A WHEY PROTEIN CONTRIBUTING
TO LOAF VOLUME DEPRESSION[1]

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ABSTRACT

Baking studies showed whey, whey ultrafiltrate, and components of peak II to have a depressing effect on loaf volume as compared to a simulated milk dialysate control. A loaf volume depressing protein was separated from whey and was identified tentatively as proteose-peptone component 5. Disc-gel electrophoresis indicated that the depressor protein was present in unheated, undialyzed whey, whey ultrafiltrate, and components of peak II. SDS-gel electrophoresis and molecular weight estimation by column chromatography averaged the molecular weight of the peak II component protein to be 14,000 to 15,000 daltons. Association of the peak II component with calcium-phosphate was observed at pH 6.6.

Inclusion of NFDM proteins in bread is considered desirable due to the improvement of nutritional quality through supplementation of limiting amino acids: lysine, methionine, and tryptophan in wheat flour (1). Additionally, the use of milk improves the handling properties of the dough as well as the bread quality, i.e., flavor, crust color, toasting characteristics, crumb structure, and texture, keeping quality and overall acceptability (2). Adequate preheat treatment of the milk prior to its drying is necessary before milk can improve the mechanical and quality aspects of bread. The minimum heat treatment for milk

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to obtain optimum loaf volume is 50.6° to 56.1°C. for 30 min. (3). This heat treatment prior to drying of the milk is thought to inactivate some factor that 1) has a deleterious effect on dough consistency causing it to be slack and unacceptable, particularly for continuous mixing, and 2) causes a depressing effect on loaf volume. Sanderson (4) stated that research by Swanson et al. suggested that these problems are caused by two individual effectors. Slackness occurs as a function of uncomplexed β-lactoglobulin and K-casein. The nature of the factor responsible for loaf volume depression is, as yet, uncertain.

The casein fraction has not been found to promote either dough slackness or loaf volume depression (2,5); therefore, many whey proteins have been studied in native, oxidized, and heat-treated states to determine whether or not they contribute to loaf volume depression. Larson et al. (3) and Gordon et al. (6) showed that the euglobulins and pseudoglobulins contained in the lactoglobulin do not depress volume in either the native or heat denatured state. Furthermore, β-lactoglobulin, bovine serum albumin, and α-lactalbumin contained in the lactalbumin fraction do not result in serious decrease in loaf dimensions when they are in the native state (5,6,7).

The early method of designating whey proteins as the lactoglobulin and lactalbumin based on precipitation by $(NH_4)_2SO_4$ at 50 and 80% saturation has led to much confusion in the search for the loaf volume depressant as neither of these is a homogeneous protein preparation. Gordon et al. (6) contended that the factor existed in the lactoglobulin. However, Baldwin et al. (5) maintained that it is in the lactalbumin.

In 1959, component 5 of the proteose-peptone fraction of milk was described by Jenness (8) as having certain loaf volume-depressing qualities. The proteose-peptones are a heterogeneous mixture of proteins varying in size from 4,100 to over 200,000 (9). They are defined functionally as those proteins not precipitated by heating milk to 95°C. for 30 min., but precipitated by 10% TCA (10).

Early studies by Stamburg and Bailey (11) suggested that the depressing factor may be dialyzable. This was disputed by Harland et al. (12). Later studies by Baldwin et al. (5) again proposed the loss during dialysis for salt and sugar removal of a low-molecular-weight factor responsible for volume depression. This study was designed to observe whey protein fractions which when separated were consistent in depressing loaf volume. A common factor in all fractions was investigated.

MATERIALS AND METHODS

Ingredients

Flour used in this study was a commercial bakery A patent which was bleached, bromated, and enriched. Protein and moisture contents were determined according to AACC methods (13) as 13.0% (N × 5.7) and 10.6%, respectively. Commercial stocks of yeast, sugar, and salt were employed in bread preparation. Liquid used in this study consisted of whey proteins and controls described under Protein Preparations.

