C-Protein from rabbit soleus (red) muscle

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(Received 31 October 1980/Accepted 19 January 1981)

A new form of skeletal-muscle C-protein has been isolated from rabbit soleus (red) muscle. This new form of C-protein has been purified to homogeneity by a procedure similar to that used to purify C-protein from white skeletal muscle. In soleus muscle, only this new form of C-protein could be detected, whereas in psoas (white) muscle, only the previously identified form of C-protein was detected. The content of C-protein in rabbit soleus muscle is comparable with that found in psoas muscle. Other rabbit skeletal muscles composed of a mixture of fibre types contained at least two forms of C-protein. C-Protein derived from red skeletal muscle bound to myosin isolated from either red or white tissue, with maximum binding occurring at a ratio of approximately 13 µg of red C-protein/100 µg of myosin. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate indicated that C-protein isolated from red skeletal muscle has a molecular weight approx. 7% greater than that of C-protein isolated from white skeletal muscle. The amino acid content of both forms of C-protein was similar but major differences in the mol % of isoleucine and threonine were found. Antiserum against C-protein from white rabbit skeletal muscle formed a single precipitin line with rabbit white C-protein on double diffusion in agar. This antiserum did not form a precipitin line when diffused against red C-protein from rabbit skeletal muscle. Also, this antiserum bound specifically to the A-band region of myofibrils isolated from psoas (white) muscle, but it did not bind to myofibrils prepared from soleus (red) muscle.

C-Protein was first detected when SDS/polyacrylamide-gel electrophoresis was performed on a myosin sample that had been prepared by conventional methods (Starr & Offer, 1971). C-Protein comprises 1–2% of the total protein content of the myofibril of rabbit skeletal muscle (Offer *et al.*, 1973). It has a molecular weight of 140 000, low α -helical content, high proline content and was assumed to be a prolate ellipsoid with a length of about 35 nm (Offer *et al.*, 1973). Antibodies produced against C-protein from white muscle have been shown to bind at 14 specific sites along each thick filament of myofibrils derived from psoas (white) muscle (Pepe & Drucker, 1975; Craig & Offer, 1976).

C-Protein binds to both the light meromyosin and S-2 subfragments of myosin (Moos, 1972; Moos

et al., 1975; Starr & Offer, 1978). Binding of C-protein to myosin alters the actin-activated ATPase activity of myosin (Offer *et al.*, 1973) and seems to disrupt the periodicity of myosin filament assembly *in vitro* (Moos *et al.*, 1975; Koretz, 1979). Moos *et al.* (1978) showed that C-protein also binds to F-actin under the appropriate conditions. Although the physiological function of C-protein has not been determined, several theories have been put forth: control of cross-bridge movement (Starr & Offer, 1978), stabilization of the thick filament (Offer, 1972), regulatory role in muscle contraction (Offer *et al.*, 1973; Moos *et al.*, 1978), structural alterations of the thick filament preceding contraction (Haselgrove, 1975), and thick-filament organization (Koretz, 1979).

C-Protein has been isolated from skeletal muscle composed predominantly of the 'white' fibre types (Offer *et al.*, 1973; Pepe & Drucker, 1975; Craig & Offer, 1976; Moos, 1972; Moos *et al.*, 1975, 1978; Koretz, 1979). It was not detected in preparations of vertebrate smooth myosin by SDS/polyacrylamide-gel electrophoresis (Wachsberger & Pepe, 1974) and

Abbreviation used: SDS, sodium dodecyl sulphate.

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has not been reported in any non-skeletal muscle systems. In the present paper, we report evidence for a new form of C-protein isolated from tissues that have the slow-twitch-oxidative fibre type that can be distinguished from the C-protein isolated from muscle containing fast-twitch-glycolytic fibres by physical, chemical and immunological properties.

Materials and methods

Source of protein and protein purification

The terms red C-protein and red myosin refer to those proteins purified from the soleus muscle of rabbit. The soleus muscle from rabbit has been reported to contain more than 95% slow-twitch-oxidative fibres (Peter *et al.*, 1972). White C-protein and white myosin refer to those proteins purified from the psoas muscle and white skeletal muscle present in the thigh and back of rabbit. The psoas muscle of rabbit is composed of 98% fast-twitch-glycolytic fibres (Weeds & Hall, 1975). Because of the small size of the rabbit soleus (approx. 3 g per rabbit), the muscle usually was frozen at -70°C until enough tissue was accumulated for a C-protein preparation. No difference was detected in the C-proteins derived from fresh or frozen tissue.

