Starch-Gel Electrophoresis of Soybean Globulins[1]

G. PUSKI and **P. MELNYCHYN**, Carnation Research Laboratories,
Van Nuys, California 91412

ABSTRACT

Soybean globulins, isolated fractions (7S, 11S, and cold-insoluble fraction), and alkylated derivatives of soybean globulins were examined by starch-gel electrophoresis in alkaline and acidic buffer systems. The alkaline gel was made with 0.0825*M* *tris*-chloride solution, pH 8.7, and contained 5*M* urea and 0.1*M* mercaptoethanol. A 0.05*M* *tris*-0.35*M* glycine-3*M* urea 0.05*M* mercaptoethanol buffer, pH 8.4, was used as the bridge solution. The acid gel was prepared in 1*N* acetic acid-5*M* urea buffer, pH 3.5, and 1*N* acetic acid was used as the bridge solution. Protein samples were dissolved in the *tris*-glycine or acetate buffers which contained 6*M* urea and 0.1*M* mercaptoethanol. Soybean globulins manifested 14 and 15 readily discernible bands in the alkaline and acidic media, respectively, as well as some poorly resolved bands. The electropherograms were unaffected by phytates. Electrophoretically, 7S and 11S fractions were heterogeneous and were not comprised of the same proteins. The cold-insoluble fraction had all the 11S components, but also contained other proteins. At least three constituents of the 11S and CI fraction possess strong basic character, since they migrated toward the cathode at pH 8.7. Reaction of soybean globulins with iodoacetate, N-ethylmaleimide, and acrylonitrile was enhanced by the presence of urea. Derivatives did not give identical electrophoretic patterns.

Soybean proteins have been studied since 1883 (1), but until recently (2) limitations in methodology precluded full realization of the number of constituent proteins in the globulin fraction. This is the fraction which has been loosely referred to as soybean globulins (3, 4, 5), soybean caseins (6), acid-precipitable soybean proteins (7), or glycinin (8, 9). It is extracted from defatted soybean flakes with water, alkaline, or salt media and isolated by precipitation at pH 4.5 or by dialysis against water.

Early workers relied almost solely on the solubility characteristics of proteins to effect fractionation. Thus Osborne and Campbell (8) and later others (10, 11, 12) isolated the principal soybean protein which had been named as glycinin (8). However, all glycinin preparations were heterogeneous (13). Smith and Rackis (14) later reported that some of the heterogeneity of glycinin was due to formation of dissociable protein-phytate complexes, and Briggs and Mann (13) also showed that a "cold-insoluble" (CI) fraction was a constituent of glycinin.

Ultracentrifugal data showed that reduced soybean globulins in alkaline buffer manifest four resolvable constituents corresponding to $S_{20,w}$ values of 2S, 7S, 11S, and 15S (15, 16), and an unresolvable fraction of $S_{20,w}$ value greater than 15S (16). The ultracentrifugal pattern was influenced by ionic strength, neutral salts, urea, and pH (9, 15, 16, 17, 18). For instance, in acidic media three resolvable constituents were found with $S_{20,w}$ values of 2S, 7S, and 13S (9), whereas in highly alkaline media, 3 to 5S components were observed (17). On the other hand, in 6*M* urea there was a preponderance of material in the 1 to 2S region and a small amount of 4S component (17).

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Some of these constituents were recently isolated by gel filtration on Sephadex (5, 6) and calcium phosphate gel columns (19).

Surprisingly, soybean globulins and isolated fractions have not been studied by gel electrophoresis, although, as our work was in progress, Shibasaki and Okubo described starch-gel electropherograms in an alkaline buffer system (2). Their procedure differs from ours and they did not describe electrophoresis in acidic media.

MATERIALS AND METHODS

Defatted soybean flakes (ADM-7B) were obtained from Archer-Daniels-Midland Co. The purified 7S component (3) was supplied by D. R. Briggs, University of Minnesota, and 11S and CI fractions (19, 20, 21) were secured from W. J. Wolf and A. C. Eldridge, Northern Regional Research Laboratories.

Hydrolyzed starch for gel electrophoresis was manufactured by Connaught Medical Research Laboratories, University of Toronto (Lot No. 249-1).

Buffer Solutions

Solution A: 0.0825*M* *tris* titrated to pH 8.7 with 6*N* hydrochloric acid.

Solution B: 0.05*M* *tris*-0.35*M* glycine-3*M* urea buffer, pH 8.4, made 0.05*M* with respect to mercaptoethanol.