Baking Study

Bread was baked according to AACC method 10-85 (13) in order to measure the varying effects on bread quality of whey proteins. A protein level of 0.37%
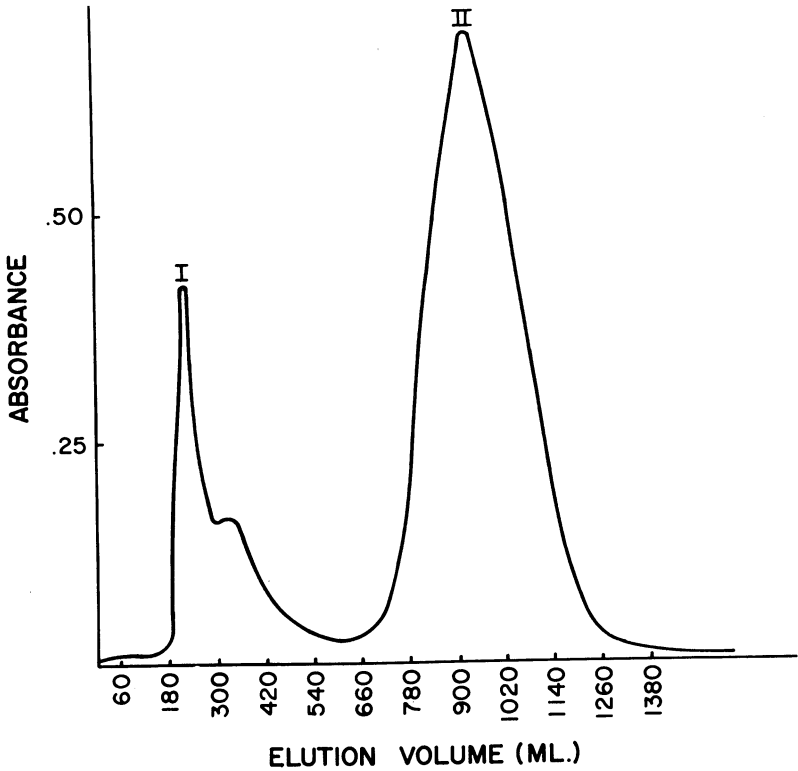
Fig. 1. Sephadex G-50 chromatography of whey ultrafiltrate.

based on the weight of the flour was selected following a pilot study in which loaves of depressed volume resulted from this protein concentration. Simulated milk dialysate at normal and increased salt concentrations was employed as a control without adjustment of salt concentration in the dough. The procedure was modified by exclusion of potassium bromate from the formula. One hour after baking, each loaf was weighed and its volume measured by rapeseed displacement on a loaf volumeter.

Protein Preparation

Whey proteins in this study were prepared by precipitation of the casein at pH 4.6 with N HCl from raw skim milk collected from the Michigan State University dairy herd. The milk was maintained at 37° C. from milking time throughout casein precipitation. The whey was separated from the precipitate and filtered through a Buchner funnel using Whatman No. 4 filter paper. Five hundred milliliters of whey at pH 4.6 was filtered on an Amicon Ultrafiltration UF-2 unit through an Amicon PM-30 membrane. Four hundred milliliters of ultrafiltrate was evaporated to a volume of 60 ml. after adjusting the pH to 6.6 with N NaOH. Adjusting of the pH to the physiological range yielded a flocculate of calcium-phosphate. Concentrated ultrafiltrate was subjected to column chromatography

using Sephadex G-50 medium gel and 0.02M NaCl for elution on a 5.0 × 60 cm. column. The chromatography resulted in separation of the ultrafiltrate into two peaks, designated peaks I and II (Fig. 1). Each peak was collected and adjusted by evaporation to 0.62% protein, which was measured by the method of Lowry et al. (14). Figure 2 presents a flow diagram of protein separation. Baking trials employed simulated milk dialysate and protein fractions according to the following:

Series I   Simulated milk dialysate (15)—buffer containing all the salts of milk
             Whey—acid precipitated

Series II   Simulated milk dialysate
             Concentrate—components retained above ultrafiltration membrane

Series III   Simulated milk dialysate
             Concentrated simulated milk dialysate
             Peak I—first components eluted during preparative chromatography
             Peak II—second component eluted during preparative chromatography

Electrophoretic Analysis

Electrophoresis was performed according to a modification of the method described by Melachouris (16) on raw whey, whey ultrafiltrate, and peak I and II components. A Buchler Polyanalyst disc-gel electrophoresis apparatus replaced the vertical flat bed used by Melachouris. The electrophoresing time at 120 v. was 45 min. for whey and whey ultrafiltrate, and 80 min. for peaks I and II. A sample volume of 70 μl of 0.62% protein solution was applied to each gel. The separated proteins were stained with amido black dissolved in 7% acetic acid. They were destained by equilibrium diffusion of unbound dye into 10% acetic acid. The presence of glycoproteins was tested using fuchsin-sulfite dye according to the procedure of Zacharius et al. (17).