C-Protein was isolated from the specified rabbit skeletal muscle by the method of Offer *et al.* (1973) with the addition of 5 mM-MgCl₂ and 5 mM-ATP to the Guba-Straub solution to enhance myosin extraction. Also, the final two purification steps after the elution of the DEAE-Sephadex resin were omitted. This same procedure was used to isolate C-protein from both bovine and avian sources.

Myosins used in these experiments were purified by low-ionic-strength precipitation of the fraction eluted with a high-salt gradient during the ion-exchange column chromatography step of the C-protein procedure. When subjected to electrophoresis in the presence of SDS, these purified myosins did not contain any detectable C-protein.

Gel electrophoresis

The low-ionic-strength Tris/glycine buffer system described by Studier (1973) was utilized with a 1 mm-thick slab gel that consisted of a 5% polyacrylamide separating gel and a 3% polyacrylamide stacking gel. SDS was present during electrophoresis; a current of 10 mA was employed for 8 h. The electrophoresis system described by Weber & Osborn (1969) also was utilized with a 1 mm-thick slab gel of 4% polyacrylamide. This SDS-containing slab gel was developed with a current of 35 mA for 12 h. Gels were stained in 0.2% Coomassie Brilliant Blue dissolved in 40% methanol and 7% acetic acid and destained for 2 h in 40% methanol and 7% acetic acid. Samples utilized for electrophoresis were prepared by boiling for 5 min in

the presence of 0.8% SDS, 1 M- β -mercaptoethanol, 0.03% Bromophenol Blue, 7% sucrose and 50 mM-4-morpholine-ethanesulphonic acid (pH 6.5).

Binding of C-protein to myosin

The binding of C-protein to filamentous myosin is the only well-characterized property of this protein. The myosin and C-protein solutions were dialysed against 100 mM-NaCl and 10 mM-sodium phosphate buffer (pH 7.0) at 4°C before each binding study. Dialysed protein solutions were mixed in Beckman cellulose propionate centrifuge tubes and diluted with dialysis buffer to a volume of 110 or 200 μl . Samples were incubated for 5 min at 23°C before centrifugation at 23°C for 10–15 min at 80 000 *g* in a Beckman Airfuge. After centrifugation, 100 μl portions were withdrawn from the supernatant and the protein content was determined by the method of Lowry *et al.* (1951). More than 98% of rabbit skeletal muscle myosin in control tubes was pelleted during centrifugation. Because of the tendency of C-protein to aggregate at low ionic strengths (Offer *et al.*, 1973), C-protein solutions were clarified after dialysis. Control tubes containing only C-protein were centrifuged under conditions identical with those previously described to determine how much C-protein sedimented in the absence of myosin aggregates. This value always represented less than 20% of the total C-protein, and was used to convert C-protein sedimented to C-protein bound.

Amino acid analysis

Samples of both red and white C-protein for amino acid analysis were purified as described above with the addition of a low-ionic-strength precipitation of the C-protein by dialysis against a 10 mM-phosphate buffer (pH 7.0). After centrifugation each protein pellet was examined by electrophoresis in the presence of SDS and found to be homogeneous. Proteins were freeze-dried and hydrolysed in 5.7 M-HCl at 100°C for 24 h or 65 h. Amino acid compositions were determined with a Durrum D-400 amino acid analyser. Analyses were performed on 24 h-hydrolysed samples from three different preparations of both red and white C-protein plus 65 h-hydrolysed samples of a single red and white C-protein preparation. The mean value and standard deviation were computed from both 24 h and 65 h analyses.

Antibody preparation and immunochemistry

An antibody to C-protein from white rabbit skeletal muscle was produced in a goat. The antigen was purified as described with addition of the low-ionic-strength precipitation step followed by chromatography on a Sephadex G-200 column. A 30 μg sample of this highly purified C-protein yielded a single band after electrophoresis on an SDS/

polyacrylamide gel. White C-protein (1 ml; 1.5 mg) was dialysed against a 100 mM-NaCl/10 mM-phosphate buffer (pH 7.0), emulsified with 1 ml of Freund's complete adjuvant, and subsequently injected into multiple sites on the hindquarters of a female goat. The animal was bled at 15-day intervals after injection. Control serum was obtained from the same goat preceding injection of the antigen.

