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Soluble Proteins of Alfalfa (*Medicago sativa*) Herbage. Fractionation by Ammonium Sulfate and Gel Chromatography

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Procedures were devised for the isolation of three soluble protein fractions: (1) fraction I protein, (2) high molecular weight fraction II proteins, and (3) low molecular weight fraction II proteins. Fraction I protein was isolated by sodium sulfate fractionation and Sepharose 6B chromatography. It was completely dissociated into two subunits at pH 11.7. The sedimentation coefficient of alfalfa fraction I protein was similar to those reported for other species. The two groups of fraction II proteins were isolated using ammonium sulfate fractionation and chromatography on Sephadex G-25 and G-150. Average sedimentation coefficients of the high and low molecular weight fraction II protein groups were 6.8 and 3.8, respectively. The high molecular weight fraction II group contained two predominant proteins while the low molecular weight fraction contained many proteins. *o*-Diphenol oxidase activity was most effectively inhibited by storing the forage under a nitrogen atmosphere and by extraction in the presence of 5.0 m*M* metabisulfite.

The soluble leaf proteins are the predominant foaming agents in legume forage crops and are responsible for pasture bloat in ruminant animals grazing alfalfa or clover pastures. In the course of our studies on pasture bloat we wished to know whether or not there are differences among soluble alfalfa leaf proteins in their ability to stabilize persistent foams in the rumen. If differences do occur, they should be indicated by certain physical–chemical properties related to surface activity, i.e., isoelectric point (Cumper, 1953), solubility, hydrophobicity, and stability of tertiary configuration (Evans et al., 1970). Isolation of the protein fractions under consideration was required for the study of these parameters.

The soluble leaf proteins are classified into two major

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groups, fractions I and II, with sedimentation coefficients of 18 S and 4–10 S, respectively. Fraction I protein, which has been intensively investigated (Kawashima and Wildman, 1970), is a single homogeneous protein and is synonymous with ribulosebisphosphate carboxylase (EC 4.1.1.39). It comprises approximately one-half of total soluble protein. A variety of procedures for isolation of fraction I protein have been published (Andrews et al., 1973; Goldthwaite and Bogorad, 1971; Gray and Kekwich, 1974; Hood, 1973; Kawashima and Wildman, 1970; Nishimura and Akazawa, 1974) using combinations of salt fractionation, differential centrifugation, gel filtration, and ion-exchange chromatography. Some properties of foams stabilized by fraction I protein have been described (McArthur and Miltimore, 1966; Jones and Lyttleton, 1972a). The fraction II proteins are a very heterogeneous group which have not been well characterized. Jones and Lyttleton (1972a) have described a scheme for the isolation of clover fraction II proteins and have compared some properties of foams stabilized by fractions I and II proteins but they did not investigate possible differences among proteins within the fraction II group.

This paper describes work on the development of procedures for isolation of soluble alfalfa leaf protein fractions for use in investigations of foaming properties. We have devised a simple procedure for the isolation of fraction I protein, obtained fraction II proteins free of fraction I protein, and resolved fraction II proteins into two groups of high and low molecular weights. Our studies on the characterization of these protein fractions in relation to foaming will be described in another paper.

EXPERIMENTAL SECTION

Field-grown alfalfa herbage (cultivars Ladak and Beaver) was harvested in the prebud stage of growth, frozen at −20°, ground in a plate grinder, cooled with liquid nitrogen (Hikichi and Miltimore, 1970), and stored in plastic bags at −20°.

Crude extracts were prepared by extracting 20–200 g of the ground alfalfa in equal amounts (w/v) of 0.1 *M* Tris-HCl (pH 7.85) or 0.125 *M* sodium tetraborate–HCl (pH 7.85) buffers containing 5.0 m*M* sodium metabisulfite. The alfalfa was stirred vigorously in the buffer for 10–15 min and strained through a Dacron cloth. The strained fluid was centrifuged at 20,000*g* for 15 min in a refrigerated centrifuge. The supernatant solution was retained and is referred to herein as the crude extract. Unless otherwise noted, buffer solutions were cooled to 4° and all operations were performed at this temperature.