Protein Molecular Weight Estimation

Sodium dodecylsulfate-gel electrophoresis and column chromatography molecular weight estimation of the peak II component was performed. The method of Weber and Osborn (18) for SDS-gel electrophoresis was employed. The SDS and 2-mercaptoethanol concentration of the sample solution applied to the gel was increased to 1%. Coomassie Brilliant Blue was employed for staining; destaining was accomplished by using 10% acetic acid equilibrium diffusion. Molecular weight estimation by gel chromatography was performed using a 1.5 × 90 cm. column packed with Bio-Gel P30, 50 to 100 mesh, elution was achieved by 0.02M NaCl. The general procedure was described by Whittaker (19). The proteins used for molecular standards were as follows: Chymotrypsinogen A (Pharmacia Biochemicals); Lysozyme (National Biochemical Corp.); and Ribonuclease A (Pharmacia Biochemicals).
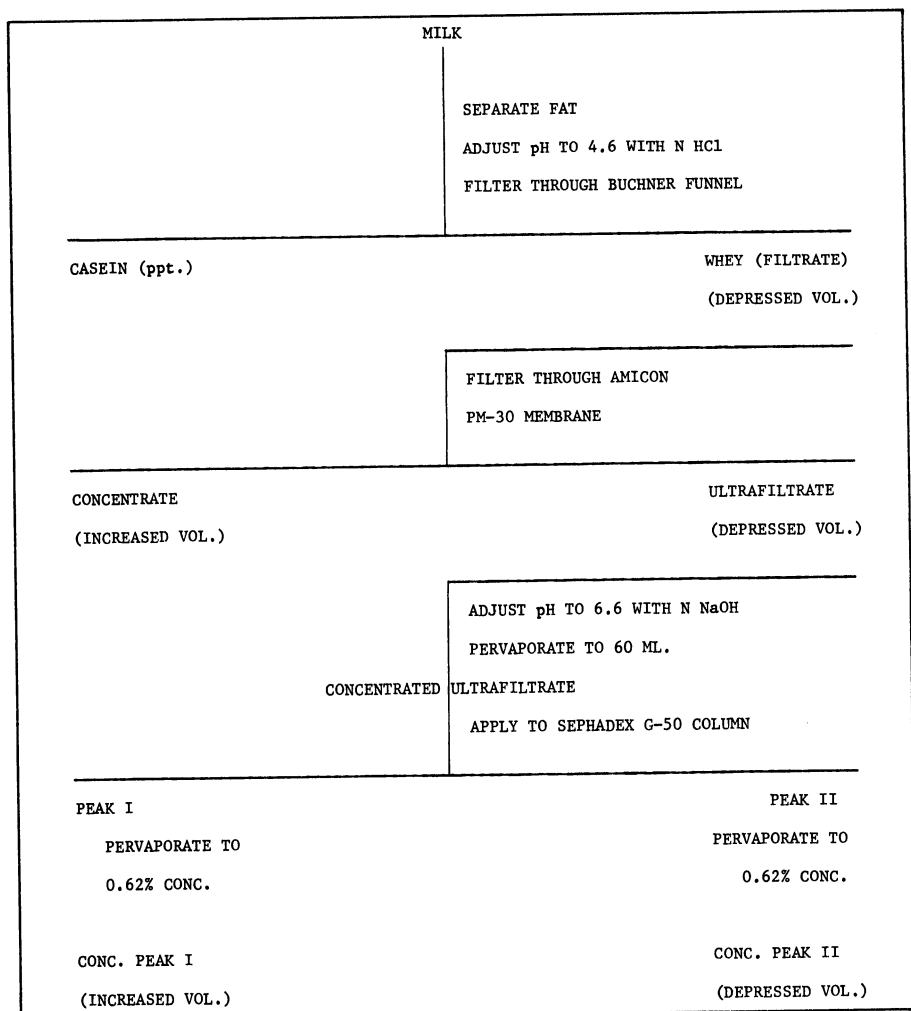
Fig. 2. Flow diagram of preparation of protein resulting in depressed volume.

