Unsaturated Fatty Acids of Butterfat

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This study was undertaken to characterize more completely the unsaturated fatty acids of butterfat. The presence of C_{10} to C_{18} monoethenoid acids was confirmed; the C_{12} and C_{14} were predominantly the cis-form, while the C_{16} and C_{18} acids had both cis and trans double bonds. The nonconjugated dienoic acids were found to be a mixture of cis-cis and either cis-trans or trans-trans isomers. Conjugated dienoic acids were identified as cis-trans and trans-trans isomers. The trienoic, tetraenoic, and pentaenoic acids had the all-cis configuration.

The composition of butterfat has been the subject of literally hundreds of investigations in the past 50 years. The presence of the normal straight chain saturated fatty acids from C_4 to C_{20}, with an even number of carbon atoms, has been fairly well established. However, in recent years, Shorland and coworkers have reported the presence of minor amounts of a number of branched-chain acids and acids with an odd number of carbon atoms (*14–20, 36–38*).

The identification and structure of the unsaturated fatty acids of butterfat have not been completely elucidated and are still the subject of extensive investigation. As early as 1912, Smedley (*40*) suggested that lower homologs of oleic acid were present. Other workers (*5, 11, 12, 27*) substantiated Smedley's postulation for the presence of decenoic acid and found evidence of C_{12}, C_{14}, and C_{16} monoethenoid acids. The double bond was claimed to be in the 9, 10 position (*26, 29*). Bertram's (*4*) discovery, in 1928, of vaccenic (trans-11-octadecenoic) acid led to further studies by others (*2, 3, 7, 9, 13, 33*) who also concluded that octadecenoic acids contained position as well as geometric isomers. Until recently, the lower unsaturated acids were thought to be exclusively cis forms. However, in 1954, Smith and coworkers (*41*) presented evidence of trans components in C_{12}, C_{14}, C_{16}, and C_{18} monoethenoid fatty acid fractions and in 1956, Backderf (*2*) found a trans-16-octadecenoic acid, a previously unreported component of butterfat.

Some early investigators concluded that the diethenoid acids of butterfat were mainly geometrical isomers of linoleic acid (*5, 6, 8, 24, 25*), while others found that these acids contained a high percentage of normal linoleic (9-cis, 12-cis octadecadienoic) acid (*44*). In 1954, Smith and coworkers (*41*) published the infrared spectra of concentrates of polyunsaturated acids from butterfat, which showed the conjugated dienoic acid to be largely cis-trans with the possibility that some trans-trans isomers were also present.

Substantially all the nonconjugated trienoic acid of butterfat has been identified as normal linolenic (9-cis, 12-cis, 15-cis octadecatrienoic) acid by the preparation of the bromo derivatives (*10, 35*).

Ultraviolet spectrophotometric evidence of a tetraenoic and a pentaenoic acid has been published (*30–32, 39, 42*). However, these acids have not been isolated and characterized.

The present study was initiated primarily to identify the polyunsaturated components of butterfat. However, in the course of the work, the monoethenoid components were also investigated. Owing to the inadequacies of the analytical methods for such complex mixtures containing geometric isomers, no attempt was made to relate quantitatively the amount of each unsaturated component in the various fractions to the amount in the butterfat.

Experimental

The butterfat employed in this investigation was prepared from butter churned from fresh pasteurized cream. The milk was obtained from the dairy herd located at the U. S. Department of Agriculture Research Center, Beltsville, Md. The herd had been fed winter rations for several months.

As many of the unsaturated fatty acids are present in minor proportions, it was necessary to concentrate them to facilitate their isolation. Four kilograms of butterfat were converted directly to the methyl esters by methanolysis; sodium methylate was used as catalyst. The methyl esters were distilled under reduced pressure to remove esters up to and including some of the esters of the C_{16} acids. The remainder were subjected to a series of crystallizations from acetone at −30° C., −45° C., and −65° C. to remove most of the palmitate, stearate, and some of the octadecenoate. The −65° C. filtrate material, 605 grams, was subjected, in 80- to 100-gram batches, to further fractionation by chromatographic adsorption on silicic acid columns. The column and technique for operation under nitrogen were essentially the same as described by Herb and Riemenschneider (*22, 23, 34*). The adsorbent mixture, 80% silicic acid–20% filter aid, was heated to 105° C. for 16 hours prior to packing in the column as a slurry in redistilled petroleum ether. The column was 6.5 cm. in diameter and 105 cm. in height and was packed with 1500 to 1600 grams of adsorbent mixture to a depth of 90 to 94 cm. Successive elutions were made with redistilled petroleum ether (boiling point 40–50° C.) containing increasing percentages of ethyl ether depending on the progress of the fractionation, which was determined by analysis of the fractions.

