acid (a ratio within the range found in orange juice) the content of amino acid fell to zero after 10 min with a corresponding decrease by ~20% in the content of D-glucose and a considerably slower loss of D-glucose thereafter. When the amino acid was omitted there was no observed loss of D-glucose during 40 min at 100°. Increasing the molar proportion of amino acid to 0.308 caused a more pronounced loss of D-glucose, falling to 40% of its original value after 40 min at 100°.

The validity of this model system, and the analytical procedure used for monitoring the loss of D-glucose, in relation to the thermal deterioration of an actual sample of dehydrated orange juice is illustrated in Figure 13. Dried orange juice powder prepared by the foam-mat process was kept at 100° and assayed by glc for content of D-glucose. The curve for loss of D-glucose showed a decrease by ~20% during the first 40 min, falling by another 20% after a total of 4 hr. The curve is observed to be closely similar to that given in the model system (Figure 12) for D-glucose and 4-aminobutyric acid present in the ratio generally found in orange juice. Greatly accelerated loss of D-glucose was observed in the dehydrated orange juice when ~1 molar equiv of 4-aminobutyric acid (with respect to D-glucose) was added before heating; the content of D-glucose fell to <10% of the original level after 40 min at 100°.

The foregoing results indicate that interactions between D-glucose and amino acids, especially 4-aminobutyric acid and L-arginine, probably play a significant role in reactions leading to deterioration of dehydrated orange juice, and demonstrate that loss of D-glucose takes place under accelerated conditions of storage deterioration. Work with model systems has established that D-glucose interacts with amino acids to give 1-(N-substituted)amino-1-deoxy-D-fructoses by way of a glycosylamine intermediate. It remains to be established, on an isolative basis, the postulated formation and subsequent decomposition of such intermediates during the initial stages of storage deterioration of dehydrated orange juice and other food products where this same degradative sequence is presumed to operate.

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Disc Gel Electrophoresis. A Technique Involving Fluorescent Staining Prior to Separation

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Disc gel electrophoresis of proteins prestained with a fluorescent indicator, Fluram, is described. Less than 1 μg of protein is readily discerned on gels. Speed of analysis is a special advantage over conventional procedures which involve staining and destaining after electrophoresis. Gels do not have to be removed from support tubes and visualization of bands under long-wavelength uv light is possible as soon as electrophoresis is completed. Proteins appear as sharp, fluorescent bands. The procedure is especially applicable at high polyacrylamide concentrations in gels.

Polyacrylamide disc gel electrophoresis is a widely used technique for protein studies. High acrylamide concentrations, sometimes desired for effective separations, result in strong gels which cannot be removed from electrophoresis tubes without fracturing the gel and altering the integrity of separated bands of protein. Moreover, fixation, staining, and destaining procedures for visualization of protein bands can be inordinately lengthy. Described herein is a fluorescent method which overcomes some of these difficulties. Using Fluram (Hoffman La Roche, Inc.), fluorophores of proteins are formed prior to electrophoresis, and after separation individual bands can be visualized without removing the gel from the tube and

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going through the cumbersome staining and destaining process.

Fluram (4-phenylspiro(furan-2(3*H*)-1′-phthalan)-3,3′-dione), formerly known as Fluorescamine, is a new reagent for the detection of primary amines and proteins (Udenfriend *et al.*, 1972). At pH 8.6–9.0 fluorophors are formed which are excited at 390 nm and emit at 475 nm. The reaction is nearly instantaneous and excess reagent decomposes to nonfluorescent products almost immediately.

MATERIALS AND METHODS

Disc gel electrophoresis was carried out primarily as described by Davis (1964) using reagents which were essentially those of Brumby and Welch (1970).

Reagents used were: gel buffer, 36.6 g of Tris and 0.23 ml of *N,N,N′,N′*-tetramethylethylenediamine (TEMED) in 8 *M* urea adjusted to pH 8.9 with approximately 48 ml of 1 *N* HCl and diluted to 100 ml volume with 8 *M* urea; stacking gel buffer, 5.98 g of Tris and 0.46 ml of TEMED in 8 *M* urea adjusted to pH 6.7 with 1 *N* HCl and diluted to 100 ml with 8 *M* urea; concentrated acrylamide, 28 g of acrylamide and 0.735 g of *N,N′*-methylenebisacrylamide diluted to 100 ml with 8 *M* urea; dilute acrylamide, 11 g of acrylamide and 1.5 g of *N,N′*-methylenebisacrylamide diluted to 100 ml with 8 *M* urea; catalyst, 0.14 g of ammonium persulfate diluted to 100 ml with 8 *M* urea; sucrose solution, 40% in distilled water (w/v).