RESULTS AND DISCUSSION

Baking Study

Table I shows loaf volume obtained for breads containing protein fractions and dialysate controls. Use of normal concentration simulated milk dialysate yielded average loaf volumes of 506 (series I), 483 (series II), and 486 cc. (series III) while the concentrated dialysate resulted in a decreased average volume of 452 cc. (series III). The whey and whey ultrafiltrate, both of which contained the depressing protein, had volumes smaller than both the normal and concentrated dialysate controls. The volume for whey-containing loaves averaged 316 cc. (series I) and the ultrafiltrate-containing loaves averaged 370 cc. (series II). The

TABLE I
Effect[a] of Various Whey Protein Fraction and
Dialysate Controls on Bread Quality

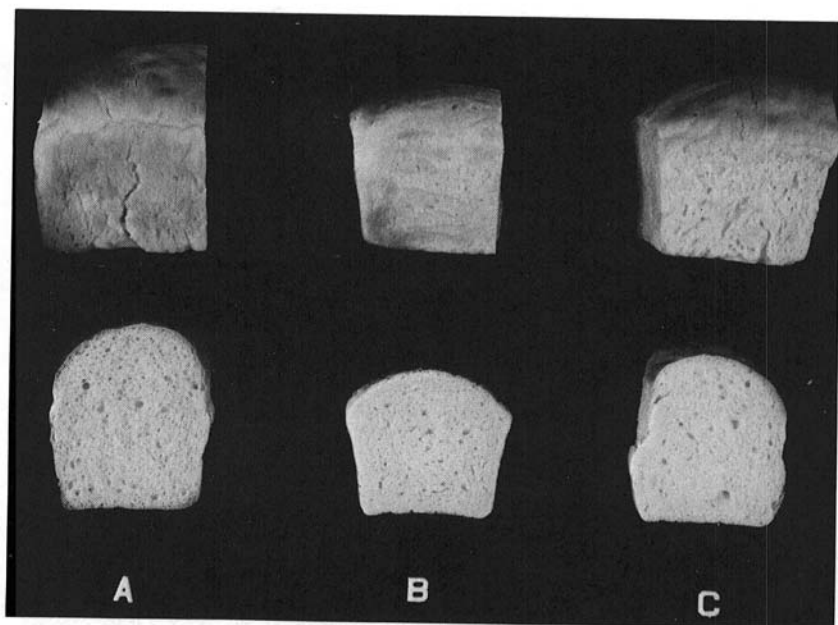| Series		Avg. Vol. cc.	Avg. Sp. Vol. cc./g.	% Volume Depression
I	Simulated milk dialysate	506	3.83	...
	Whey	316	2.83	37.5
II	Simulated milk dialysate	483	3.64	...
	Concentrate	495	3.65	− 2.4
	Ultrafiltrate	370	2.66	23.4
III	Simulated milk dialysate	486	3.64	...
	Conc. sim. milk dialysate	452	3.40	...
	Peak I	525	3.96	− 7.9
	Peak II	415	3.08	14.7

[a]Based on triple replications.

Fig. 3. Effects on bread quality of peak I (A), peak II (B), and unconcentrated simulated milk dialysate (C).

concentrate, as separated from whey, should have contained no depressing protein and yielded an average volume of 495 cc. (series II) which was larger than the controls from series II and III. The loaves prepared with peak I components had the greatest average value of 525 cc. (series III) which was considerably greater than any of the controls. The loaf containing the peak II components averaged 415 cc. (series III), showing depression as compared to all controls.
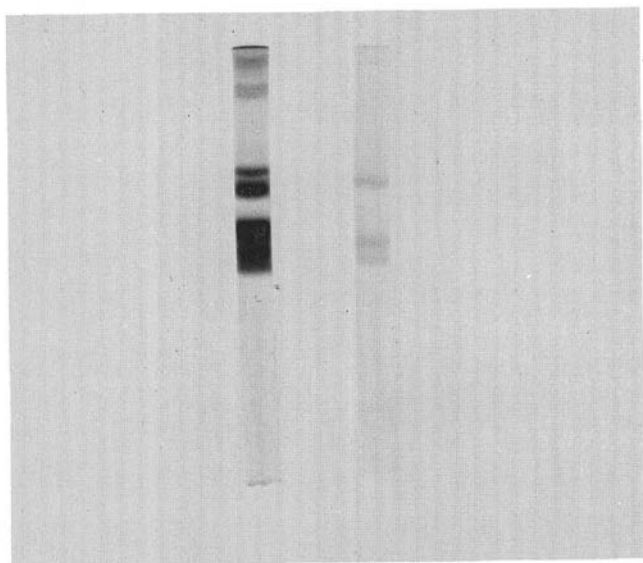
Fig. 4. Polyacrylamide disc-gel electrophoresis patterns of whey (left) and whey ultrafiltrate (right).