Fractions from this large column having similar composition, whether from the same or separate runs, were combined for further fractionation either by adsorption on a smaller silicic acid column, low pressure distillation, or urea - adduct stepwise crystallization. The smaller chromatographic adsorption columns were of the same general type as the larger column and were packed so that there was a ratio of 30 or more grams of silicic acid–filter aid mixture per gram of sample.

The urea adduct stepwise crystallization procedure was the same as that described by Allen (*1*). Fifteen grams of the esters were dissolved in 150 ml. of methanol, 15 grams of urea was added, and the mixture heated until the urea dissolved. The solution was held

Table I. Spectrophotometric Analysis of Butterfat and Concentrate from Butterfat

Acids	Butterfat (I.V. 30.2)	Concentrate (I.V. 101.0)
	Acid in Sample, %	
Conjugated		
Dienoic	0.43	2.23
Trienoic	0.02	0.07
Nonconjugated		
Dienoic	1.50	8.70
Trienoic	0.59	3.70
Tetraenoic	0.23	1.40
Pentaenoic	0.20	1.20
Monoenoic	29.1	68.80
Saturated (by difference)	65.9	8.90

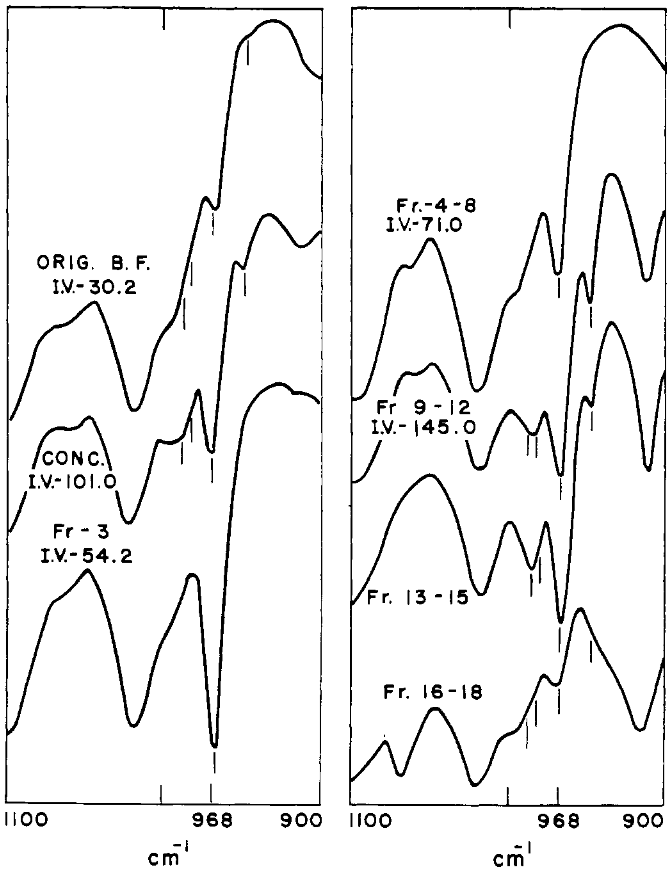

Figure 1. Infrared curves of butterfat methyl esters, concentrate of unsaturated esters, and fractions from a chromatographic separation of the concentrate. Ordinate is transmittance with zero displaced

at room temperature until the complex crystallized; the complex was removed by filtration. The filtrate was treated with 12 grams of urea to remove another fraction of the esters. The urea treatments were repeated until all the esters were recovered.

Methods of Analyses. The iodine values were determined by the Wijs method (30 minutes). Polyunsaturated acids were determined by the micro method of Herb and Riemenschneider (*21*), using a Beckman Model DU spectrophotometer. Infrared data were obtained with a Perkin-Elmer Model 21 spectrophotometer using essentially the procedure of Swern *et al.* (*43*).