Double-diffusion analysis was performed on agar plates equilibrated in 150 mM-NaCl/10 mM-sodium phosphate buffer, pH 7.4, and the proteins were dialysed against phosphate-buffered saline. The agar plates were developed for 10 h at 23°C, then they were washed for 30 h in 150 mM-NaCl/10 mM-sodium phosphate, pH 7.4, at 23°C, and precipitin lines were detected by reaction with Coomassie Brilliant Blue.

Indirect immunofluorescence was performed as previously described (Bechtel, 1979) on myofibrils isolated from specific rabbit skeletal muscles. The antiserum was diluted 1:170 in phosphate-buffered saline before incubation with the myofibrils.

Materials

A-50-120 DEAE-Sephadex, acrylamide, bis-acrylamide, 4-morpholine-ethanesulphonic acid, Coomassie Brilliant Blue, ATP and SDS were all purchased from Sigma Chemical Co. Sephadex G-200 was obtained from Pharmacia, Ouchterlony plates were purchased from Sebia and Freund's complete adjuvant (type H37 Ra) was purchased from Difco Chemical Co.

Results

Purification of C-protein from soleus muscle

C-Protein from soleus (red) muscle of rabbit was purified by first isolating a crude myosin fraction from a high-ionic-strength extract. The crude myosin was then applied to a column of DEAE-Sephadex, eluted with a salt gradient and fractions were examined by SDS/polyacrylamide-gel electrophoresis. Red C-protein was present in the initial elution peak and the highly purified myosin was eluted with the salt gradient (Fig. 1). The fraction of material eluted from the DEAE-Sephadex column in the first peak was comparable for the two tissue types (Table 1).

Gel electrophoresis

Purification of C-protein from rabbit soleus muscle (Fig. 2) can be followed from the whole-tissue homogenate (lane C) to the crude myosin fraction (lane B), and final product (lane A). Likewise, purification of C-protein from white skeletal muscle is shown in Fig. 2, lanes (D), (E), and (F). Protein purified from red muscle has an

Fig. 1. Purification of rabbit skeletal-muscle C-protein from soleus (red) and psoas (white) rabbit muscle by chromatography on DEAE-Sephadex

Samples were dialysed against 0.15 M-potassium phosphate buffer (pH 7.0) and 215 mg of protein from soleus muscle and 450 mg of protein from psoas muscle were applied to identical columns of DEAE-Sephadex equilibrated with the same buffer. Proteins were eluted with a linear KCl gradient (----). Fractions were collected, and the A_{280} was determined for psoas (O) and soleus (Δ) muscle samples.

apparent higher molecular weight than C-protein from white tissue (lanes A and F), and, in addition, the white muscle whole-tissue homogenate (lane D) does not contain red C-protein nor does the soleus muscle whole-tissue homogenate contain much white C-protein (lane C).

To substantiate the differences between the two C-proteins, a direct comparison of the mobility of the proteins on Tris/glycine-buffered SDS/polyacrylamide-gel electrophoresis (Studier, 1973) was performed as shown in Fig. 2 (lanes G, H and I). The red (lane I) and white (lane G) C-protein do not have the same mobility and when these two proteins are mixed together, they retain different mobilities (lane H). This difference in mobility was further examined by subjecting the samples to electrophoresis in the high-ionic strength phosphate/SDS buffer system of Weber & Osborn (1969). Again, the results (Fig. 2, lanes J, K and L) indicated that red C-protein (lane L) has a mobility approx. 7% less than that of white C-protein (lane J) and that, after being mixed together, the two forms were readily separated (lane K).

