Hydrolysis of Calf Skin Tropocollagen by Pepsin, Trypsin, Chymotrypsin, and Elastase

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To determine the influence of proteolytic enzymes upon collagen, acid-soluble calf skin collagen was treated with pepsin, trypsin, chymotrypsin, and elastase. The dialyzable materials were separated by two-dimensional electrophoresis and chromatography. Differential staining showed four tyrosine-containing components following trypsin digestion, three tyrosine-positive areas in the pepsin and chymotrypsin digests and only one in the elastase digest. The major components were acidic. Changes in the protein subunits were monitored using disk gel electrophoresis. Chymotrypsin and elastase treatment significantly changed the banding patterns. Only small amounts of carbohydrate were shown to be present, and stained in the familiar α-, β-, and γ-component pattern, with two additional bands at the solvent front. The effectiveness of the enzymes in preventing protein aggregation was: pepsin < trypsin < chymotrypsin < elastase.

Collagen is an integral part of all bones, tendons, teeth, and skin, as well as a supporting element for all organs and tissues of the body. Therefore, any changes in body collagen could affect many vital processes. Because of the universal presence of collagen in muscle, it has obvious implications in meat science.

The nature and structure of collagen were thoroughly reviewed by Harrington and von Hippel (1961), while Harding (1965) reviewed the crosslinks of collagen. Several studies on the effects of proteolytic enzymes on acid-soluble calf skin collagen have recently been reported (Drake *et al.*, 1966; Kühn *et al.*, 1966; Rubin *et al.*, 1965). Rubin *et al.* (1965), Drake *et al.* (1966), and Kühn *et al.* (1966) have shown that proteolytic enzymes are able to change the aggregation properties of the enzyme-treated collagen, as well as the α- to β-component ratio. Furthermore, the dialyzable materials following enzymatic treatment have been shown to be acidic in nature and to contain large amounts of tyrosine (Drake *et al.*, 1966; Rubin *et al.*, 1965).

Hodge *et al.* (1960) and Bensusan and Scanu (1960) indicated that tyrosine was a key component governing the ability of neutral salt solutions of collagen to form fibrils when warmed to 35° C. Recently, Dabbous (1966) followed the effects of a tyrosinase on soluble collagen by monitoring the fluorescence spectra of enzyme-treated and untreated collagen. The results showed a shift in the fluorescence maximum from 305 mμ for the untreated sample to a broader maximum between 304 and 350 mμ for the enzyme-treated sample.

The goals of the present study were to separate the dialyzable materials resulting from pepsin-, trypsin-, chymotrypsin-, and elastase-treatment of acid-soluble calf skin collagen by two-dimensional high-voltage paper electrophoresis and chromatography. Changes in the treated protein were monitored using disk gel electrophoresis and thermal gelation techniques.

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MATERIALS AND METHODS

The acid-soluble calf skin collagen utilized throughout this study was extracted and purified according to the procedure outlined by Rubin *et al.* (1965). The reagents were all of reagent grade. The water was distilled and deionized. The dialysis tubing was treated as described by Drake *et al.* (1966). The enzyme reaction media was calcium acetate (0.05 to 0.1*M*) adjusted to the desired pH with NH_4OH for all enzymes except pepsin. The reactions for pepsin were carried out in 0.05% acetic acid at pH 3.5.

Enzymatic reactions and recovery of the dialyzates were carried out as described by Drake *et al.* (1966), except that the digestion period lasted 24 hours. Following digestion, the reaction mixtures and controls were dialyzed against fresh reaction medium for 24 hours at 4° C.

Nitrogen determinations were made by the micro-Kjeldahl procedure and protein concentrations were determined by this method and by comparison with absorbancies at 230 mμ. Hydroxyproline determinations were made according to Woessner (1961). Chromatographic tests for tyrosine, ninhydrin-positive areas, and peptides were by the procedures of Easley (1965). Proteins and carbohydrates on the disk gels were detected by the methods described by Smithies (1955) and Keyser (1964), respectively.

Disk gel electrophoresis used the method of Nagai *et al.* (1964) with the following modifications: No sample gel was used and the upper gel was not photopolymerized. The ammonium persulfate concentration was 0.4% and cyanogum (E-C Apparatus Corp.) was used instead of acrylamide and *N,N'*-methylenebisacrylamide. The upper gel was 3.1 and the lower gel was 7.5% cyanogum. The denatured protein was layered on the upper gel in concentrated sucrose to prevent mixing with the running buffer.