Isolation of Fraction I Protein. Crystalline sodium sulfate was added to the crude extract to 11% (w/v) concentration. The precipitate was sedimented by centrifugation and discarded. The supernatant was made to 25% sodium sulfate and the protein precipitate was collected by centrifugation. These operations with sodium sulfate were performed at room temperature. After redissolving in a minimal amount of 0.02 *M* sodium dihydrogen phosphate buffer (pH 6.8) the protein was applied to a column of Sepharose 6B (5 × 90 cm, void volume 525 ml). Ascending elution was with the same buffer. The eluate fractions were examined by electrophoresis and the fractions which contained only fraction I protein were pooled, made to 65% (w/v) concentration with ammonium sulfate, and stored at 4°. Fraction I protein, free of fraction II proteins, was eluted on the leading edge of the major protein peak.

Isolation of Fraction II Proteins. Crystalline ammonium sulfate was added to crude extract to a concentration of 27–30% (w/v) and let stand for 2 hr. After centrifugation (30,000*g* for 15 min) the precipitate was discarded. The supernatant was made to 65% (w/v) ammonium sulfate, let stand 3 hr, and centrifuged as above. This precipitate was dissolved in a minimal amount of 0.1 *M* Tris-HCl buffer (pH 7.85) containing 5.0 m*M* sodium metabisulfite and 1 m*M* dithioerythritol, applied to a column of Sephadex G-25 coarse (5 × 50 cm, void volume 475 ml), and eluted with the same buffer. The pooled eluate fraction containing protein was retained, made to 65% ammonium sulfate, let stand 3 hr, and centrifuged. The precipitate was redissolved in a minimal amount of G-150 buffer (0.02 *M* Tris-HCl–0.02 *M* sodium chloride–0.02% sodium azide, pH 7.85), applied to a column (5 × 90 cm) of Sephadex G-150, and eluted in 13-ml fractions. Ascending elution was in the same buffer. The void volume of the column, determined with dextran blue, was 615 ml.

Protein composition of the eluate fractions was determined by electrophoresis and the fraction II proteins, free of fraction I, were pooled into two groups, the high and low molecular weight groups.

A batch treatment with DEAE-Sephadex was employed to concentrate the fraction II proteins and to remove nonprotein contaminants. Two grams of DEAE-Sephadex A-50, swollen and washed in G-150 buffer, was placed in a 1000-ml separatory funnel over a glass wool plug. Pooled eluate fractions from the Sephadex G-150 column were added to the funnel, adsorbed onto the DEAE-Sephadex, and washed with G-150 buffer until the eluate was free of substances absorbing at 280 nm. To elute the protein, the DEAE-Sephadex was washed by shaking with 30 ml of G-150 buffer containing 0.75 *M* sodium chloride and allowed to stand for 30 min before draining. This washing procedure was repeated three times with additional 20-ml volumes of the same eluent and then twice with 20 ml of G-150 buffer containing 1.0 *M* sodium chloride. These six washings were pooled, made to 65% (w/v) with ammonium sulfate, and allowed to stand overnight. The protein precipitate was collected by centrifugation, redissolved in 20 ml of G-150 buffer, and dialyzed against two changes of 0.002 *M* sodium dihydrogen phosphate buffer (pH 7.0) containing 0.1 m*M* dithioerythritol.

Analytical Procedures. Electrophoresis was in polyacrylamide gels (5.25% T, 4.75% C) in 0.1 *M* Tris-glycine buffer (pH 8.9). The electrophoresis chamber was the thin slab apparatus of Reid and Bieleski (1968). A constant voltage of 80 V (12.5 mA) was applied for 10 min followed by 160 V (25 mA) for 60 min. Gels were stained with 0.1% Coomasie Blue R-250 dissolved in methanol–acetic acid–water (25:7:68, v/v/v) and destained overnight in the same solvent.

