in the number of measurable SH groups of the 12S and 11S proteins (Fig. 2). The proteins were so modified by the heat treatment that the SH groups became unstable to alkali in the 11S protein and more resistant to change in the 12S protein in comparison to the native protein. This indicates that the reactions of SH groups in the presence of alkali are markedly dependent on structure and may explain in part the observed differences between the different proteins.

Table I shows the results of disulfide group measurement with native and heat-treated proteins before and after exposure for 15 min to pH 12.0. Native and heat-treated proteins differed very little in total half-cystine (-S-) contents after exposure to pH 12.0. Any difference in -S- groups distribution that may have resulted from heating 11S and 12S proteins was eliminated by the alkali treatment. The distribution of -S- groups between the surface and

**TABLE I**
**Distribution of Half-Cystine (-S-) and Cysteine (SH) Groups in Native and Heat-Treated Proteins After Exposure to pH 7.0 or to 12.0 for 15 min**

| Protein | Surface[a] -S- (m/m)[b] | Interior[c] -S- (m/m) | Total[d] -S- (m/m) | SH[e] (m/m) |
|---|---|---|---|---|
| 11S-Soja | | | | |
| Native, pH 7.0 | 8.4 | 23.4 | 31.8 | 5.6[f] |
| Native, pH 12.0 | 17.5 | 0.5 | 18.0 | 6.7 |
| Heated, pH 12.0 | 15.0 | 3.1 | 18.1 | ··· |
| 12S-Rapeseed | | | | |
| Native, pH 7.0 | 7.1 | 6.2 | 13.3 | 2.6[f] |
| Native, pH 12.0 | 9.2 | 0.0 | 9.2 | 3.9 |
| Heated, pH 12.0 | 9.4 | 1.1 | 10.5 | 1.5 |
| Immunoglobulin G | | | | |
| Native, pH 7.0 | 12.0 | 26.7 | 38.7 | ··· |
| Native, pH 12.0 | 17.2 | 16.0 | 33.2 | 0.8 |
| Hemoglobin | | | | |
| Native, pH 7.0 | 1.0 | 0.0 | 1.0 | 0.0 |
| Native, pH 12.0 | 1.3 | 0.0 | 1.3 | 0.0 |

[a]Total number of SH determined after reduction with mercaptoethanol in the absence of 6*M* guanidine-HCl minus the number of SH determined without reduction.
[b]m/m: Concentrations in mole-per-mole protein.
[c]Total -S- minus surface -S-.
[d]Same as "a" except that reduction was accomplished in the presence of 6*M* guanidine-HCl.
[e]Sulfhydryl determined without reduction in the presence of sodium dodecyl sulfate.
[f]Data from Simard and Boulet (1978b).