Breads prepared with peak I and II component proteins and proteins of whey, as observed from the concentrate and peak I, resulted in maintenance or improvement of loaf volume when compared with all controls except the dialysate control of series I. The concentrated peaks I and II, when compared with the concentrated simulated milk dialysate, showed an improvement in the loaf volume resulting from peak I proteins and a depression from the peak II protein (Fig. 3). Because of difficulties with electrophoretic experiments some depressing effect due to high salt concentration in the peak II fraction was suspected, and a dialysate control was prepared in which the salt concentration was twice that possible in the peak II fraction.

Electrophoretic Analysis

Polyacrylamide gel electrophoresis was employed to assess the protein complement of whey fractions yielding depressed loaf size. One zone was found consistently in each electropherogram of fractions that resulted in bread of depressed dimensions. Electropherograms of whey, used as a standard of identification, showed that the depressing protein migrated slightly behind β-lactoglobulin. The whey ultrafiltrate showed the depressing protein expressed as the second band from the bottom in the gel pattern (Fig. 4). Peak I components did not depress loaf volume nor was there evidence of the depressing protein, whereas peak II showed only a single band, slightly slower than the front-running band of peak I (Fig. 5). Disc gels of the peak II fraction initially showed poor electrical conductivity as evidenced by a slower migration of the marker dye, bromphenol blue. This problem was due to the slow elution of the protein during column chromatography and thus its contamination by salts present in the concentrated ultrafiltrate. Removal of the salts from the samples by dialysis
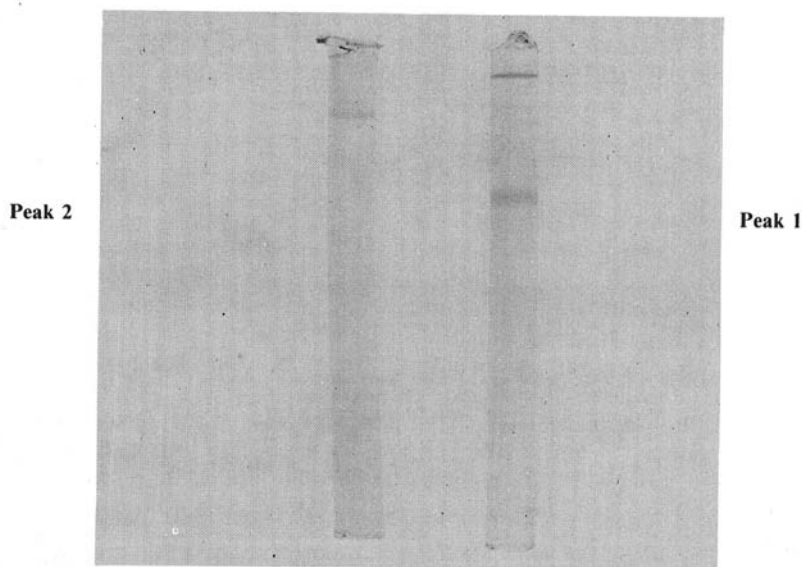
Fig. 5. Polyacrylamide disc-gel electrophoresis patterns of whey proteins fractionated from whey ultrafiltrate on Sephadex G-50. Peak I (right) and peak II (left) as in Fig. 2.

resulted in a loss of the band from the electropherogram. A consistent rate of migration of frontal bands of components of peak II was eventually achieved by dilution of the protein and electrophoretic analysis at 0.62% protein rather than 1% concentration which had been initially attempted. The disc gel pattern of peak II, the depressing protein, was not amenable to identification. The whey pattern in the general location of the peak II band was diffused. Because the peak II band stained with fuchsin-sulfite dye, it is suggested that the depressant factor is a glycoprotein.