Sedimentation coefficients were measured with a Beckman Model E analytical ultracentrifuge operating at 20° and 50,740 rpm (Chervenka, 1969). The protein concentration was 2–4 mg/ml in 0.002 *M* sodium dihydrogen phosphate buffer (pH 7.0). Spectrophotometric measurements were made on Unicam SP 1800 and Beckman DB spectrophotometers. Protein concentrations were measured by the Lowry (Munro and Fleck, 1969) method.

RESULTS AND DISCUSSION

Fraction I Protein. Our procedure for isolation of fraction I protein is a relatively simple method which uses salt fractionation and gel filtration. This procedure permits the preparation of relatively large amounts of fraction I protein with ease. The preparations appeared homogeneous on polyacrylamide electrophoresis but the more powerful technique of isoelectric focusing indicated a trace of a second protein band. The ratio of absorbances at 280/260 nm was 1.6–1.7, slightly less than values as high as 1.75–1.80 reported by others (McArthur et al., 1964; Jones and Lyttleton, 1972a). For greater purity a further purification by ion exchange would be desirable but this degree of purity was considered acceptable for our purposes.

Identification of the protein as fraction I protein was confirmed by analytical ultracentrifugation. The protein had an $s_{20,buffer}$ value of 17.9, a value consistent with $s^0_{20,w}$

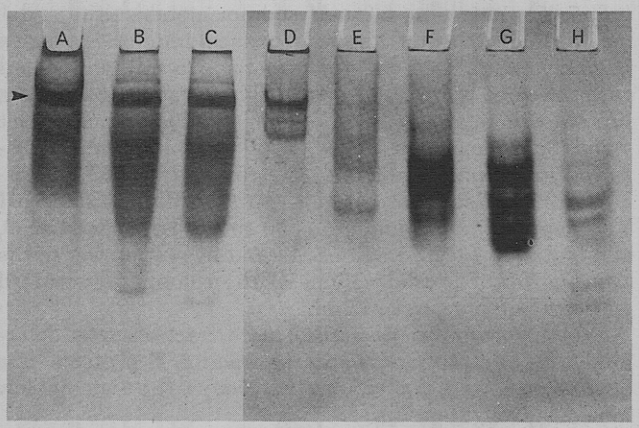

Figure 1. Polyacrylamide gel electrophoresis of alfalfa soluble proteins: (A) crude extract; (B) 30–65% ammonium sulfate cut; (C) after Sephadex G-25 chromatography. Samples in wells D to H, respectively, are fractions 60–72, 73–81, 82–102, 103–114, and 115–126 from Sephadex G-150 chromatography. Fraction I protein is indicated by the pointer to the left of well A. The faint band just above may be the dimer of fraction I protein. All other proteins are fraction II.

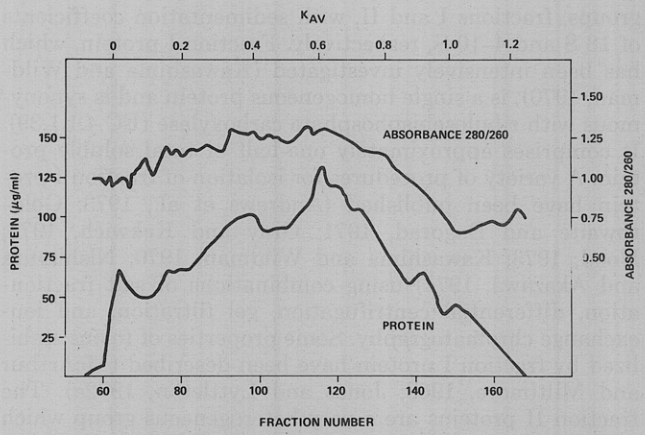

Figure 2. Chromatography of alfalfa fraction II proteins on Sephadex G-150. The lower line shows protein concentration and the upper line shows the ratio of absorbances, 280/260 nm.