Solution C: 0.05*M* *tris*-0.35*M* glycine-6*M* urea buffer, pH 8.4, made 0.1*M* with respect to mercaptoethanol.

Solution D: 1*N* acetic acid.

Solution E: 1*N* acetic acid-6*M* urea buffer, pH 3.5, made 0.1*M* with respect to mercaptoethanol.

Staining Reagent

A 0.1% solution of Amido Black 10B (Buffalo Black) in 15% acetic acid solution.

Destaining Solution

Glycerol solution, 5%, in 2% acetic acid.

Phosphorus Determination

The Fiske and Subbarow (22) method was used as modified by Joe *et al.* (23).

Preparation of Soybean Proteins

Isolated soybean globulins and CI fraction were prepared as shown in Figs. 1 and 2, respectively. These methods vary only slightly from those given in the literature (2, 4, 12, 13).

Phytate removal was attempted by: (a) dialysis of soybean globulins at pH 6.5 against water followed by treatment with Dowex 1-X10 (14); (b) dialysis against 1*N* sodium chloride (24); and (c) washing the acid-precipitated proteins with saturated ammonium sulfate solution, and subsequent exhaustive dialysis against water (24).

Preparation of Alkylated Derivatives of Soybean Globulins

Cyanoethyl derivatives were made according to the method described by Weil and Seibles (25).

The iodoacetate and N-ethylmaleimide (NEMI) derivatives were pre-

pared as follows: A 5% protein solution at pH 8 was made 0.1*M* with respect to mercaptoethanol, stirred for 1 hr. at room temperature, then made 0.11*M* with respect to the reagent (iodoacetate or NEMI), which was added

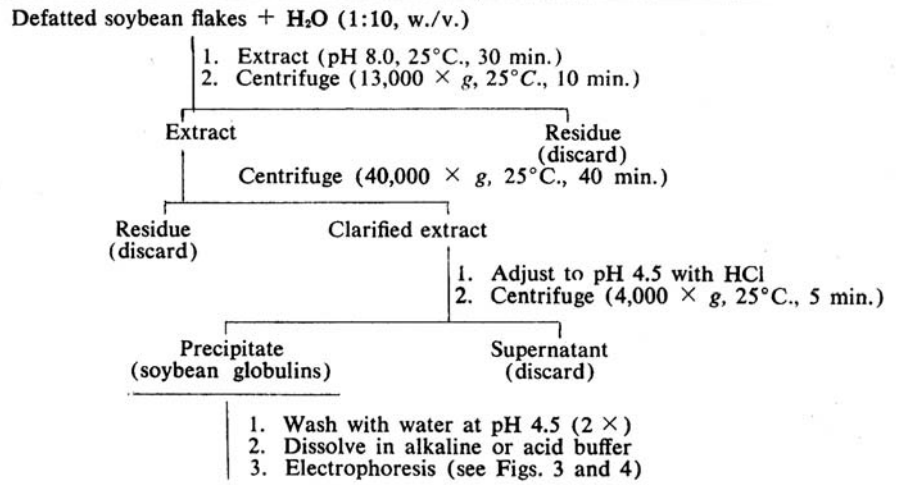


Fig. 1. Isolation of soybean globulins.