Myosin binding

Binding of C-protein to myosin was examined because this is the best characterized functional property of C-protein *in vitro*. The binding assay involved incubation of C-protein with insoluble filamentous myosin, followed by sedimentation of the C-protein-myosin complex. When the amount of

Table 1. *Purification of C-protein from red and white skeletal muscle*

Rabbit muscle	Muscle tissue (mg total wet wt.)	Guba-Straub extract (mg of protein)	0.5 M-KCl resuspension (mg of protein)	1.41–1.66 M-(NH ₄) ₂ SO ₄ fraction (mg of protein)	First peak from DEAE-Sephadex column (mg of protein)
Soleus (red)	26 000	1500	740	200	4
Psoas (white)	26 000	1400	560	250	5

Fig. 2. *Gel electrophoresis in the presence of SDS*

Gel sections (A)–(F) and (G)–(I) were formed in a Tris/glycine buffer system with a 5% polyacrylamide separating gel and 3% polyacrylamide stacking gel. Gel sections (J)–(L) were formed in a phosphate buffer system with a 4% polyacrylamide gel. Gel lane (A), red C-protein (1.5 μ g); (B), 1.41–1.66 M-(NH₄)₂SO₄-precipitated fraction of partially purified red myosin (18 μ g); (C), soleus muscle homogenate (30 μ g); (D) psoas muscle homogenate (30 μ g); (E), 1.41–1.66 M-(NH₄)₂SO₄-precipitated fraction of partially purified white myosin (22 μ g); (F), white C-protein (1 μ g); (G), white C-protein (1 μ g); (H), white C-protein (1 μ g) plus red C-protein (1.5 μ g); (I), red C-protein (1.5 μ g); (J), white C-protein (2 μ g); (K), white C-protein (2 μ g) plus red C-protein (3 μ g); (L), red C-protein (3 μ g). Protein standards used: mol.wt. 200 000, rabbit skeletal-muscle myosin heavy chains; 100 000, α -actinin; 69 000, bovine serum albumin (all shown $\times 10^{-3}$ in the Figure).

red myosin was kept constant while the concentration of red C-protein was increased, the binding of C-protein to red myosin filaments increased until a plateau value was obtained (Fig. 3b). A similar result (Fig. 3a) was obtained for binding of white C-protein to white myosin filaments. These experiments indicated that C-protein from red skeletal muscle could bind to red myosin in a manner comparable with binding of white myosin by C-protein derived from white skeletal muscle.

The binding of red and white C-protein to red and white myosin was examined (Table 2) and the results indicate that both red and white C-proteins can bind to both red and white myosin in a similar manner. This could indicate that protein-binding sites are similar, but caution should be used in the interpretation of these experiments because of the simplistic nature of the binding assay.

Conditions for the myosin-binding assay have been shown to favour formation of long myosin

filaments, which are structurally homologous to the natural filament (Moos *et al.*, 1975). The myosin-binding assay was not significantly affected by pH variation between 6.5 and 7.5. Control experiments, in which supernatant and pellet fractions from the binding study were examined by gel electrophoresis, indicated that a constant myosin/C-protein ratio was presented in pellets under conditions in which C-protein was in excess (plateau region, Figs. 3a and 3b).

Amino acid analysis

To probe further into the similarities and differences between the two forms of C-protein, amino acid compositions of highly purified samples of both proteins were determined (Table 3). White C-protein had an amino acid composition (Table 3) similar in many respects to that reported by Offer *et al.* (1973), in which a high proline content was noted, and C-protein from red skeletal muscle also showed

Fig. 3. Binding of rabbit red and white C-protein to their respective myosins

Proteins were incubated in 100 mM-NaCl/10 mM-sodium phosphate buffer, pH 7.0. Bound C-protein was calculated by determining the protein content of the supernatants. Soluble C-protein represents the amount of C-protein calculated to be in the supernatant in the absence of myosin. (a) Binding of different amounts of C-protein from white skeletal muscle to 360 mg of myosin from white muscle; (b) binding of different amounts of C-protein from red skeletal muscle to 320 mg of myosin from red muscle. See the Materials and methods section for details of calculations and procedures.

a high proline content as well as a number of other similarities to white C-protein. However, major differences (more than two standard deviations) were found in contents of isoleucine and threonine in addition to other smaller differences.

Immunodiffusion and indirect immunofluorescence

An antibody against a highly purified preparation of white C-protein was produced, and the immunological properties of the two C-protein forms were compared. Analysis by the double-diffusion method resulted in formation of a single precipitin line, when the antiserum (Fig. 4, well 1) was diffused against white C-protein (well 4). This antiserum did not form a detectable precipitin line when diffused against red C-protein (Fig. 4, wells 2 and 3). No precipitin line was observed in other double-diffusion experiments, in which red C-protein concentration was varied from 0.1 to 10 μg per well. When more than 10 μg of red C-protein was used, a small precipitin line was observed.