values of 18.2–18.7 reported for alfalfa (McArthur et al., 1964) and other species (Kawashima and Wildman, 1970). Several reports have indicated that the sedimentation coefficient of fraction I protein has a very low concentration dependency (McArthur et al., 1964; Kawashima and Wildman, 1970). However, Hood (1973) reported an $s^0_{20,w}$ value of 25.3 for alfalfa fraction I protein. His relatively high sedimentation coefficient value apparently arose from an appreciable concentration dependency.

Limited studies were performed on the dissociation of fraction I protein at alkaline pH in phosphate buffers. Subunits were detected by analytical ultracentrifugation. Partial dissociation occurred at pH 11.3 and complete dissociation occurred at pH 11.7. Two fragments, with observed sedimentation coefficients of 1.2 and 4.7 S, appeared when fraction I was dissociated. These results are in general agreement with the subunit structure of fraction I protein from other species except that our observed sedimentation coefficient for the smaller subunit was lower than values reported for other species (Kawashima and Wildman, 1970). Hood (1973) reported the appearance of three subunits when alfalfa fraction I protein was exposed to 0.1% sodium dodecyl sulfate. In our studies, we sometimes observed three fragments and assumed this to be due to incomplete dissociation.

Fraction II Proteins. In previous work (Howarth et al., 1973) when alfalfa crude extract was chromatographed on Sepharose 6B fraction I protein trailed into the region where fraction II proteins were eluted, probably because of the large amount of fraction I protein in the crude extract. Several other gel permeation media were tried with similar results. We therefore employed ammonium sulfate precipitation to achieve a preliminary incomplete removal of fraction I protein prior to chromatography on Sephadex G-150. The crude extract was made to 27–30% (w/v) with crystalline ammonium sulfate to precipitate the majority of fraction I protein. There was partial loss of several larger fraction II proteins and this became significant if the ammonium sulfate concentration was high enough to precipitate all fraction I protein. The optimum ammonium sulfate concentration varied from 27 to 30% with different sources of alfalfa. At the optimum ammonium sulfate concentration 50% of the protein in the crude extract was precipitated. Excessive amounts of fraction II proteins were precipitated in 30% ammonium sulfate below pH 7.8 and large amounts of these proteins were precipitated at pH 5.7. Therefore, it was necessary to use a relatively concentrated extraction buffer to provide sufficient buffering capacity against the organic acids in the alfalfa and the ammonium sulfate.

Fraction I protein could also be precipitated by adjusting the crude extract to pH 5.2 and standing at 4° overnight. However, the ammonium sulfate fractionation was more convenient for our purpose and allows recovery of native fraction I protein, if desired, whereas pH precipitation irreversibly denatures fraction I protein.

Although precipitation of fraction II proteins removed some low molecular weight, nonprotein constituents, an appreciable amount of phenolic glycoside was precipitated with fraction II proteins in the 65% ammonium sulfate. Therefore, the redissolved 30–65% ammonium sulfate fraction was chromatographed on Sephadex G-25 to remove these phenolic compounds from the proteins as early as possible, thus decreasing the possibility of oxidation of phenols to quinones and covalent bonding to proteins. During passage through G-25 the protein fraction was easily visible as a light brown band. Another colorless component, perhaps nucleic acids, eluted along the leading edge of the protein fraction. The phenolic compounds were easily visible by their yellow color. If the protein fraction was rechromatographed on G-25, additional yellow compounds dissociated from the proteins and eluted behind the proteins. The phenolic constituents were partially adsorbed onto the Sephadex. After the proteins were eluted, adsorbed phenols were removed from the Sephadex by washing the column with 0.1 *N* acetic acid followed by reequilibration with buffer. Electrophoresis of the protein preparation before and after passage through Sephadex G-25 (Figure 1, wells B and C) showed that no protein was lost by this treatment.