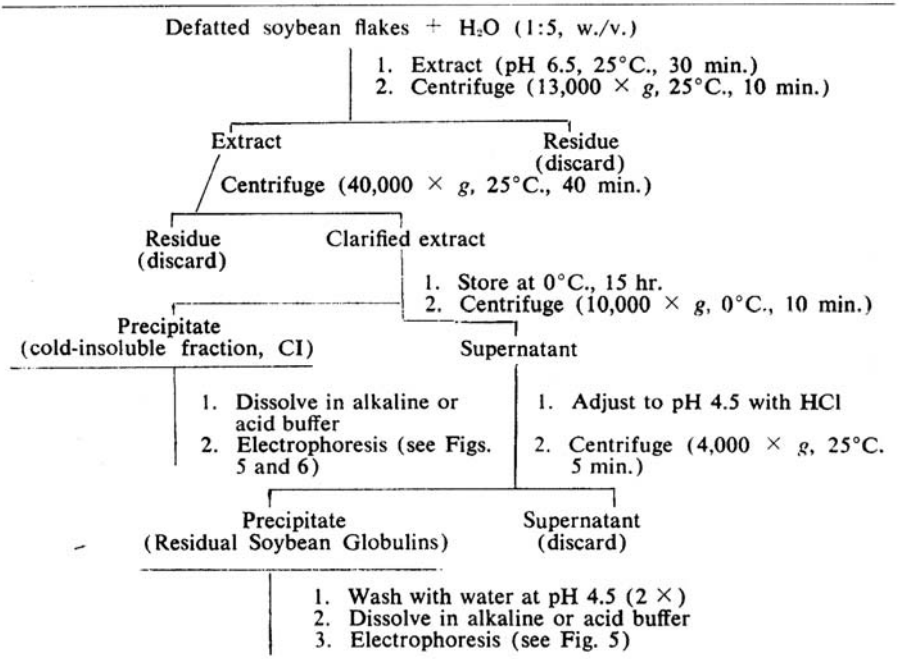


Fig. 2. Isolation of CI fraction and residual soybean globulins.

in a crystalline state. A constant pH was maintained and reaction was conducted for 1 hr. Reduction and alkylation were then repeated as outlined above. The proteins were precipitated at pH 4.5, centrifuged, washed once with deionized water, and dissolved in the respective buffers (solution C or E) for electrophoretic analysis.

The above derivatives were also prepared in the presence of 6*M* urea, which, however, after reaction was removed by exhaustive (48-hr.) dialysis against deionized water prior to precipitation of proteins.

Gel Electrophoresis—Alkaline Gel

A vertical starch-gel-electrophoretic apparatus was used (26). The basic procedure was as described by Smithies (27, 28) and Wake and Baldwin (29), but application of the technique to soybean proteins necessitated sufficient variation in methodology to warrant detailed description of the procedure. Two electrophoretic buffer systems were developed, one at an alkaline and another at an acid pH.

The alkaline gel was prepared by addition of 75 g. of hydrolyzed starch to 500 ml. of solution A. The slurry was heated and stirred continuously with a heavy-duty mechanical stirrer. As the viscosity of the starch increased, stirring action also was increased to prevent charring. Heating was discontinued when the mixture attained a temperature of 72°C. (about 5 min.), then 170 g. urea and 4 ml. mercaptoethanol were immediately added, and stirring was continued until all urea dissolved. The mixture was transferred into a 4-liter suction flask and evacuated with a water-aspirator until entrained air was exhausted. Meanwhile, the gel form was readied; it was overlaid with a fiber-glass net, the end plates were attached, and the net was moistened with solution C. (Fiber-glass net was used to facilitate removal of the gel on conclusion of the electrophoresis.) The cover plate (26) was covered with Saran film to prevent its adherence to the gel, except in the area of the slot formers, which was sprayed with a silicon release spray (Dow Corning).

The starch sol was then poured into the gel form, and the cover plate was positioned and weighted. The starch sol was allowed to set for at least 20 hr. at 25°C., after which time the cover plate and end plates were removed, but the Saran film was left on the gel. The slots were filled with solution containing 1 to 3% protein (dissolved overnight in solution C); covered with molten petroleum jelly (m.p. about 50°C.); the entire gel surface was covered with Saran.

The apparatus was then set up for electrophoresis as described by Smithies (26). We differed in our operation in using solution B as bridge solution, flannel cloth instead of paper as the connecting bridge, and platinum instead of silver-silver chloride electrodes. The apparatus was kept in a room maintained at 4°C. and thermal convection was minimized by circulating cold water (4°C.) through the cooling plate. A voltage of 12.5 volts/cm. was applied for 7 hr. During this time, the solvent front traveled about 25 cm.

At the conclusion of electrophoresis the cover film and petroleum jelly seal were removed and ends of the gel were trimmed. With the fiber-glass net, the gel was removed from the apparatus; it was then placed on a flat

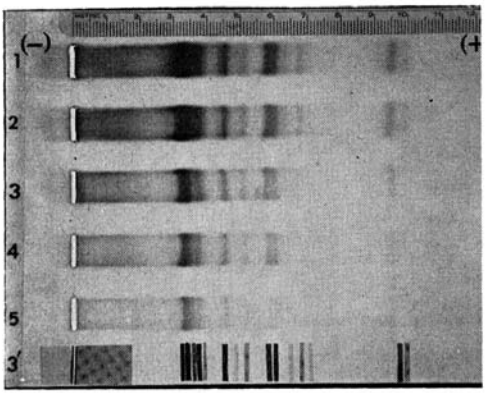
surface and sliced into three longitudinal sections; a piano wire was used for cutting, and different-sized rods for guiding. The sections were separated by forcing water between the slices. The middle section was saved and stained for 30 min., then destained by repeated washings with the destaining solution. When the background was sufficiently clear, the gel was placed on a smooth glass surface and covered with a sheet of fine-textured paper soaked in washing solvent. Air bubbles between the surfaces were carefully removed and the gel was air-dried for about 2 days. The paper cover was then moistened somewhat and removed, and drying was continued. The dried gel was stored between sheets of Saran film.