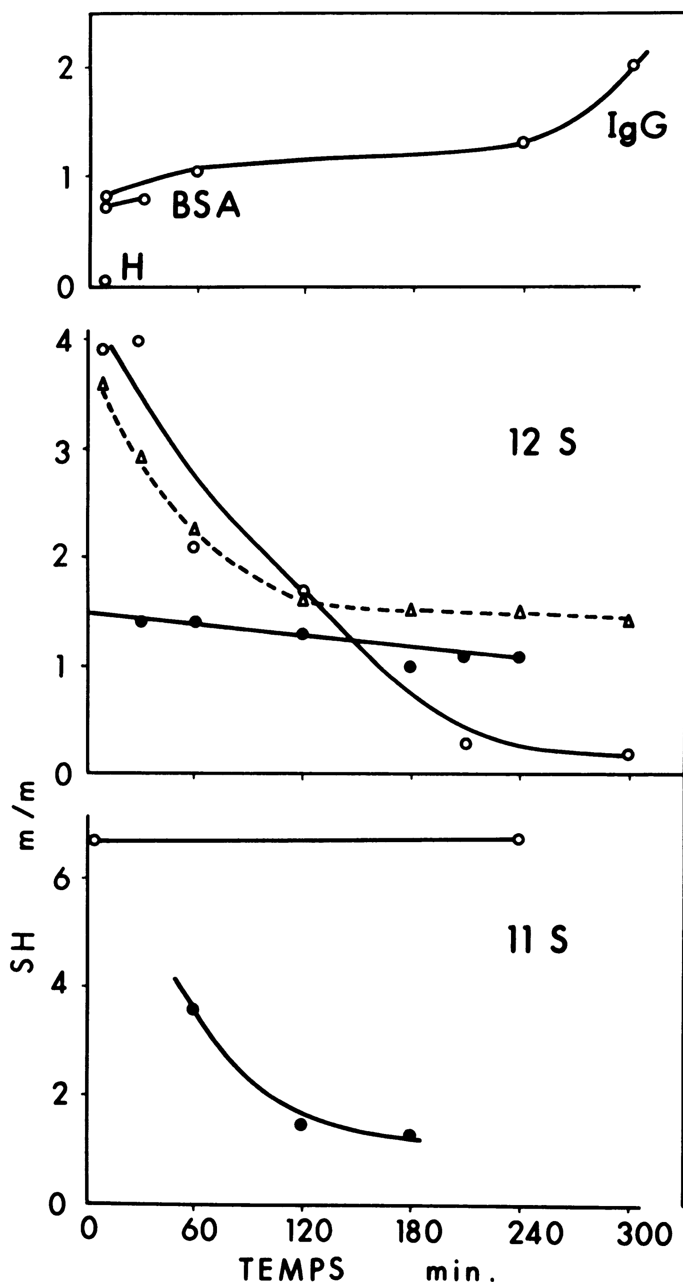


**Fig. 2.** Changes in sulfhydryl contents of bovine serum albumin (BSA), immunoglobulin G (IgG), 12S rapeseed protein, 11S soybean protein, and hemoglobin (HG) with time, following addition of alkali to pH 12.0. (———) or pH 10.0 (- - -), o or △ = native protein, • = heat-denatured protein.

the interior of the protein molecule was markedly modified (Table I) in favor of surface groups by the alkali treatment. Unfolding of the protein molecules with alkali was sufficient to expose most of the SS groups of the 11S and 12S proteins, but not with IgG, which retained more than half of its interior -S- groups. Figure 3 shows the effect of the concentration of guanidine-HCl on the apparent -S- content of the proteins. At pH 7.0, the protein molecules apparently unfolded at similar guanidine-HCl concentrations with the IgG and the 12S proteins, ie, about 2 mole/l and at higher concentration (4 mole/l) with the 11S protein. At pH 12.0, only IgG requires a relatively high level of guanidine-HCl (3*M*) for complete exposure of -S- groups.

Table I and Fig. 3 show that the treatment with alkali for 15 min decreased the total number of measurable -S- groups in all the cystine-containing proteins. Measurements of free SH groups (Fig. 2 and Table I) showed an increase of slightly over one group per mole in 11S and 12S proteins, following the alkali treatment. These new groups may have originated from SS groups as a result of hydrolysis such as $R\text{-}S\text{-}S\text{-}R + OH^- \; RS^- + RSOH$. But this appeared as a relatively small contribution to the total loss of -S- groups, which was 13.8 for 11S, 4.1 for 12S, and 5.5 for IgG. Hemoglobin contained only one -S- and no SH group.

In a review of sulfur chemistry of proteins, Cecil and McPhee (1959) concluded that the main mechanism for the alkaline decomposition of cystine and cysteine residues in proteins is that of $\beta$-elimination with the formation of the $\alpha$-amino acrylic acid derivative dehydroalanine. This was confirmed more recently by the work of Nashef et al (1977).

Dehydroalanine is a highly reactive intermediate compound found to react with other amino acid residues to form the new amino acids lanthionine, lysinoalanine, and aminoalanine (Friedman 1979, Nashef et al 1977).