The protein fraction from Sephadex G-25 was chromatographed on Sephadex G-150 to remove residual fraction I protein from fraction II proteins and to separate fraction II proteins into two groups on the basis of molecular weight. Figure 2 shows an elution profile from the G-150 column based upon analysis of the eluate by the Lowry method. A number of peaks, incompletely separated, were observed. Figure 1 shows the electrophoretic analyses of selected eluate fractions. Fraction I protein eluted in the first peak while the remainder of the profile contained fraction II proteins, free of fraction I protein. Approximately 15 fraction II protein bands are easily distinguishable. These will be described in more detail in a subsequent report. Careful control of buffer flow rate was essential because at high flow rates fraction I protein trailed badly into the fraction II proteins. The first peak contained a variety of fraction II proteins in addition to fraction I, probably resulting from aggregation of fraction II proteins during passage through

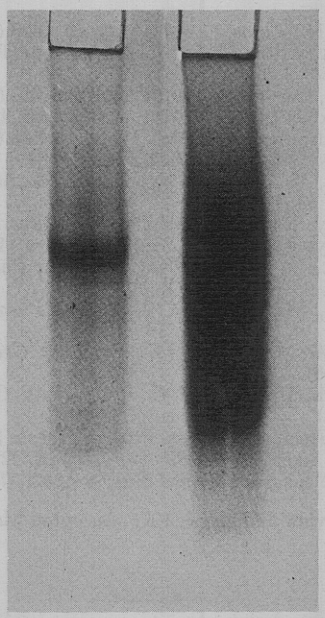

Figure 3. Polyacrylamide gel electrophoresis of fraction II proteins. The high molecular weight group is shown on the left and the low molecular weight group is on the right.

the Sephadex G-150 and disaggregation during electrophoresis. Since phenols give color development in the Lowry procedure, the amount of protein is overestimated in fractions near the bed volume. No protein was detected by electrophoresis beyond fraction 132.

The ratio of absorbances (A) at 280 and 260 nm was low at the first peak, reached a maximum of 1.3 in the second and third peaks, and declined rapidly to values less than 1.0 toward bed volume. The decrease in ration A_{280}/A_{260} between fractions 120 and 130 was not accompanied by increased absorbance at 330 nm suggesting that the nonprotein material with a low A_{280}/A_{260} ratio was not flavonoid.

The work of Kawashima et al. (1967) who chromatographed tobacco leaf proteins on Sephadex G-200 is pertinent to our results. Their elution profile was similar to ours except that they consistently observed four major fraction II protein peaks whereas we have seen three. Our failure to observe the same number of peaks may be due to the use of proteins from a different plant, or the use of a larger diameter column with loss of resolution. They have characterized some tobacco fraction II proteins using immunological and enzymatic methods.

After electrophoretic analysis the Sephadex G-150 eluent fractions, containing fraction II proteins free of fraction I protein, were pooled into two groups: (1) the high molecular weight fraction II proteins eluting into tubes 70 to 100, and (2) the low molecular weight fraction II proteins eluting into tubes 100 to 130. DEAE-Sephadex A-50 was initially chosen to concentrate the fraction II proteins but it also removed nonprotein contaminants from these two pooled fractions.