Gel Electrophoresis—Acid Gel

In preparation of the acid gel for electrophoresis, the following modifications were made from the procedure described for the alkaline gel. Solution D was used, and the gel contained 72 g. starch, 185 g. urea, no mercaptoethanol. Solution E was used to dissolve the protein samples and to moisten the fiber-glass net. Solution D was used as bridge solution. A voltage of 11 volts/cm. was applied during electrophoresis.

RESULTS AND DISCUSSION

Electropherograms of soybean globulins, shown in Figs. 3 and 4, reveal



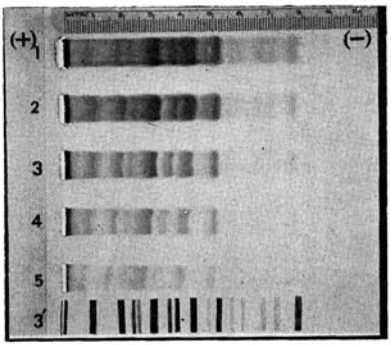


Fig. 3 (left). Starch-gel electropherograms and schematic diagram of soybean globulins in 0.0825*M* *tris*-chloride buffer, pH 8.7, containing 5*M* urea and 0.1*M* mercaptoethanol (7 hr. at 12.5 volts/cm.): 1, 4%; 2, 2%; 3, 3′, 1%; 4, 0.5%; 5, 0.25% protein, respectively.

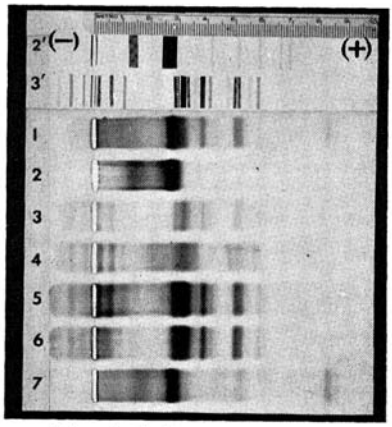
Fig. 4 (right). Starch-gel electropherograms and schematic diagram of soybean globulins in 1*N* acetic acid-5*M* urea buffer, pH 3.5 (7 hr. at 11 volts/cm.): 1, 4%; 2, 2%; 3, 3′, 1%; 4, 0.5%; 5, 0.25% protein, respectively.

many bands stained to various intensities. These differences may be indicative of concentration of the respective protein constituents or may be a manifestation of adsorbability of the stain by the proteins. Patterns were reproducible for different preparations, even though an indeterminant number of proteins were poorly resolved. Fourteen bands were observed in the alkaline system, whereas 15 were detected in the acid gel. Resolution was best when urea was incorporated into gel and buffers; otherwise, many of the proteins did not penetrate the gel matrix. In addition, in the alkaline gel, it was

necessary to use reduced proteins and maintain them in a reduced state; otherwise, some protein stayed at the starting slot. Presumably this behavior is due to polymer formation through disulfide bonds (4, 30). However, in the acid gel, which did not contain mercaptoethanol, both reduced and non-reduced proteins could be analyzed simultaneously. Apparently reoxidation of free sulfhydryl groups is not favored, or occurs very slowly under the acidic conditions used for electrophoresis.

In the alkaline gel (Fig. 3) there were some poorly resolved constituents which migrated toward the cathode. Migration in this direction at pH 8.7 indicates a high positive charge density on the proteins and implies that some of them have pronounced basic character. Since reverse migration occurred only when urea was incorporated in the buffers, Shibasaki and Okubo suggested that the phenomenon was due to complex formation between the proteins and isocyanate derived from urea (2). It is not likely that isocyanate formation would occur under the conditions of electrophoresis (31) and, even if isocyanates did form, and react, the proteins would not be expected to possess basic character. Furthermore, this phenomenon should be manifested by all protein fractions and not restricted only to the CI or 11S (Fig. 5). These basic proteins should be regarded as indigenous constituents of soybean globulins which are resolved only when urea is incorporated into the system.