Molecular interactions of proteins at high concentrations are largely caused by noncovalent forces (or bonds) like electrostatic, hydrogen and hydrophobic, and possibly by intermolecular SH-SS exchange. Intramolecular SH-SS exchange may indirectly affect molecular interactions by modifying surface characteristics of the protein molecules.

The results obtained in this work show that SH groups are rapidly destroyed at pH 12.0 and that SS groups decreased rapidly following addition of alkali.

Losses of cystine in soybean protein solutions treated with alkali at pH 13.3 are progressive with time until only 27% of the original number remains (de Groot and Slump 1969). In addition, Friedman (1979) showed that all cystine and half-cystine are eventually lost in 1% wheat gluten and in lactalbumin solutions exposed to pH 11.2 for 3 hr at 65°C. The loss of SS probably was due in part to hydrolysis but mainly to other types of reactions such as the known $\beta$-elimination. Intra- and intermolecular SH-SS interchange may not play any direct role in the viscosity increase in alkaline protein solutions.

Between pH 10.0 and 12.0, all of the lysine and part of the arginine residues lose their charges. Under these conditions, intra- and intermolecular electrostatic charge repulsion increases, which should favor molecular dissociation and unfolding. This was observed in diluted solutions of 11S soybean protein (Diep and Boulet 1977, Diep et al 1982) and is again supported by the very large increase in viscosity at pH 12.0 as compared to pH 10.0, which was observed in this work (Fig. 1).

Protein unfolding appears to be a prerequisite for the viscosity increase of protein solutions at high pH. Under conditions of high protein concentrations and exposed hydrophobic regions, the molecules tend to associate into a micellelike or progel state (Catsimpoolas and Meyer 1970) and may eventually aggregate into a gel lattice. This extreme situation was observed with a 10% BSA

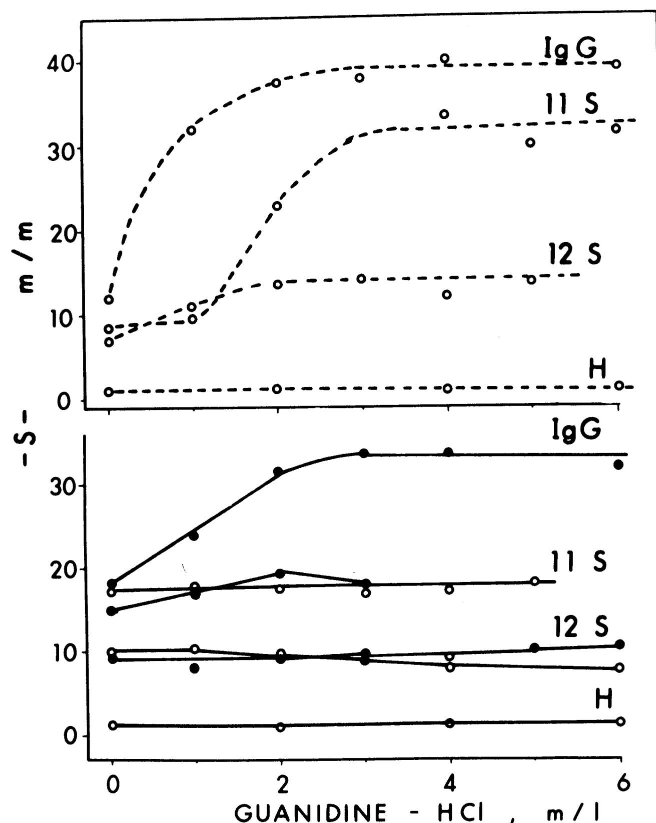


**Fig. 3.** Effect of guanidine-HCl concentration on the apparent half-cystine (-S-) content of 12S rapeseed protein, 11S soybean protein, and immunoglobulin G: o = native protein, • = heat-denatured proteins. - - - = protein at pH 7.0; ——— = protein exposed to pH 12.0 during 15 min, then returned to pH 7.0.