Interpretation of Infrared Data. The infrared region from 900 to 1100 cm.$^{-1}$ (11.11 to 9.09 microns) is shown in the figures. The pertinent bands for interpretation of geometrical configuration are at 948, 968, 982, and 988 cm.$^{-1}$ (10.55, 10.33, 10.18, and 10.12 microns). The 968-cm.$^{-1}$ band is characteristic of a trans bond in a mono unsaturated ester or of a trans bond in a nonconjugated polyunsaturated ester. In this paper, the former will be referred to as an isolated trans bond and the latter as a nonconjugated trans bond. The band at 988 cm.$^{-1}$ and the doublet at 948 and 982 cm.$^{-1}$ were established by Jackson and coworkers (*28*) as characteristic of trans-trans conjugated diene and cis-trans conjugated diene, respectively. Unresolved absorption in the 982 to 988-cm.$^{-1}$ region in conjunction with a definite band at 948 cm.$^{-1}$ was considered evidence of both cis-trans and trans-trans conjugated diene.

The infrared curves are in per cent transmittance. The spectra of different fractions are presented in order to show the relative effect of various techniques in fractionating mixtures containing geometric isomers of the unsaturated components. The intensity of the trans bands shown in the curves for the fractions represent only visual comparisons of the trans components in these fractions and are not intended to show a quantitative interrelationship.

In every investigation where a number of fractionating techniques and manipulations are applied to unsaturated fatty acids and esters there is always the possibility of artifacts being formed. Precautions were exercised at every stage of the separation to minimize this possibility by preventing exposure to air and excessive heating. In no instance in the course of employing fractionating procedures for obtaining enrichment of the components was there observed an unexpected increase in trans components as might be the case if they were being produced as artifacts. Hence, the trans material did not appear to result from manipulative or chemical procedures. Likewise, other workers (*9*, *41*) found no evidence that similar techniques produced trans isomers as artifacts.

Results and Discussion

Further fractionation of the low pressure distillates or the precipitates from the fractional crystallization has not been attempted here. However, iodine values, saponification equivalents, and ultraviolet and infrared spectrophotometric analyses were obtained on these products. These analyses indicated that the unsaturated constituents of the distillates were principally esters of C_{10}, C_{12}, C_{14}, and C_{16} monoene acids, the esters of C_{12} and C_{14} acids being predominantly of the cis configuration. The major components of the precipitates from the fractional crystallization were methyl palmitate, methyl stearate, and methyl oleate.

Principal attention was given to the identification of the unsaturated components of the filtrate obtained from the −65° C. crystallization. The ultraviolet spectrophotometric analysis of this filtrate material (−65° C. concentrate) and of the original butterfat are given in Table I. The infrared spectra are shown in Figure 1. The original butterfat contained small amounts of conjugated diene and traces of conjugated triene esters. Nonconjugated esters with 2, 3, 4, and 5 double bonds were present. The total polyunsaturated acids present in butterfat is about 3%. A small amount of isolated or nonconjugated trans isomers are evident from the infrared spectrum. This is estimated to be not more than 5%. The −65° C. concentrate material represented about a sixfold concentration of polyunsaturated esters.

Table II. Analyses of Fractions from Chromatographed Concentrate

Fraction	Wt., G.	I.V.	Conjugated Diene	Nonconjugated Diene	Nonconjugated Triene	Nonconjugated Tetraene	Nonconjugated Pentaene
			Acid in Sample, %				
1–2	0	...	...	...	...	...	...
3	6.3	54.2	0.003	TR.	...	...	...
4–5	17.9	70.6	0.004	TR.	...	...	...
6–7–8	24.0	71.0	0.009	0.85	...	...	...
9	15.3	79.6	1.05	2.41	...	...	...
10	10.6	114.4	3.96	23.9	...	...	...
11	9.1	149.8	7.16	56.2	0.35	...	...
12	1.7	161.1	4.59	55.7	2.72	...	...
13	0.4	...	11.2	34.3	8.20	...	...
14	0.2	...	15.5	39.8	22.7	...	...
15	1.1	...	...	14.3	48.0	9.0	...
16	1.9	...	...	...	63.5	17.9	...
17	1.0	...	...	...	68.2	24.7	...
18	1.5	296.6	2.66	...	7.7	11.4	35.0
Residue	9.5	Viscous material containing 13% unsap.					

The infrared spectrum of the concentrate showed the presence of conjugated cis-trans and trans-trans isomers and an effective enrichment of the isolated or nonconjugated trans compounds as compared with the original butterfat.

The analyses and infrared spectra of the fractions from a typical chromatographic separation of the concentrate are illustrated in Table II and Figure 1. Fraction 3 contained saturated and monoene ester while fractions 4 to 8 consisted mainly of monoene esters with a lesser amount of saturated esters. The infrared curves show that fraction 3 contained a greater concentration of isolated trans bonds than fractions 4 to 8. This suggests that some fractionation of the trans from the cis monone esters had resulted.