Indirect immunofluorescence was performed on myofibrils isolated from the soleus (red) and psoas (white) muscle. The antibody to white C-protein specificity bound only to myofibrils isolated from white muscle (Fig. 5a). White C-protein antibody localized on specific portions of the A-band and did not bind to the M-line region, a result in agreement with previous studies (Pepe & Drucker, 1975; Craig & Offer, 1976). When the same concentration of antibody was incubated with myofibrils isolated

Fig. 4. Immunoprecipitation of red and white C-proteins with antiserum to white C-protein

Immunodiffusion was performed on an agar plate equilibrated in 150 mM-NaCl/10 mM-sodium phosphate (pH 7.0). After samples were applied, the agar plate was developed for 10 h at 23°C and then stained for protein. Well (1) contains 5.0 μl of goat anti-(rabbit white C-protein); well (2) contains 3 μg of red C-protein 1 mg/ml; well (3) contains 6 μg of red C-protein; well (4) contains 3 μg of white C-protein (0.7 mg/ml). See the Materials and methods section for further details.

Table 2. *Binding of C-protein to myosin*

Duplicate samples were incubated in 100mM-NaCl/10mM-sodium phosphate (pH 7.0) at 25°C and binding of C-protein was determined as described in the Materials and methods section. Red and white rabbit skeletal-muscle myosins were purified by chromatography on DEAE-Sephadex.

Myosin		C-Protein		Bound C-protein (μ g)	C-Protein bound (μ g/100 μ g of myosin)
Type	(μ g)	Type	(μ g)		
White	360	White	200	51	14
White	320	Red	93	51	16
Red	100	Red	54	13	13
Red	330	White	105	37	11

Table 3. *Amino acid composition of red and white C-protein*

Results are means $\pm$ s.d. for four hydrolysates from three different C-protein preparations from both red and white rabbit skeletal muscle. The contents of tryptophan and half-cystine were not calculated.

Amino acid	Content (mol %)	
	White	Red
Lysine	8.93 $\pm$ 0.40	9.26 $\pm$ 0.22
Histidine	1.72 $\pm$ 0.08	1.51 $\pm$ 0.08
Arginine	5.19 $\pm$ 0.30	4.76 $\pm$ 0.17
Aspartic acid	9.88 $\pm$ 0.37	10.70 $\pm$ 0.46
Threonine	5.78 $\pm$ 0.20	6.90 $\pm$ 0.24
Serine	6.17 $\pm$ 0.86	5.83 $\pm$ 0.29
Glutamic acid	12.40 $\pm$ 0.31	11.94 $\pm$ 0.30
Proline	6.92 $\pm$ 0.22	7.04 $\pm$ 0.48
Glycine	8.53 $\pm$ 0.99	6.99 $\pm$ 0.16
Alanine	7.36 $\pm$ 0.35	7.96 $\pm$ 0.21
Valine	8.45 $\pm$ 0.71	6.82 $\pm$ 0.26
Methionine	1.31 $\pm$ 0.73	1.77 $\pm$ 0.16
Isoleucine	4.26 $\pm$ 0.47	6.15 $\pm$ 0.21
Leucine	6.79 $\pm$ 0.21	6.41 $\pm$ 0.24
Tyrosine	2.71 $\pm$ 0.41	3.51 $\pm$ 0.16
Phenylalanine	3.52 $\pm$ 0.20	3.17 $\pm$ 0.17

from soleus (red) muscle, specific localization was not observed (Fig. 5b). Incubation of antiserum with 10 μ g of white C-protein followed by centrifugation at 10000g and subsequent incubation of supernatant with white myofibrils resulted in blocking of the specific A-band localization of the antibody. The anti-(rabbit white C-protein) antibody was chosen for the immunological characterizations due to the high purity and availability of white C-protein, which contained no detectable red C-protein. An antibody produced against red C-protein exhibited an identical localization pattern to that produced by antibody made to white C-protein, but this antibody stained psoas (white) myofibrils with nearly the same intensity as soleus (red) myofibrils. Though the antibody against red C-protein will be a valuable