Preparation of Gels. Acrylamide separating and stacking gels (21%) were prepared using various proportions of the above listed reagents (v/v): separating gel, gel buffer–concentrated acrylamide–catalyst (1:6:1, respectively); stacking gel, stacking gel buffer–dilute acrylamide–catalyst–sucrose solution (1:2:1:4, respectively).

A measured volume (1 ml) of separating gel mixture was placed in a 0.5 cm i.d. × 7 cm electrophoresis tube and overlayered with 20–30 μl of water to give a flat surface to the gel. After polymerization the nonpolymerized top layer was removed and 0.2 ml of stacking gel added. The stacking gel was water layered as previously mentioned.

Preparation of Protein for Electrophoresis. Bovine serum albumin (BSA) and α-lactoglobulin were obtained from a commercial source. All other milk protein standards were supplied by M. P. Thompson (U. S. Department of Agriculture, Eastern Marketing and Nutrition Research, Philadelphia, Pa.). Each protein (1 mg) was dissolved in 0.25 ml of buffer solution (40 g of sucrose and 21 g of urea added to 100 ml with 2% Na_2CO_3 in 0.1 *N* NaOH adjusted to pH 8.6 with HCl). Using a syringe to obtain instantaneous mixing, 100 μl of Fluram solution (1 mg/ml in acetone) was added to 100 μl of protein solution in 2-ml vials. One to fifty micrograms of the Fluram stained samples were applied to electrophoresis tubes.

Serum protein and casein were recovered from skim milk (37°) by filtration after adjusting to pH 4.6 with HCl. The filtrate was used as the serum or acid whey protein fraction. The precipitate from the above was resolubilized in 0.1 *N* NaOH, reprecipitated with HCl and again resolubilized in 0.1 *N* NaOH to yield acid-precipitated casein solution. The whey protein and casein solutions were allowed to react with Fluram in a manner similar to that used for the protein standards to yield final protein concentrations of 1–2 μg/μl.

Electrophoresis. The buffer used for electrophoresis was 6 g of Tris buffer and 28.8 g of glycine per liter of water further diluted one part in seven parts of water just prior to electrophoresis. Separations were conducted on a Conalco Model 12 apparatus with a Conalco Model 1400 power supply. The system was subjected to a current of 2.5 mA until the sample had moved through the stacking gel and then 5 mA was applied for separation. Electrophoresis was complete when the marker was 1 cm from the bottom of the tubes. Total time involved was less than 1 hr.

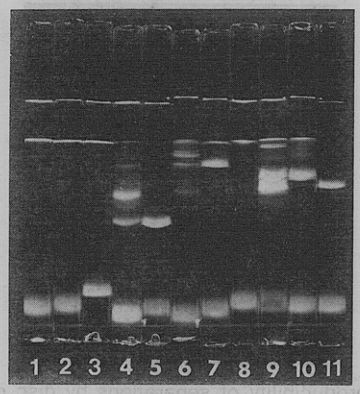

Figure 1. Disc gel electrophoresis of milk proteins prestained with Fluram: (1) euglobulin; (2) pseudoglobulin; (3) γG-globulin; (4) acid whey; (5) α-lactalbumin; (6) β-lactoglobulin; (7) bovine serum albumin (BSA); (8) κ-casein; (9) acid-precipitated casein; (10) β-casein; (11) α_s-casein.

After electrophoresis, samples were made visible and photographed in a black box under long-wavelength ultraviolet light. Photography was accomplished using conventional photographic equipment fitted with a Wratten 2A filter.

RESULTS AND DISCUSSION

Figure 1 shows the electrophoretic patterns for milk protein standards, whey protein, and acid caseins prestained with Fluram. Although some differences are evident, migration patterns were comparable to those reported for milk proteins made visible by conventional staining techniques after electrophoresis (Manning, 1969; Morr and Lin, 1970; Morr *et al.*, 1971). The caseins in particular migrated in the same order when prestained as they do when traditionally subjected to electrophoresis, but they did not move as far as might have been expected. This is attributed to the high acrylamide concentration in the gels used in this study.

The immune globulins (tubes 1, 2, 3) of milk serum and κ-casein did not migrate, due probably to their sizes and the high acrylamide concentration. Relative to each other, prestained bovine serum albumin (BSA) and the β-lactoglobulins migrated in reverse order to that reported when staining is done after electrophoresis. This inversion may reflect an effect of Fluram, but it could also involve urea which is not normally used for whey protein separations.

Reaction with Fluram followed by electrophoresis resulted in the detection of impurities in the standards, particularly in BSA and α_s-casein. Moreover, the whey proteins were observed to be contaminated with the caseins and *vice versa*. Comparison with conventional staining–destaining techniques would be desirable to determine if Fluram is an especially sensitive reactant for determining the electrophoretic homogeneity of protein samples.