Ultraviolet spectrophotometric analysis of fractions 9 to 12, both before and after isomerization, showed the presence of conjugated and nonconjugated diene esters and some nonconjugated triene esters. The infrared spectrum confirmed the presence of cis-trans and suggested the possible presence of trans-trans conjugated diene esters and nonconjugated diene containing some trans double bonds. The probable presence of trans-trans conjugated diene is more clearly shown by the infrared curves for fractions 13 to 15, which also contained a small amount of nonconjugated tetraene esters as indicated by the ultraviolet analysis.

Examination of the infrared spectra of fractions 9 to 12 and 13 to 15 shows that the unresolved absorption at 982 to 988 cm.$^{-1}$ favors the 982 cm.$^{-1}$ band in fraction 9 to 12 and the 988 cm.$^{-1}$ band in fraction 13 to 15. This, when considered with the intensity of the 948 cm.$^{-1}$ band in their respective spectra, indicates that some fractionation of the conjugated cis-trans from the conjugated trans-trans isomers has been achieved. Further evidence for the separation of these isomers is shown by the relative amounts of conjugated dienes removed in fractions 9 to 14 (Table II). Fractions 16 to 18 consisted essentially of triene, tetraene, and pentane esters having the all-cis configuration, as the small band at 968 cm.$^{-1}$ represents less than 3% trans material calculated as methyl elaidate.

Separation of Cis and Trans C_{18} Monoene Esters. A composite of fractions similar in composition to fraction 3 (Table II) was rechromatographed. The infrared spectra are shown in Figure 2. Fractions *A* and *B*, which are essentially all saturated as indicated by the iodine values, contained only a slight amount of trans material. Fraction *C* had a high percentage of isolated trans isomers, while Fraction *D*, which has an iodine value close to that of methyl oleate 85.1 (theory 85.6), contained no trans isomers. This shows the feasibility of this procedure for separating trans from cis monoene esters.

Separation of Trans-Trans and Cis-Trans Diene Esters. A sample of esters, which had an analysis similar to that of the composite of fractions 9 to 12 (Table II), was subjected to urea-adduct stepwise crystallization. The infrared curve of the composite is designated *O* in Figure 3. The conjugated trans-trans isomers were found mainly in precipitate fraction *A*, while the conjugated cis-trans isomers were found predominantly in precipitate fraction *C*. Precipitate fraction *B* and the final filtrate *D* contained very small amounts of both. Most of the isolated or nonconjugated trans material concentrated in fractions *A* and *B*. The final filtrate *D*, by ultraviolet spectrophotometric analysis, was rich in dienes; hence, even though there is evidence of small amounts of trans isomers, the presence of cis-cis nonconjugated linoleate is almost certain.

Separation of Triene and Tetraene Esters. Attempts to fractionate material comparable to fractions 16 and 17 (Table II) were only partially successful. Rechromatographing produced fractions containing 88 to 89% triene and 7 to 8% tetraene esters with no trans bonds, and fractions containing about 45% tetraene and 49% triene esters which had no trans bonds. The x-ray diffraction pattern of a completely hydrogenated portion of a fraction, which contained a high percentage of triene, showed only the presence of esters of C_{18} acids. Similar treatment of a fraction which contained a high percentage of tetraene indicated that the tetraene was an ester of a C_{22} acid although the presence of a C_{20} tetraene was not excluded.

Pentaene Esters. A composite of fractions comparable to fraction 18 (Table II) was rechromatographed. A fraction was obtained that appeared to be an ester of a C_{22} pentaenoic acid as judged by the iodine value, 368.3 (theory 368.5), and from a hydrogen number of 67.71 (iodine equivalent 374.8). However, the absorptivity determined by

