PHYSICAL AND CHEMICAL ASSESSMENT OF THE CHANGES IN QUALITY CHARACTERISTICS OF STORED INSTANT NAVY BEAN POWDER

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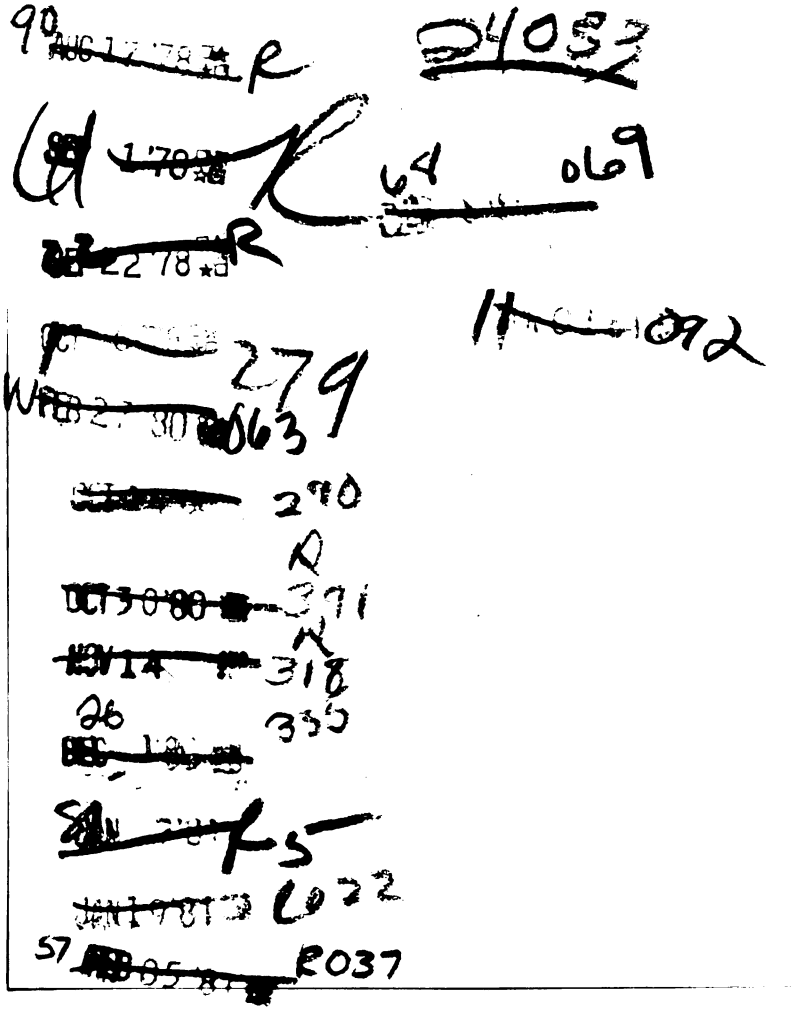

ABSTRACT

PHYSICAL AND CHEMICAL ASSESSMENT OF THE CHANGES IN QUALITY CHARACTERISTICS OF STORED INSTANT NAVY BEAN POWDER

By

Mark Howard Love

Storage stability has been a major concern in the development of instant dry bean powders. Earlier work had shown changes in the lipid and protein fractions; however, the exact chemical nature of these changes had not been completely characterized. Certain quality characteristics which are also subject to change on storage include solubility, color, texture, and flavor. Some of these have been studied previously. In the process of establishing optimum storage conditions for any new product, it is important to determine the effects of storage environment; temperature, atmosphere, and water activity, a_w; on product quality.

This investigation was designed to measure the effect of storage atmosphere and a_w on the quality indices for instant navy bean (Phaseolus vulgaris) powder. A one month accelerated storage study at 37°C was initiated to assess the extent of lipid oxidation, the changes in the solubility of protein, the changes in the content of reducing sugars, the extent of non-enzymatic browning, and the changes in the color of the product. The product was freshly prepared and stored

separately at 0.11 or 0.23 a_w in an air and a nitrogen atmosphere at each a_w. Periodically, samples were analyzed by physico-chemical methods to measure changes in the quality indices. Initial values for all parameters were measured before storage, and the moisture, soluble protein, total lipid, relative fluorescence, browning index (BI), and relative reflectance were measured every four days during the study. At the conclusion of the study, the samples were again analyzed for all of the indices to assess the changes which had occurred.

Changes in the unsaturate to saturate ratio (U/S) for the three major classes of lipids; neutral, phosphatidyl choline, and phosphatidyl ethanolamine; and the phosphorus content of the total lipid extract indicated that at 0.11 a_w (below the monolayer a_w) greater amounts of lipid oxidation occurred in the neutral lipids and phospholipids of air stored samples than in the nitrogen stored samples. These indices also showed that phospholipids were slightly protected when the product was stored at a higher a_w (0.23), while the neutral lipids were not protected from autoxidation.

The reducing sugar, relative reflectance, and BI values showed that the 0.23 a_w samples browned to a greater extent than those samples stored at 0.11 a_w. The nitrogen atmosphere samples stored at the monolayer (0.23 a_w) showed greater losses of reducing sugar, darker color, and higher BI than the samples did which had been stored in air at the same a_w.