An Aminco-Bowman spectrophotofluorometer was used for comparison of the relative fluorescence intensity of the enzyme-treated protein. Standard solutions of

L-tyrosine and L-tryptophan in dilute NaOH were also compared with the native soluble collagen. All fluorescence maxima are expressed relative to L-tyrosine at a maximum of 303 mμ (Teale and Weber, 1959), which was used to calibrate the instrument. Tryptophan standards showed a fluorescence maximum at a wavelength of 330 mμ, which falls within the range of 320 to 350 mμ reported by Teale and Weber (1959).

High-voltage paper electrophoresis was performed in an apparatus similar to that used by Katz *et al.* (1959). An acetic acid–formic acid buffer, pH 2 (Efron, 1960), was employed with Whatman 3 MM chromatographic paper. The voltage was approximately 70 volts per cm. developing about 5 to 6 ma. per cm. for 25 minutes (Gross, 1955). Following electrophoresis, the paper was dried and subjected to ascending paper chromatography in a butanol–acetic acid–pyridine–H_2O (15:3:10:12) solvent system (Randerath, 1964). Chromatography required approximately 6 hours, after which the paper was dried and color reactions were developed as described by Easley (1965).

Thermal gelation and fibril formation were initiated by mixing equal volumes of soluble collagen in 0.05% acetic acid and a NaH_2PO_4-Na_2HPO_4-NaCl buffer of pH 7.4 and an ionic strength of 1.2*M* (0.45*M* NaCl). Short-term gelation studies were continuously monitored at 230 mμ in a Beckman DU-2 monochronometer with a Gilford automatic cuvette positioner and absorbance converter (Gilford Instrument Co.) connected to a Sargent recorder (Model L SR, E. H. Sargent and Co.). The temperature during short-time gelation was approximately 33° C. Long-term gelation was conducted by placing the protein-salt mixtures in an incubator at 35° C. for 24 hours.

Disk electrophoresis of the protein samples used in these long-term gelation experiments were conducted as described above; however, the protein was treated as follows: Samples of the protein-salt mixture were dialyzed free of salts before thermal gelation and used as controls. Following thermal gelation of other samples, the precipitated protein was removed by filtration. The filtrate was dialyzed free of salts against 0.05% acetic acid and concentrated by pervaporation. These concentrated solutions were then subjected to disk gel electrophoresis to observe changes in the banding patterns.

RESULTS AND DISCUSSION

Protein Purity. Hydroxyproline analysis indicated 14.2%, in good agreement with the values reported by Woessner (1961) and Neuman and Logan (1950). Specific fluorometric analysis verified the purity of the collagen preparation, which exhibited a fluorescence maximum at 300 mμ, approximately the same wavelength as L-tyrosine (Figure 1). Thus, the collagen preparation appeared to be free from tryptophan-containing proteins.

A sample of collagen prepared in the same manner but lyophilized and then redissolved in 0.05% acetic acid prior to measurement of fluorescence showed a shift in the fluorescence maximum to 323 mμ. Although the reason for the shift is unknown, the fluorescing residues of collagen may be slightly altered by lyophilization of the protein.

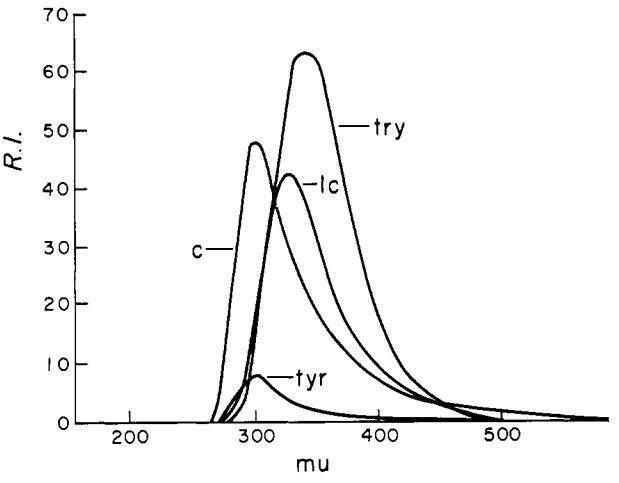

Figure 1. Fluorescence spectra of tyrosine, tryptophan, and lyophilized and nonlyophilized acid-soluble calf skin collagen excited at wavelength 280 mμ

R.I. Relative intensity
tyr. Tyrosine (0.5 μg. per ml.)
try. Tryptophan (0.5 μg. per ml.)
c. Nonlyophilized collagen
lc. Lyophilized collagen

Dialyzates. Composite two-dimensional chromatograms of the dialyzates were prepared. The composite consists of two chromatograms, one stained with ninhydrin and one with a chlorination stain. Chlorination staining is indicated only if it differed from ninhydrin.