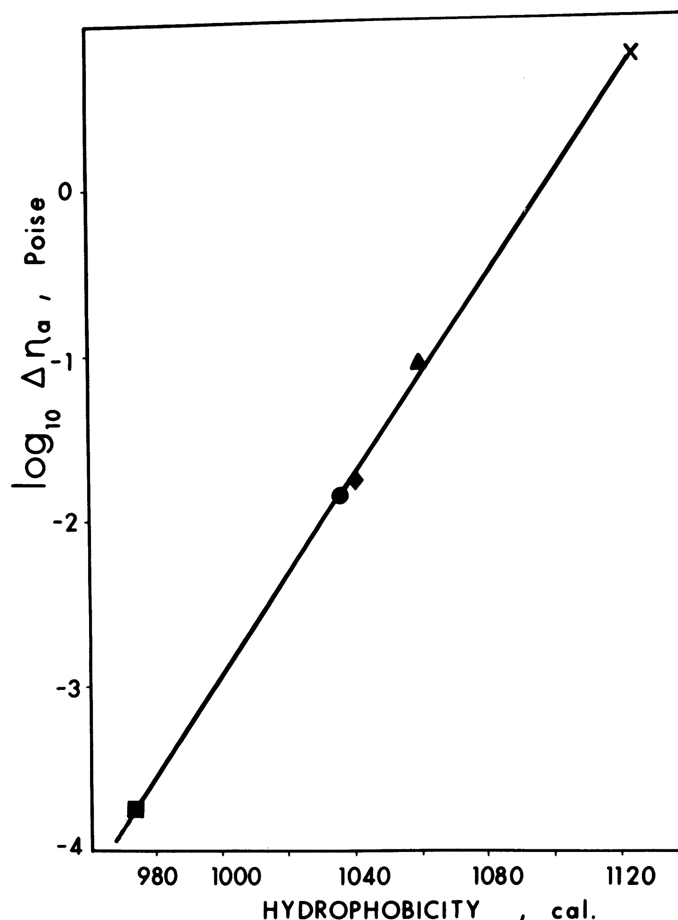


**Fig. 4.** Relation between the slope of the viscosity curves ($\Delta\eta_a$) and Bigelow's hydrophobicity values of 11S (■), 12S (●), IgG (◆), HG (△), and BSA (x) proteins. The $\Delta\eta_a$ value was the initial slope of the curve: the first 2 hr with 12S, IgG and HG, 15 min for BSA. The $\Delta\eta_a$ for the 11S protein was estimated.

solution at pH 12.0 that was rapidly transformed into a solid gel. The importance of hydrophobic interactions on the rate of development of viscosity in the protein solutions at pH 12.0 is shown in Fig. 4; the rate of increase of viscosity with time increases in log relationship with Bigelow's hydrophobicity value (Bigelow 1967) of the different proteins.

As the protein molecules unfold and their hydrophobic interior is exposed to the environment, they tend to associate with a rate related to the hydrophobic character of the protein. Denatured 11S and 12S proteins deviate from this relationship as other proteins may also do, because other protein characteristics may influence unfolding. Ishino and Okamoto (1975) have shown, with alkali-denatured proteins, that the increase in viscosity at pH between 11.5 and 12.8 was accompanied by an increase in the amount of heptane retained by the protein. This was taken to mean that at these pHs, hydrophobic regions of the protein become exposed and that hydrophobic bonds may play a part in establishing a three-dimensional network.

Intramolecular covalent SS bonds are, of course, a factor of structure stability, but in alkaline solution, these may eventually be replaced by other types of covalent bonds such as those established between $\alpha$-amino acrylic residues and other residues such as lysine. Establishment of these bonds would be structure-dependent, which may explain the difference in viscosity changes between the heated and the native proteins.

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