A comparison of the indices of change in the product protein and other quality characteristics indicated that the samples which had undergone the greatest amount of non-enzymatic browning (0.23 N) also had altered *in vitro* digestibility of the protein. This same protein *in vitro* digestibility data showed that there was less cysteine and methionine in the 0.23 N sample. The sample stored in nitrogen below the monolayer had losses of cysteine and methionine, but these losses were less than those recorded in their air atmosphere counterpart.

Based on the results of this study and those from the literature, recommendations for the optimum storage of instant navy bean powder would include processing to a moisture content between 4 and 5%, packaging in an inert atmosphere, nitrogen, with an antioxidant, butylated hydroxy toluene, and storage at temperatures not greater than $20^{o}C$; however, storage at less than $0^{o}C$ would not be necessary.

PHYSICAL AND CHEMICAL ASSESSMENT OF THE CHANGES IN QUALITY CHARACTERISTICS OF STORED INSTANT NAVY BEAN POWDER

By

Mark Howard Love

A DISSERTATION

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DEDICATION

What little we know, what little power we possess, we owe to the accumulated endeavors of our ancestors. Mere gratefulness would already oblige us to study the history of the endeavors, our most sacred heirlooms. But we are not to remain spectators. It is not enough to appreciate and admire what our ancestors did, we must take up their best traditions, and that implies expert knowledge and craftmanship, science and practice.

--George Sarton, The History of Science and New Humanism, 1956.

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INTRODUCTION

Michigan navy bean (Phaseolus vulgaris V.) is one of the most important crops in Michigan agriculture. In 1973, the production was 42% of the total United States of America dry bean crop and contributed $173 million to the Michigan farm economy (USDA, 1974 and USDA-SRS 1974). Traditionally this commodity has been purchased in the canned form as pork and beans or soup and as dry beans. Morley (1966) indicated that the canned vegetable with greatest sales was the canned bean and of all items packed in cans beans ranked fourth in total sales.

With current consumer interest in convenience foods and an erosion of the dry bean market, researchers began projects designed to provide consumers and institutions with acceptable convenient forms of dried beans (Rockland 1966, Hoff and Nelson 1966, and Bakker-Arkema et al. 1966). Among the products developed was a cooked instant dry navy bean powder at Michigan State University. It was processed by retort cooking followed by spray-drying (Bakker-Arkema et al., 1966 and Bakker-Arkema et al., 1967) or drum drying (Bakker-Arkema et al., 1967). That concept was similar to the product produced by Morris (1961). The product produced by the drum dehydration process had good reconstitutability, good flavor acceptance, a light tan or brown color (rated highly acceptable), and negligible free starch content.

With the development of this product numerous, potential markets were opened to further the consumption of dry beans (White and Kon, 1972). Included among these markets were uses in instant bean soup mixes, instant bean puree or as an ingredient in convenience main dish products.

Instant dry bean powder has a protein content of 23.4% (dry weight) (Iriarte, 1969). The amino acid profile which is limiting in methionine provides relatively large amounts of lysine (Bressani and Elias, 1968 and Iriarte, 1969). While evaluating the effects of processing on the instant product, Miller et al. (1973) showed that the drum dried product had retention of thiamin, niacin, pyridoxine, and folacin equivalent to or better than the soaked-blanched and canned product and better retention than the cooked and canned product. These studies emphasize the nutritional contribution of the instant dry bean powder to the diet.

One major concern in the development of the instant dry bean powder has been the storage stability. Counter (1969) noted that the major changes in storage were in the lipid and protein fractions. His evidence also showed that while total reducing sugars changed on storage, the changes measured were not significant. Other major quality characteristics which could change on storage include solubility, reconstitutability, color, texture, and flavor. One important factor in the development of optimum storage conditions is the determination of the effects of storage environment; temperature, atmosphere, and equilibrium moisture content (a_w); on the deteriorative storage processess.

Hiller et al. (1973) reported on vitamin changes during processing and short term storage. Counter (1969) observed changes in four parameters (lipid content, color, protein solubility, and total reducing sugar content) to assess the effect of processing on the rates of product deterioration. He did not relate the changes to the a_w of the product.

From this background, the present investigation was initiated to measure the effect of storage atmosphere and a_w on the lipid and protein content under accelerated storage conditions. Physico-chemical parameters of color, protein solubility, reducing sugar content and lipid oxidation were measured to correlate changes in these quality characteristics with an in vitro assessment of protein quality. The study was carried out on instant dry bean powder processed from Michigan navy beans which was stored at two a_w's (0.11 and 0.23) under air or nitrogen atmospheres at each a_w. The storage study was conducted at 37°C for one month. At intervals, measurements were made of changes in color and protein solubility, of the development of non-enzymatic browning pigment, of the extent of lipid oxidation and changes in protein quality in control and storage samples.