Figures 2, 3, 4, and 5 show tracings of the composite chromatograms minus the control. Earlier work in this laboratory has shown that most of the spots on the composite chromatograms were due to nitrogenous material originating from the dialysis tubing (Crevasse and Pearson, 1969), whereas the composite minus the control shows the dialyzable material due to enzymic hydrolysis. Comparison of the electrophoretically slower moving components—i.e., spots directly above the origin (0)—reveals that the leading spot resulting from trypsin (Figure 3), elastase (Figure 5), and chymotrypsin (Figure 4) digestion has a similar R_f value. The average R_f values from duplicate samples of each enzymatic digest were 0.72, 0.73, and 0.73 for trypsin, elastase, and chymotrypsin digests, respectively. However, only the spot from trypsin digestion was positive to tyrosine staining.

Chymotrypsin digests, when analyzed by electrophoresis in only one dimension, showed a positive tyrosine staining area near the origin. However, two-dimensional analysis of the chymotrypsin digests did not show a positive tyrosine test in the area above the origin.

The second leading spot above the origin in the elastase (Figure 5), chymotrypsin digests (Figure 4), and the spot above the origin in the pepsin digest

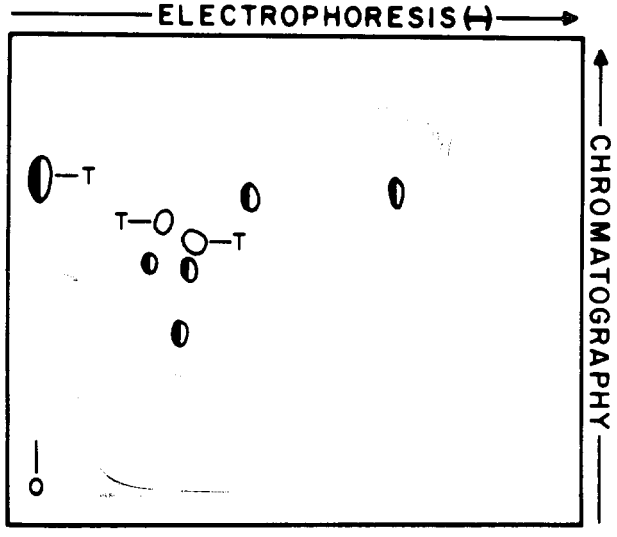

Figure 2. Two-dimensional chromatogram of dialyzable materials from pepsin-digested acid-soluble calf skin collagen after 24-hour digestion at 20° C.

Material in dialyzates:
◐. Positive chlorination staining where different from ninhydrin-positive areas
O. Ninhydrin-positive areas
T. Positive tyrosine test
δ. Point of sample application
Chromatogram is a composite of two chromatograms—one stained for tyrosine and with ninhydrin and the other with a chlorination stain

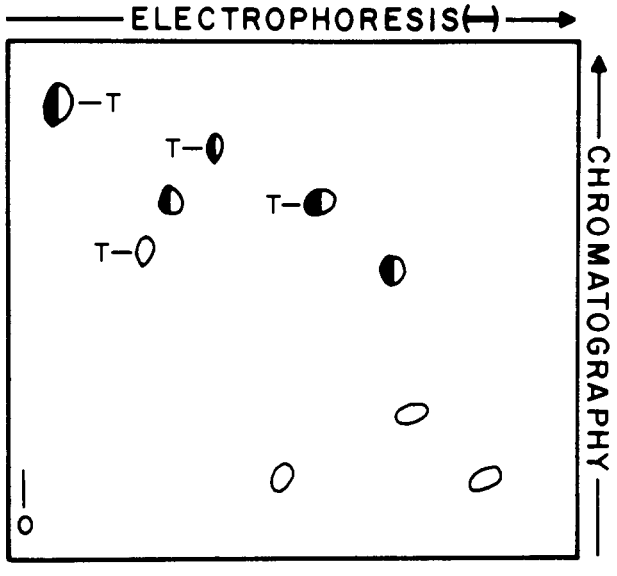

Figure 3. Two-dimensional chromatogram of dialyzable material from trypsin-digested acid-soluble calf skin collagen