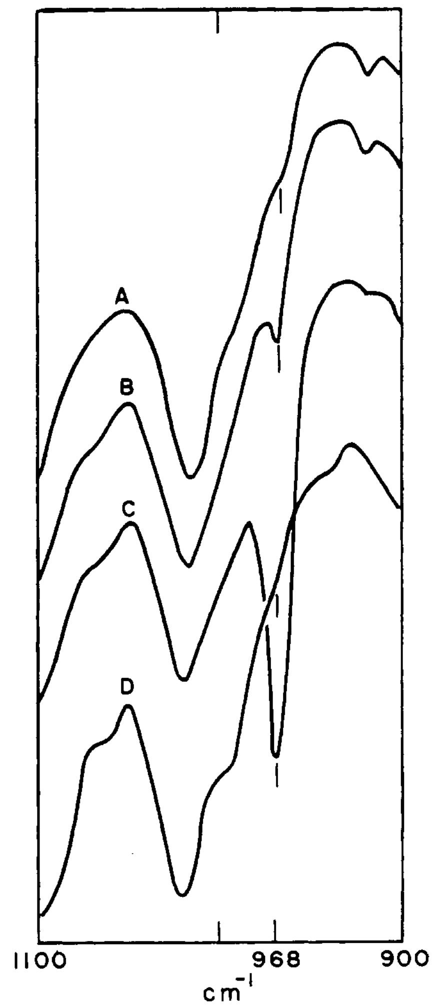

Figure 2. Infrared curves demonstrating the fractionation of butterfat cis monoethenoid from trans monoethenoid esters. Ordinate is transmittance with zero displaced

A. I.V. 8.0, mostly saturated esters
B. I.V. 8.7, mostly saturated esters
C. I.V. 62.8, saturated + cis and trans methyl oleate
D. I.V. 85.1, essentially pure cis methyl oleate

ultraviolet spectrophotometric analysis was high for this ester, $a_{346\,m\mu} = 56.4$, an indication that the fraction probably contained the esters of both C_{22} and C_{20} pentaenoic acids [published values *(27)* $a_{346\,m\mu} = 48.3$ and 83.6, respectively]. From the spectrophotometric data, the fraction was estimated to contain 77% esters of C_{22} and 23% esters of C_{20} pentaenoic acids. The calculated iodine number of such a mixture would be 376.0, which is in good agreement with that found by hydrogenation.

An x-ray diffraction study of a completely hydrogenated portion of this particular fraction and known mixtures indicated the presence of more than two components, which made the interpretation of chain length inconclusive.

The infrared spectrum, Figure 4, indicated that the pentaenes had the all-cis configuration.

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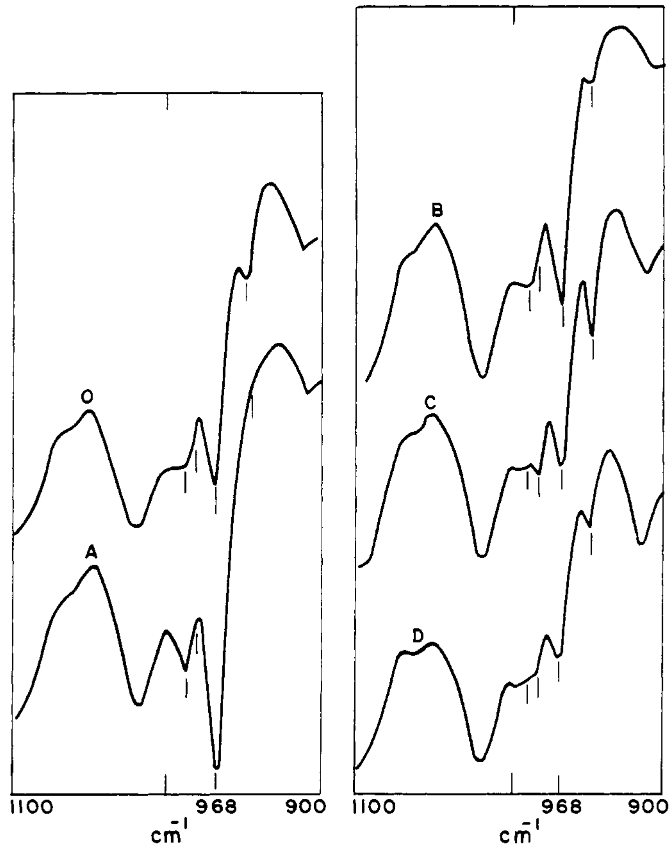

Figure 3. Infrared curves demonstrating the separation of cis-trans from trans-trans conjugated methyl linoleate by step-wise urea adduct crystallization. Ordinate is transmittance with zero displaced

O. Fraction similar to Fr. 9–12 (Table II)
A. 1st ppt. fraction
B. 2nd ppt. fraction
C. 3rd ppt. fraction
D. Final filtrate

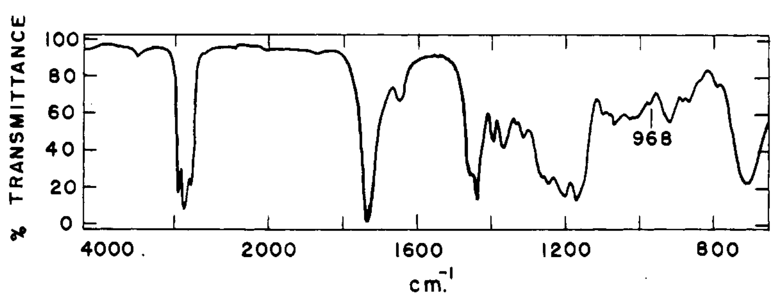

Figure 4. Infrared curve of methyl pentaenoate from butterfat. Determined as a liquid