When a solution containing pooled high molecular weight fraction II proteins was applied to the DEAE-Sephadex some nonprotein material was not firmly adsorbed and appeared in the eluate. This eluate had maximum absorbance at 276 nm, A_{280}/A_{260} of 1.2, and no absorbance between 220 and 245 nm. The same material was eluted while washing the DEAE-Sephadex with G-150 buffer and was also eluted before the protein during washing with G-150 buffer containing 0.75 M sodium chloride. Although this DEAE-Sephadex treatment removed nonprotein material from these fraction II proteins, the A_{280}/A_{260} was improved only marginally from 1.22 to 1.28, since the nonprotein material removed by DEAE-Sephadex had A_{280}/A_{260} of about 1.2. When a preparation of the high molecular weight fraction II proteins was not passed through DEAE-Sephadex but rather was precipitated in 65% (w/v) ammonium sulfate and dialyzed, the A_{280}/A_{260} ratio was increased from 1.09 to 1.34. The supernatant after addition of 65% ammonium sulfate contained material with maximum absorbance at 274 nm and A_{280}/A_{260} of 1.2 but no absorbance between 220 and 245 nm.

When a solution containing pooled low molecular weight fraction II proteins was applied to the DEAE-Sephadex, some nonprotein material was not adsorbed and appeared in the eluate. This eluate had maximum absorbance at 269 nm, no absorbance between 220 and 240 nm, and A_{280}/A_{260} of 0.77. The adsorbed proteins were removed from the DEAE-Sephadex by elution with G-150 buffer containing 0.75 M sodium chloride. This procedure with DEAE-Sephadex increased the A_{280}/A_{260} of the low molecular weight fraction II proteins from 1.08 to 1.16. After precipitation of these proteins in 65% (w/v) ammonium sulfate and dialysis their A_{280}/A_{260} was 1.46. During elution of this protein fraction from the DEAE-Sephadex, the presence of another nonprotein contaminant was indicated by lower A_{280}/A_{260} in the last eluate (A_{280}/A_{260} of 0.97) than in the first eluate (A_{280}/A_{260} of 1.36). Thus, two major nonprotein contaminants were present in the low molecular weight fraction II proteins before passage through DEAE-Sephadex. One contaminant was not retained by the DEAE-Sephadex and the other was adsorbed more strongly than the protein. Both contaminants had A_{280}/A_{260} less than 1.0.

Apparently these dialyzable substances, removed from the fraction II proteins by DEAE-Sephadex, were now dissociating from the proteins because they had not been previously removed by ammonium sulfate precipitation or Sephadex G-25.

Figure 3 shows acrylamide gel electrophoresis of the high and low molecular weight fraction II proteins. The high molecular weight group is characterized by two predominant protein bands, while the low molecular weight proteins are more heterogeneous. Some proteins in the low molecular weight fraction have a low net charge/mass ratio because they had slower electrophoretic mobility than the high molecular weight proteins.

Analytical ultracentrifugation showed that $s_{20,buffer}$ of the high molecular weight fraction II proteins was 6.8 and that of the low molecular weight group was 3.8. These observed sedimentation coefficients correspond to approximate average molecular weights of 125,000 and 40,000, respectively.

Inhibition of *o*-Diphenol Oxidase (EC 1.14.18.1). Endogenous phenolics may be oxidized by *o*-diphenol oxidase producing quinones and brown pigments which form covalent bonds with proteins (Anderson, 1968), thus altering physical properties of the proteins (Jones and Lyttleton, 1972b). We were therefore concerned with inhibition of this enzyme before and during extraction.

Our conditions for storage of the alfalfa herbage were very effective in preventing oxidation. The freeze-ground alfalfa was stored in plastic bags and the evaporation of liquid nitrogen after grinding provided a nitrogen atmosphere. When stored under such conditions there was no apparent oxidation of phenols for up to 1 year but if a package was opened and closed several times to remove material, then oxidation occurred at freezer temperature and the extracts became brown.

The rate of browning in alfalfa extracts prepared without *o*-diphenol oxidase inhibitors was relatively slow compared to some other forage species, i.e. red and white clover. This may be due to relatively low *o*-diphenol oxidase activity or possibly low concentrations of *o*-dihydroxyphenols in alfalfa. In our experience, metabisulfite (5.0 mM) has been more effective than polyvinylpyrrolidone, diethyldithiocar-

bamate, or ascorbate in the inhibition of *o*-diphenol oxidase of legume forages.