Digestion at 20° C. for 24 hours
See Figure 2 for legend

(Figure 2) have R_f values that are comparable. The average R_f values were 0.60, 0.62, and 0.66 for the elastase, chymotrypsin, and pepsin digests, respectively. Only the spot from the pepsin digest gave a positive tyrosine test, and, therefore, is probably distinctly different from the other spots. The proximity of the two spots described above in the various digests, plus the

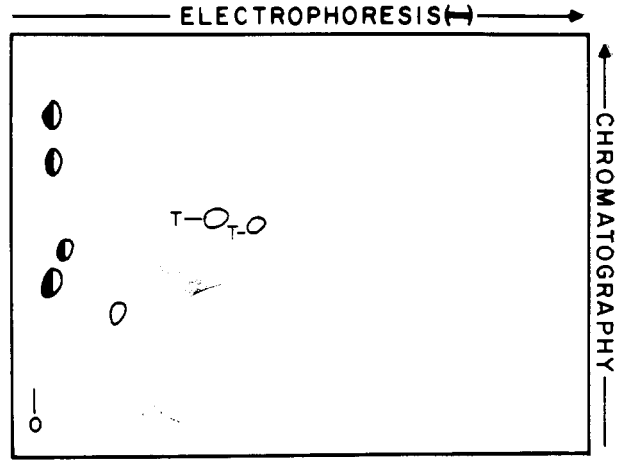

Figure 4. Two-dimensional chromatogram of dialyzable material from chymotrypsin-digested acid-soluble calf skin collagen

Digestion at 20° C. for 24 hours
See Figure 2 for legend

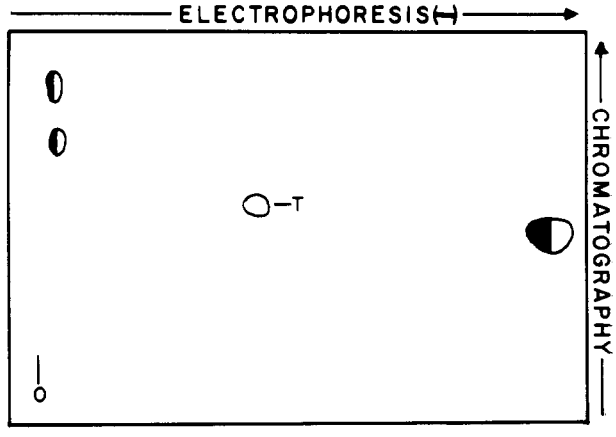

Figure 5. Two-dimensional chromatogram of dialyzable material from elastase-digested acid-soluble calf skin collagen

Digestion at 20° C. for 24 hours
See Figure 2 for legend

apparent lack of electrophoretic mobility at pH 2.0, indicates that these compounds are acidic in nature and may differ by only a few amino acid residues. The remaining spots above the origin in the chymotrypsin digest have no counterparts in the other digests, and consequently appear to be unique to chymotrypsin.

The electrophoretically mobile tyrosine staining areas in the various digests differed in both mobility and R_f values between digests, as well as in the number of positive areas discernible. The tyrosine-positive areas in the chymotrypsin digests (Figure 4) have R_f values similar to that of the spot for the elastase digest (Figure 5). The R_f values were 0.46 for the spot in the elastase digest and 0.50 and 0.47 for the two spots in the chymotrypsin digest.

The chymotrypsin digest has an extra positive tyrosine area and two other spots directly over the origin. The elastase digest differs in that it has a large chlorination positive area at the leading edge of the electrophoretic

front. Other than the similarities previously discussed, the pepsin and trypsin digests showed no similar tyrosine-staining areas. The remaining spots appear to have no counterparts, when all chromatograms are compared.

The repeatability of the electrophoretic runs was very good when duplicate samples were analyzed on the same paper. However, day-to-day repeatability was variable with respect to the relative mobility of a given spot. This was probably due to variation in the temperature during the run and to the degree of wetness of the paper, as well as to fluctuations in current flow. Control samples for all enzymes and substrates were run at the specific pH of the reaction media. All the controls were very similar, and indicated that the pH of the reaction media had no effect on the collagen substrate.