Molecular Weight Estimation of Peak II by SDS-Gels and Column Chromatography

The SDS-gel electrophoresis analysis yielded an estimated molecular weight of 14,500 to 15,000 daltons. Column chromatography of the peak II protein component showed it to have a Ve/Vo of 2.32 (Table II) or very similar to the elution volume of Lysozyme, indicating a molecular weight of approximately 14,500 to 15,000 daltons. The peak II protein showed a strong tendency to adsorb to a Sephadex column packing, a characteristic of glycoproteins.

Tentative Identification of the Peak II Component

The tendency of the depressing protein to coprecipitate with calcium-phosphate as well as its glycoprotein nature suggested its identity as a proteose-peptone. Since proteose-peptones are a heterogeneous group of proteins, a problem of identification of the depressing protein remained. Molecular weight estimations of the depressor by SDS-polyacrylamide gel electrophoresis and column chromatography indicated a value of 14,000 to 15,000 daltons, thus implicating component 5 (9) as the depressing protein.

Summary and Discussion

In an attempt to isolate a loaf volume depressant factor, whey was divided by

TABLE II
Proteins Used as Standards in Column Chromatography
Molecular Weight Estimation of Peak II; Molecular Weights; Ve/Vo

Sample	Molecular Weight	Ve/Vo
Chymotrypsinogen A	25,000	1.94
Lysozyme	14,000	2.34
Ribonuclease A	13,700	2.43
Peak II		2.32

ultrafiltration into retentate and ultrafiltrate, the latter of which contained three proteins as determined electrophoretically and yielded bread of reduced dimensions. The ultrafiltrate was fractionated by column chromatography into two peaks; peak I components showed two zones and appeared to have an enhancing effect on loaf volume. The peak II components showed a single zone and had definite depressing effects. It was tentatively identified as proteose-peptone component 5. This would support earlier contentions by Jenness (8) as to its volume-depressing characteristics. The proteose-peptone component 5 was tested early in this study for deleterious effects on bread volume but purified preparations of the protein are extremely difficult to process. A method devised by Kolar (9), requiring no heat treatment, yielded protein without depressing effects. The exact nature of this protein is still unknown and needs considerable attention. Proteose-peptones are known to associate strongly with calcium-phosphate (9) in the whey system; this would explain the failure to remove the protein if the pH was adjusted to 6.6 prior to ultrafiltration. Component 5 is a glycoprotein which could explain peak II's pattern of adsorption to the Sephadex column and its positive testing for carbohydrate. The sugar moiety should be investigated to determine if it plays a role in the depression mechanism; perhaps it interferes with bonding in the gluten structure. Component 5, according to Kolar (9), contains no cysteine, indicating that loaf volume depression is probably not a function of sulfhydryl-disulfide interchange. The contention of Larson et al. (7) that the weakening of the gluten structure in depressed volume bread is not a function of disulfide interchange is thus supported.

In comparison of the amino acid composition of proteose-peptone component 5 and the gluten proteins, they are both found to be high in glutamine, proline, and nonpolar amino acids. This could infer that component 5 interfered with hydrogen or hydrophobic bonding which joined the gluten proteins into strong sheets comprising the structural network of the dough and the loaf. If an interference in the bonding forces occurred on a molecular level, areas of weakness in the dough would result wherein the expanding forces of the gas could cause limited cell collapse. Concomitantly, other air cells would also experience lower total extension because of slippage at the molecular level of the protein sheets due to a weaker intermolecular bonding of the gluten.

In working with the peak II protein it was found to be easily "lost," that is, when dialyzed, the peak II fraction no longer exhibited volume depression and the characteristic single band was absent in electrophoretic analysis. There was no evidence of the protein when the column was monitored at 280 nm. and protein determinations showed lower protein contents. This is in accord with statements by Baldwin et al. (5) relative to a low-molecular-weight substance

which is lost during dialysis as possibly being responsible for volume depression. In an early pilot study, bread prepared from a common lot of whey, one part of which had been dialyzed, the other not, showed no depression in the dialyzed portion but exhibited considerable depression in volume in the latter. Kolar (9) noted a dialysis loss of component 8 while preparing it for free boundary electrophoresis. The components 8-fast and 8-slow should be investigated to determine if they also contain depressing characteristics.

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