Fig. 5. *Indirect immunofluorescence localization of goat anti-(rabbit white C-protein) on soleus and psoas rabbit muscle myofibrils*

Upper panels are the phase-contrast photographs, and lower panels are photographs of the immunofluorescence localization. Myofibrils isolated from psoas muscle (a) or soleus muscle (b) were first incubated with goat anti-(rabbit white C-protein) serum diluted 1:170 and subsequent incubation with fluorescein-conjugated rabbit anti-(goat immunoglobulin G) 1:20. It was consistently observed that the Z-line was more prominent in the soleus muscle myofibrils (a) than in the psoas muscle myofibrils (b). See the Materials and methods section for further details. Magnification is 1900 $\times$.

tool in studies involving the myogenesis and tissue distribution of C-protein, the lack of specificity of this antibody made it undesirable for use in probing the structural differences between red and white C-protein. This lack of specificity could be caused by contamination of red C-protein preparation with highly antigenic white C-protein (Offer, 1976) or could indicate a degree of homology between the two forms of C-protein.

Comparative studies

C-Protein was purified from bovine latissimus dorsi muscle, chicken breast muscle (95% white-fibre

type) and chicken thigh and leg muscle (mixed fibre types). The C-protein preparation isolated from chicken thigh and leg muscles migrated as two bands on SDS/polyacrylamide gels. C-Protein from chicken breast migrated as one band that corresponded to the slower migrating electrophoretic form of C-protein from chicken leg and thigh muscle. The preparation of C-protein from latissimus dorsi (mixed-fibre type) migrated as two bands. The C-proteins prepared from both bovine and chicken skeletal muscle had similar purification characteristics to the rabbit C-protein preparations and possessed myosin-binding properties. Goat and anti-(rabbit white C-protein) serum did cross-react (precipitin line by double-diffusion analysis) weakly with C-protein preparations derived from chicken breast and bovine skeletal muscle.

These data indicate that multiple forms of C-protein exist in the skeletal muscle from other species. Of interest was the observation that the form of C-protein with the slower electrophoretic mobility varies with species. Red C-protein isolated from rabbit skeletal muscle migrates slower than white C-protein, whereas the opposite appears to be the case in chicken skeletal muscle.

Discussion

Evidence that the protein isolated from crude myosin extracted from rabbit soleus muscle is a form of C-protein includes the following points. The protein was isolated by the C-protein purification scheme, the tissue content of this protein in red skeletal muscle is similar to that of the C-protein content in white skeletal muscle, the protein exhibits myosin-binding properties similar to white C-protein and the protein possesses the characteristically high proline content of C-protein derived from white muscle.

Data that support the hypothesis that this protein is a unique form of C-protein are the following. Differences in electrophoretic mobility were obtained on two different SDS/polyacrylamide-gel electrophoresis systems, significant differences in amino acid composition and the specificity of the white C-protein antiserum.

Existence of multiple forms of contractile proteins that are associated with specific fibre type have been well documented. The actin-binding proteins, troponin (two subunits) (Head *et al.*, 1977; Wilkinson, 1978) and α -actinin (Suzuki *et al.*, 1973), have been found to exist in multiple forms specific for both fibre types. In thick filaments, isoenzymes of the S-1 region of myosin have been extensively used to discern fibre types (Seidel, 1967), and recent immunological evidence indicates that there may be fibre-type-specific forms of the rod portion of myosin (Gauthier & Lowey, 1979). The present study is the

first example of a myosin-binding myofibrillar protein having forms specific for red or white muscle.

C-Protein preparations from bovine and chicken skeletal muscle exhibited two polypeptides with different electrophoretic mobilities indicating there may be two or more forms of C-protein in other vertebrates. There are no reports of C-protein being present in any tissues other than skeletal muscle. In the light of the present study, failure to detect C-protein could be a result of tissue such as cardiac and smooth muscle, or non-muscle systems containing other forms of C-protein. Different physical and immunological properties exhibited by the two forms of C-protein in skeletal muscle could complicate the identification of other forms of C-protein.

We are grateful for the fluorescence-microscopy expertise supplied by F. Richardson. This work was funded by the Iowa Agriculture and Home Economics Experiment Station, Ames, IA (Project 2127, Paper no. J9783).

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