Electropherograms of purified 7S, 11S, and CI fractions in alkaline and acidic systems are shown in Figs. 5 and 6. In the alkaline gel, the 7S



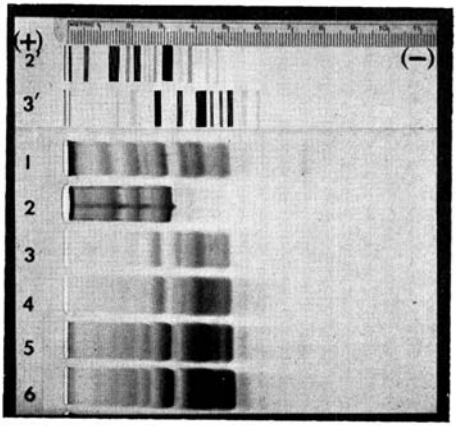


Fig. 5 (left). Starch-gel electropherograms and schematic diagrams of soybean globulins in 0.0825*M* *tris*-chloride buffer, pH 8.7, containing 5*M* urea and 0.1*M* mercaptoethanol (7 hr. at 12.5 volts/cm.): 1, unfractionated soybean globulins; 2, 2′, 7S; 3, 3′, 11S (Eldridge's); 4, 11S (Wolf's); 5, CI (Wolf's); 6, CI (Carnation's); 7, residual soybean globulins.

Fig. 6 (right). Starch-gel electropherograms and schematic diagrams of soybean globulins in 1*N* acetic acid-5*M* urea, pH 3.5 (7 hr. at 11 volts/cm.): 1, unfractionated soybean globulins; 2, 2′, 7S; 3, 3′, 11S (Eldridge's); 4, 11S (Wolf's); 5, CI (Wolf's); 6, CI (Carnation's).

component exhibits two major and five minor bands, whereas in the acid gel three major and nine minor bands can be seen. The 11S component mani-

fested 18 bands in the alkaline and 10 bands in the acid gel. The constituents of the 7S fraction are not identical with those of 11S; therefore, these fractions cannot exist in equilibrium as suggested by Kretovich *et al.* (32, 33). Differences between the two 11S preparations are attributable to the fact that they were prepared by different methods. The CI fraction shows all the bands present in the 11S component and, in addition, includes some 7S and other proteins.

One might expect to see many bands in both the 7S and 11S moieties, since Roberts and Briggs (3) reported that the 7S fraction may consist of a minimum of five different peptide chains and Mitsuda *et al.* (34) found at least three different N-terminal amino acids in the 11S fraction. Our results show that both of these components are, in fact, even more complicated than heretofore realized. Even though only a few different N-terminal groups have been identified, there are a large number of different polypeptides in the 7S and 11S moieties.

At present it is impossible to correlate the bands on the electropherograms with specific soybean proteins. Interpretation is complicated by the fact that urea is a dissociating agent (17, 19, 35), so some bands may be attributed to protein complexes at various stages of association or dissociation. The same argument can be applied to the effect of mercaptoethanol. However, we believe that most of the bands must be attributed to discrete proteins, since length of time in buffer solution (as long as 4 days) did not show any change in the electropherograms. This conclusion is also supported by the observation that individual constituents isolated by chromatography in 4*M* urea and 0.01*M* mercaptoethanol gave a single band on gel electrophoresis (36).

Since there is still the possibility that some of the bands may be due to complexes containing varying amounts of proteins and phytates, soybean globulins were examined after phytate removal. Earle and Milner (37) observed that phytate phosphorus represents about 80% of the total quantity of this element in soybeans, so phosphorus content is a good approximation of phytin content. Our analytical data indicated that more than 70% of the total phosphorus was removed (Table I), but the electrophoretic patterns did not change (Fig. 7). The similarity in appearance between the treated and nontreated soybean globulins suggests that either urea alone is a protein-

TABLE I

PHOSPHORUS CONTENT OF ISOLATED SOYBEAN GLOBULINS AFTER VARIOUS TREATMENTS

SAMPLE	TOTAL P	P REMOVED BY TREATMENT
	g./16 g. N	*% of total P*
Soybean globulins, control	0.526	.....
Soybean globulins dialyzed *vs.* water at pH 6.5, then treated with Dowex 1-X10 (14)	0.145	72.5
Soybean globulins dialyzed *vs.* 1*N* NaCl (24)	0.166	68.5
Soybean globulins washed with saturated ammonium sulfate at pH 4.5, and exhaustively dialyzed *vs.* water (24)	0.156	70.2

phytate-dissociating agent, or the remaining phosphorus is sufficient to stabilize the complex.