Thermal Gelation. Figure 6 shows the results of short-time studies on the ability of the enzyme-treated protein to gel on warming to 33° to 35° C. in a phosphate-NaCl buffer at an ionic strength of 0.6*M* and a pH of 7.4. Preliminary experiments at various protein concentrations confirmed the results of Gross and Kirk (1958), who demonstrated the dependence of the lag phase upon initial concentration. The curves in Figure 6 show an increase in the lag phase following pepsin or trypsin treatment of the protein. Chymotrypsin treatment resulted in a curve with a very gradual slope. The elastase-treated protein never formed a gel or fibrous precipitate during the duration of these short-term experiments. On the other hand, the untreated sample readily formed a slightly opaque gel, indicated by the sharp slope of the curve.

Listed in inverse order of their ability to prevent gelation, the enzymes would be arranged as follows: pepsin < trypsin < chymotrypsin < elastase.

Disk Gel Electrophoresis. Figure 7 shows the results of disk gel electrophoresis of a control sample and the enzyme-treated collagen. The control sample (BSC) shows the typical α, β, γ banding pattern of denatured collagen. The protein concentrations varied between samples, as indicated by the wider, more intensely staining bands. Nevertheless, it is clear that elastase (BERx) and chymotrypsin (BCRx) treatment increased the amount of the α-component apparently at the expense of the β-component. Similar results were obtained by Bornstein *et al.* (1966) for chymotrypsin-treated, acid-soluble rat skin collagen.

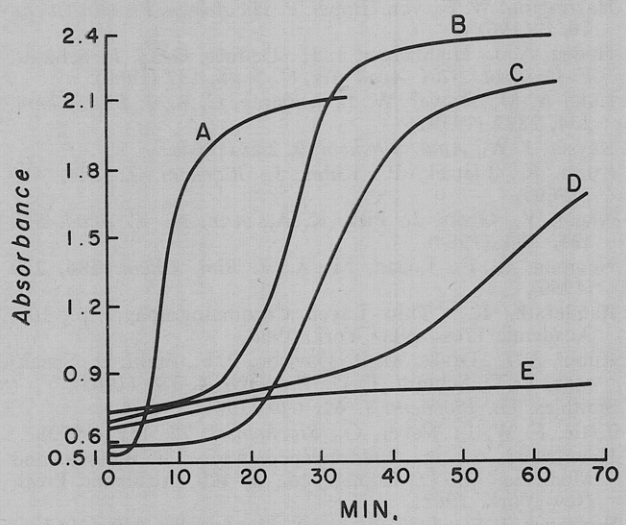

Figure 6. Thermal gelation curves of lyophilized, enzyme-treated, acid-soluble calf skin collagen.

Thermal gelation occurred at 33°C. using a 0.6*M* phosphate-NaCl buffer at pH 7.4. Absorbance (O.D.) monitored at 230 mμ

A. Untreated protein
B. Pepsin-treated
C. Trypsin-treated
D. Chymotrypsin-treated
E. Elastase-treated

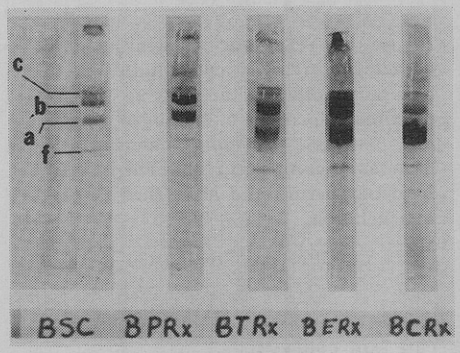

Figure 7. Disk gel patterns of enzyme-treated, acid-soluble calf skin collagen

Protein treated with enzymes at 20°C. for 24 hours
BSC. Untreated collagen
BPRx. Pepsin-treated collagen
BTRx. Trypsin-treated collagen
BERx. Elastase-treated collagen
BCRx. Chymotrypsin-treated collagen
Banding patterns
a. α-component
b. β-component
c. γ-component
f. Buffer front
Collagen samples heat-denatured at 40–45° C. for 20 minutes prior to electrophoresis

The trypsin-treated sample (BTRx) shows little or no change from the control, exhibiting approximately equal proportions of the α- and β-components. This seems to hold true also for the pepsin-treated sample (BPRx).

Disk gel electrophoresis of the enzyme-treated samples before and after long-term thermal gelation experiments resulted in the banding patterns shown in Figure 8. Electrophoresis was utilized to determine what components were removed from solution during thermal gelation. The gels were overloaded with the protein in each case in an attempt to show other fast-moving components that may result from enzyme treatment. In photographing, the α-, β-, and γ-components appeared highly stained and distorted, so that drawings are used to present the banding patterns herein.