The ratios A_{280}/A_{260} of our fraction II preparations were lower than we had hoped to achieve but could have been increased by accepting lower yield of protein during treatment with DEAE. However, the major contaminant may not have been phenolic for it had maximum absorbance at 272–274 nm, little absorbance at 330 nm, and no absorbance between 220 and 240 nm.

Chromatography on Sephadex gave partial removal of phenolic compounds from proteins and suggested the occurrence of reversible adsorption of phenols to proteins, possibly through hydrogen bonding.

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Composition of San Francisco Bay Brine Shrimp (*Artemia salina*)

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Brine shrimp from San Francisco Bay have been analyzed for proximate composition, including moisture, protein, crude fat fiber, and ash; 11 vitamins; 9 minerals; amino acids; cholesterol; distribution of lipid fractions; and amounts of fatty acids in the various lipid fractions.

Dried brine shrimp have long been used as food for aquaria but few reports of their composition exist. In work in progress in our laboratories, juvenile lobsters have been shown to attain excellent growth rates when fed a diet consisting solely of live brine shrimp. Also, Serfling et al. (1974) have described methods for feeding both live and frozen brine shrimp which result in good larval survival. In view of these findings, this study was undertaken to determine the composition of brine shrimp with the expectation that such knowledge would be useful in formulating diets for lobsters and other crustacea.

EXPERIMENTAL SECTION

Live brine shrimp (San Francisco Bay Brand, Metaframe Co.) were drained of water in a nylon fish net and then washed and filtered in acetone. This rinse was carried out in order to remove as much water as possible from between the swimmerets of the brine shrimp so that moisture determinations would be more accurate. The rinse was not of sufficient duration to extract any compounds from the brine shrimp. After the acetone had evaporated the shrimp were frozen and lyophilized. Moisture content was based on the original weight and that of the freeze dried material.

Amino acid composition of acid-hydrolyzed samples was done in a conventional manner using a Technicon amino acid analyzer. Cysteine was determined as cysteic acid (Moore, 1963) and tryptophan was determined by the colorimetric method of Spies and Chambers (1948).

The carotenoid index (C_I), a relative means of expressing content of carotenoids, was determined by the method of Kelly and Harmon (1972) which employs the following calculation (the absorbance (A) being that of a cyclohexane solution of extracted carotenoids):

$$C_I = (A_{474nm} \times 100)/(\text{g wet wt sample} \times \% \text{ dry wt})$$

Crude fat was determined by Soxhlet extraction with chloroform and methanol (2:1). Characterization of the lipid fraction was done by column chromatography, thin-layer chromatography, and gas–liquid chromatography of methyl esters. In general, the procedure of Medwadowski et al. (1967) was followed except that the chloroform soluble fraction of the crude fat was passed initially through a Sephadex G-25 column and the operating temperature for the gas–liquid chromatography was 190°. The methyl esters were identified and quantified according to Bartlett (1966) and Panos (1965). Cholesterol was determined by the Liebermann–Burchard method using alkaline-hydrolyzed crude fat samples (Stadtman, 1957).

Aliquots of the lyophilized brine shrimp were sent to a commercial laboratory (Ralston Purina Research 900, St. Louis, Mo.) for vitamin, mineral, and proximate analysis.

RESULTS AND DISCUSSION

As can be noted from the data in Table I, brine shrimp have a high protein content on a dry weight basis. Ash is also high as would be expected from the fact that these animals have a mineralized exoskeleton. The crude fat value is higher than that reported by Enzler et al. (1974) for Mono Lake brine shrimp. The difference could be due to variations in habitat, diet, age, or a combination of these factors. There is considerable difference in the carotenoid index between fresh and freeze-dried brine shrimp, indicating that processing changes the extractability of carotenoids. This could be of importance in formulation of diets for crustaceans requiring carotenoids.

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