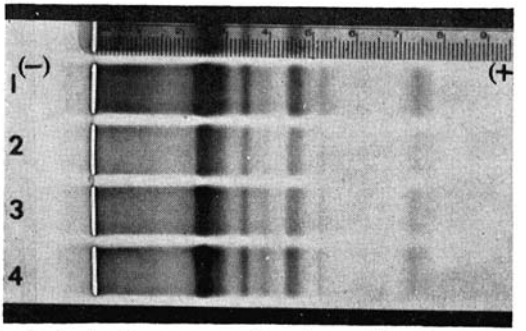
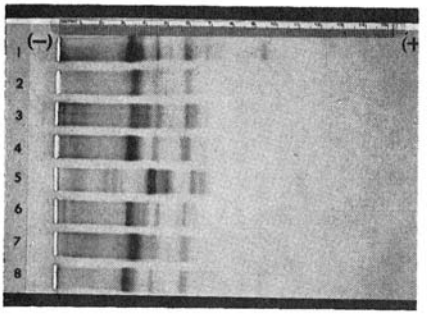


Fig. 7. Starch-gel electropherograms of soybean globulins in 0.0825*M* *tris*-chloride buffer, pH 8.7, containing 5*M* urea and 0.1*M* mercaptoethanol (7 hr. at 12.5 volts/cm.), before and after removal of phytates: 1, untreated control; 2, dialyzed *vs.* water at pH 6.5, then treated with Dowex 1-X10 (14); 3, dialyzed against 1*N* NaCl (24); 4, washed with saturated ammonium sulfate at pH 4.5, and exhaustively dialyzed against water (24).

As this paper is concerned with application of gel electrophoresis to studies of soybean proteins, and since protein studies inevitably involve formation of derivatives, alkylated soybean globulins were examined. Products of reactions with iodoacetate, acrylonitrile, and N-ethylmaleimide were prepared in the presence and absence of 6*M* urea. The results, given in Figs. 8 and 9, show how the alkylating agent and solvent systems affect the patterns



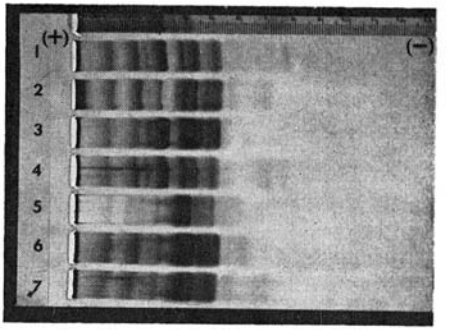


Fig. 8 (left). Starch-gel electropherograms of soybean globulins in 0.0825*M* *tris*-chloride buffer, pH 8.7, containing 5*M* urea and 0.1*M* mercaptoethanol (7 hr. at 12.5 volts/cm.): 1 and 8, soybean globulins, control; 2, 3, and 4, cyanoethyl, iodoacetate, and N-ethylmaleimide derivatives, respectively, prepared in the absence of 6*M* urea; 5, 6, and 7, cyanoethyl, iodoacetate, and N-ethylmaleimide derivatives, respectively, prepared in the presence of 6M urea.

Fig. 9 (right). Starch-gel electropherograms of soybean globulins in 1*N* acetic acid-5*M* urea, pH 3.5 (7 hr. at 11 volts/cm.): 1, soybean globulins, control; 2, 3, and 4, cyanoethyl, iodoacetate, and N-ethylmaleimide derivatives, respectively, prepared in the absence of 6*M* urea; 5, 6, and 7, cyanoethyl, iodoacetate, and N-ethylmaleimide derivatives, respectively, prepared in the presence of 6*M* urea.

obtained. It is apparent that, compared with the control, urea enabled all of the reagents to be more effective alkylating agents. This effect could be

attributed to the influence of urea upon conformational structure of the proteins making the functional groups more amenable to react. Moreover, these agents are not identical in reactivity. Alkylation of a mixture of proteins, such as soybean globulins, makes it impossible to define specific effects of each reagent.

For a better understanding of soybean proteins, it is necessary to isolate individual protein constituents. Starch-gel electrophoresis is a good analytical tool to demonstrate the effectiveness of the various purification steps, or to observe any modification of the proteins.

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