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AMINO ACIDS IN SOYBEANS

Amino Acid Composition of Soybean Protein Fractions

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Soybean meal is of great economic importance because of its use in human foods and because it is a source of high-quality protein in formulating animal feeds. Amino acid analyses were made on soluble soybean protein and on acid-precipitated and heat-coagulated fractions. Mostly ion exchange chromatographic methods were used. The heat-coagulated fraction contained larger amounts of nearly all of the nutritionally essential amino acids than the total water-soluble protein. The amounts of amino acids in the water-soluble and acid-precipitated fractions were similar to reported values for the meal.

More than 6,000,000 tons of soybean meal are sold annually in mixed feeds. To improve feed efficiency, to stimulate use of isolated soybean protein, and to increase the consumption of meal products in foods, more exact information is required about the amino acid composition of soybean protein fractions. Such information is important because of the nutritionally essential nature of some amino acids. The variation of their requirement by different animal species (*2*) and the increasing evidence that the amount and kind of nonessential amino acids present affect the total amino acid required (*1*), are further reasons why exact composition needs to be determined.

Analyses of soybean protein by recently developed ion exchange chromatographic methods would be of value for comparison with previously reported analyses of the amino acid composition of whole soybean protein made mostly by microbiological methods. Insofar as is known, no amino acid analyses of different protein fractions from the soybean have been made. For the above reasons, the total water-soluble protein, the heat-coagulated fraction of the protein, and the acid-precipitated fraction were analyzed for their amino acid content.

Materials and Procedure

Preparation of Protein Fractions. Three protein isolates were investigated for their amino acid composition: the total water and dilute alkali-soluble protein, hereafter called soluble protein, representing more than 95% of the total protein in the soybean (*10, 12*); the acid-precipitable protein (*11*), representing about 60% of the soluble protein; and the heat-coagulable protein of whey (*13*), which is 7 to 9% of the soluble protein.

Soluble protein was prepared by extracting 20 grams of hexane-defatted meal at room temperature with 400 and 200 ml. of water successively, then twice more with 200 ml. of water adjusted to pH 8.0 with sodium hydroxide. The combined solutions were dialyzed for about 80 hours at 5° C. with several changes of distilled water, and the volume was reduced by pervaporation at room temperature. This solution was dialyzed again, further reduced in volume by pervaporation, and finally dried by lyophilization.

Acid-precipitated protein was prepared by a double extraction with water to meal ratios of 10 to 1 and 5 to 1, and the protein was precipitated from the combined extracts at pH 4.4 with hydrochloric acid. The curd was washed twice and dried by lyophilization.

Heat-coagulable protein, sometimes called albumin, was prepared from whey recovered from the acid-precipitated protein. The whey was adjusted to pH 8.0 to precipitate phosphorous compounds (*13*), heated for 15 minutes at 75° C., dialyzed, and then clarified by centrifugation. When the solution was adjusted to pH 5.0, the heat-coagulable protein was precipitated, recovered in a centrifuge, and dried by lyophilization. Whey proteins prepared by minor variations of this procedure gave the same amino acid composition.

Elementary Composition of Protein Fractions. Table I gives the elementary analysis of the protein samples analyzed for amino acids, as well as the variety of soybeans and crop year from which the samples were obtained. Total nitro-

Table I. Source and Elementary Composition of Various Protein Samples Analyzed

Variety	Crop Year	Preparation	Percentage[a] Nitrogen	Phosphorus	Sulfur	Ash
Lincoln	1952	Soluble protein	15.8	0.42	0.81	1.9
Hawkeye	1955	Soluble protein	16.1	0.52	0.93	2.0
Lincoln	1952	Heat-coagulated	16.1	...	...	...
Hawkeye	1950	Heat-coagulated	15.9	0.0	...	...
Adams	1954	Heat-coagulated	15.9	0.0	0.89	0.1
Adams	1955	Heat-coagulated	15.9	0.0	1.05	0.2
Lincoln	1952	Acid-precipitated	16.5	1.2	0.90	2.6

[a] Calculated on dry basis. Average values for at least two determinations.