The band at the bottom of each gel reflects primary amine contamination of the buffer used in prestaining. Phosphate, borate, and carbonate buffers were checked and showed no bottom band. When urea was added the band appeared. The band has an advantage. It is visible when the apparatus is operating and acts as a marker enabling the operator to determine when electrophoresis is complete.

Figure 2 is presented as evidence that the Fluram method yields reproducible results with respect to the number and relative intensities of migrated bands. Shown are whey protein and casein fractions recovered from two samples of skim milk. Two portions of whey protein and

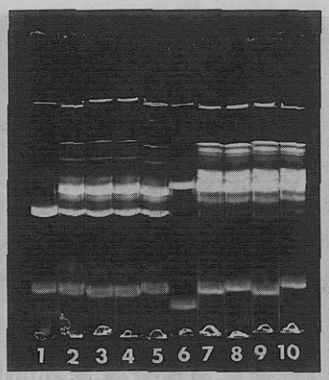

Figure 2. Reproducibility of separations by disc gel electrophoresis of milk proteins prestained with Fluram: (1) α-lactalbumin; (2–5) acid whey; (6) α_s-casein; (7–10) acid-precipitated casein.

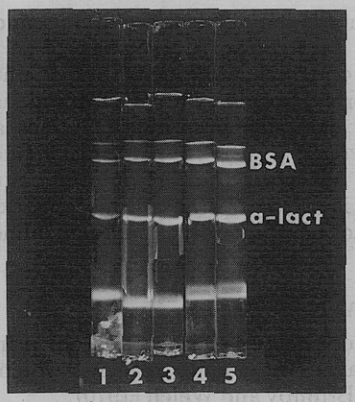

Figure 3. Sensitivity of disc gel electrophoresis of BSA–α-lactalbumin mixture prestained with Fluram. Each protein: (1) 1.25 μg; (2) 2.5 μg; (3) 5 μg; (4) 10 μg; (5) 15 μg.

casein from each skim milk had been prestained separately. No differences were found.

Figure 3 allows judgment about the minimum quantity of protein that can be detected on disc gels. Shown are gels to which varying quantities of BSA and α-lactalbumin had been applied after staining with Fluram. Protein amounts ranged from 1.25 to 15 μg. Both BSA and α-lactalbumin were easily discerned at the lowest level of application and, quite likely, amounts well below 1 μg could be detected.

Based on the study reported herein, prestaining with Fluram offers distinct advantages when compared to conventional methods which involve staining after electrophoresis. Included are speed of analysis, ability to use high acrylamide concentrations in gels, clarity of separations, and improved sensitivity. Assuming gels are made in advance, total time for analysis, including photography, takes less than 1 hr.

The marker and smaller protein bands diffuse and become less distinct with time. However, if photographed within a few hours a permanent record is obtained. When photography is not possible, gels can be stored in a refrigerator where band diffusion is delayed and fluorophor clarity and intensity can be maintained several days without significant changes.

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Role of Calcium in Activating Soybean Lipoxygenase 2

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Lipoxygenase 2 is activated by Ca^{2+}, but does not show optimum activity at a particular Ca^{2+} concentration as does crude soybean lipoxygenase. Still, the Ca^{2+} activation probably is due to Ca^{2+}–linoleate interaction rather than to Ca^{2+}–lipoxygenase 2 interaction. Such a conclusion is based on findings that (1) Ca^{2+} activation was not due to inhibition of lipoxygenase self-catalyzed destruction, (2) lipoxygenase 2 is active without added Ca^{2+}, (3) ultracentrifugal analysis indicates disappearance of a floating fraction when Ca^{2+} is mixed with linoleate, (4) Ca^{2+} does not activate lipoxygenase 2 when it acts on methyl linoleate, and (5) Ca^{2+} causes visible precipitates with linoleate. We find that Ca^{2+} activation of lipoxygenase 2 occurs only in the absence of Tween 20 and that, as a consequence, the differentiation of lipoxygenase 2 and 3 based on Ca^{2+} activation needs to be reexamined.

Restrepo *et al.* (1973) and Koch *et al.* (1971) reviewed the literature on Ca^{2+} activation of lipoxygenase. Restrepo *et al.* (1973) found that lipoxygenase 1 was inhibited but lipoxygenase 2 was activated by Ca^{2+} and concluded that Ca^{2+} was interacting with sodium linoleate rather than with lipoxygenase to cause the activation. They based their conclusion on studies with crude soybean extract showing an optimum activity when linoleate concentration was equal to Ca^{2+} concentration. We now investigate this activity relationship by using purified lipoxygenase 2.

Smith and Lands (1972) studied the self-catalyzed destruction of soybean lipoxygenase. The activation due to Ca^{2+} could be explained as an inhibition of the self-catalyzed destruction, and we report on studies investigating that possibility. While investigating the role of Ca^{2+} in

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