Examination of the drawings (Figure 8) shows that chymotrypsin and elastase treatment prevented relatively large amounts of the α-component from being removed from solution, as well as smaller amounts of the β-component, whereas the control sample and pepsin- and trypsin-treated samples indicate that nearly all of the α- and β-components were removed by thermal gelation.

In the light of these results, as well as those of the short-term thermal gelation experiments, it is apparent

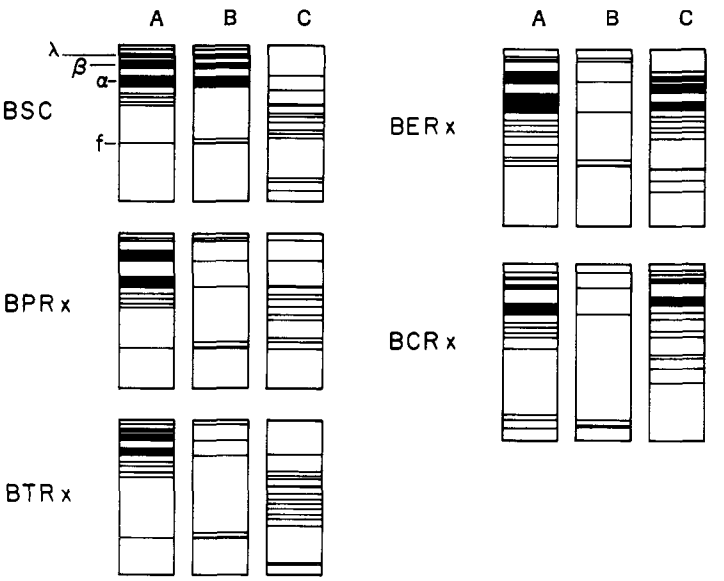

Figure 8. Schematic drawings of enzyme-treated acid-soluble calf skin collagen showing approximate location of fast-moving, weakly staining bands between α-component and buffer front before and after thermal gelation

Banding patterns following carbohydrate staining are also shown
BSC. Untreated acid-soluble calf skin collagen
BPRx. Pepsin-treated
BTRx. Trypsin-treated
BERx. Elastase-treated
BCRx. Chymotrypsin-treated
A. Strong and weak staining bands before thermal gelation
B. Carbohydrate staining patterns
C. Weak and strong staining bands after thermal gelation

that chymotrypsin and elastase must cleave certain key residues from the collagen molecule to prevent it from participating in the gelation process. The increased amount of the α-component resulting from the action of these enzymes indicates that perhaps the β-component or its crosslink are key factors in the polymerization process. However, a more plausible view would be that these enzymes cleave residues mainly from the α- and β-components, preventing them from participating in the gelation process. While the evidence presented here is not overwhelming, it is indicative of the role of α- and β-components of collagen in gelation phenomenon.

Lightly staining, fast-moving components were found when the gels were overloaded with the protein. In general, the banding patterns between treatments were very similar to the control sample. Whether or not these bands resulted from the release of fast-moving peptides following thermal denaturation of the collagen or are indicative of contamination remains obscure. Purification procedures and subsequent analysis of the protein as previously described indicated no contamination by proteins containing tryptophan. A partial amino acid analysis of the protein (Crevasse, 1967) was in good agreement with values in the literature. The significance of these lightly staining components is unknown and must await more detailed study.

Figure 8 also shows in graphic form the banding pattern of the protein when stained for carbohydrates. The bands were so lightly staining that they defied photography; consequently, these results are shown as drawings in column *C*. The banding pattern matched the α-, β-, and γ-components in most cases. However, all samples showed two other positive bands, one at the buffer front and one just behind it.

The fact that carbohydrate staining was extremely weak even though the gels were overloaded with protein further attests to the purity of the protein preparation. In all of the disk gel patterns discussed, enzyme blanks were included in each run, but did not contribute to the banding pattern.

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Received for review February 1, 1968. Accepted December 9, 1968. Approved as Journal Article 4205, Michigan Agricultural Experiment Station. Investigation supported in part by Public Health Service Research Grant No. UI-00191 and in part by Public Health Service Grant No. 8 TOI UI-01031 from the National Center for Urban and Industrial Health. The authors gratefully acknowledge the financial assistance of the Food Research Division, Armour and Co., Oak Brook, Ill.