LITERATURE REVIEW

Navy Beans

Michigan navy beans (*Phaseolus vulgaris*) are botanically classified in the family *Leguminosae*. Anatomically, they are dicoteledonyous reproductive organs which have a protein content in excess of 20%. On a worldwide basis, legumes represent a major source of protein in the diet of humans. Altschul (1966) estimated that the world population received more than 8.5M tons of protein per year from legume sources. Navy beans and related dry beans have no oil product to add to their processing value as is the case with soybeans. Traditionally, there has been little if any interest in processing legumes to improve their protein value. The world situation has changed and as Silbernagel (1971) indicated, world problems, especially the protein deficiency in developing nations, have stimulated a great deal of international interest in the nutritional value of legumes. Further, he suggested that the increased public awareness, in the United States of America, of health benefits and the economic importance of quality protein have generated a great deal of interest in legumes. Bressani *et al.* (1963) indicated that legume seeds were an important source of protein in the diets of Latin Americans of low economic status, providing 20-30% of the protein for these persons.

The protein content of navy beans is relatively high. Rutger (1971) reported a range of 19-31% protein for 343 varieties which he analyzed. The average protein content was 24.6%. Silbernagel (1971) pointed out that while beans are relatively high in total protein content, their actual nutritional value as a total source of human dietary protein needs is limited to approximately 8%, because of the low content of sulphur containing amino acids, methionine and cystine. Bressani (1963) reported that methionine was the first limiting amino acid and that leucine and tryptophan were second and third. If the sulphur amino acid content is increased by supplementation, tryptophan becomes the limiting amino acid. To attain the ideal pattern Silbernagel (1971) indicated that the bean protein would have to be supplemented with sulphur amino acids to three times the natural levels and with tryptophan to twelve times the natural level. These facts give an indication of the imbalance in the essential amino acid pattern for navy beans. Iriarte (1969) found during studies on the nutritive value of instant navy bean powder that the protein content of the powder was 23.4% by Kjeldahl analysis.

Antinutritional Factors

Earlier in this century Waterman and Johns (1920) showed that proteins in raw navy beans were not capable of producing normal growth in rats. They attributed this problem to the content of cystine, which they held to be the limiting essential amino acid. Work later in the century revealed the presence of naturally occurring toxins, phytohemagglutinous and antitryptic factors. Liener (1966) pointed out that

the first toxic protein substance isolated from a plant source was Ricin, which was discovered in 1888 and shown to be a phytohemagglutinin. Further, he stated that Sumner revealed in 1919 that jack bean contained a similar substance. Since that time researchers have found toxic principles in navy, lima, kidney, and soy beans and lentils which all contain trypsin inhibitors. Kakade and Evans (1963) reported that autoclaving for 5 minutes at 121°C was sufficient heating to destroy the antitrypsin factors in navy beans. Later work (Kakade and Evans, 1965) reported the isolation and study on an antitrypsin factor and a hemagglutinin in navy beans. These factors were proteinaceous in nature and rapidly inactivated by heating at 121°C. A five minute autoclaving at 121°C was sufficient to improve the biological value of navy beans. These researchers along with Liener (1966) postulated the presence of other antinutritional factors (toxic materials) in legumes which had not been characterized.

Miller et al. (1973) studied the vitamin retention of instant bean powder during processing. Their data indicated that processing to instantize the powder resulted in greater retention of these nutrients compared to a conventionally canned product. Although the variability of the content of the vitamins (niacin, thiamin, pyridoxine and folacin) can be considerable, dry beans represent a good source of these vitamins. This fact is a further indication of the nutritional worth of beans in the dietary. The reality of the variability of these constituents from lot to lot makes comparison of processing effects difficult, nevertheless, concern for their retention is an important consideration

when evaluating the effects of processing for instantization on the nutritional value of any food.

Other important constituents in beans are the carbohydrates. In addition to the polysaccharide component, starch, the Michelite variety of Michigan navy beans contains the disaccharide sucrose (2.4%); the trisaccharide, raffinose (0.8%); and the tetrasaccharide, stachyose (3.4%) (Lee et al., 1970). While the monosaccharides, fructose and glucose, were not reported as present in the whole, raw bean, Counter (1969) showed their presence in the instant product.

Instant Dry Navy Bean Powder

Spurred by the growing world interest in legume protein and its processing and the attempt to provide consumers and institutions convenient, dry bean products, researchers initiated projects to develop new and convenient forms of dry beans, dry limas (Rockland, 1966), accelerated processing (Hoff and Nelson, 1966) and instant navy bean powder (Bakker-Arkema et al., 1966). The last cited product was based on the concept of Morris (1961) for the production of a convenient, instant legume powder. Ultimately, the processing methods took two routes: spray drying (Bakker-Arkema et al., 1966 and Bakker-Arkema, et al. 1968) or drum dehydration (Bakker-Arkema et al., 1967). The studies of Bakker-Arkema et al. (1966), indicated that while the spray-dried product had slightly better reconstitutability, taste panels could not distinguish between spray dried or drum dried products. There was a preference expressed for the retort cooked powder over that

made from beans cooked at atmospheric pressure. This product also had better rehydration characteristics. The drum-dried product had a lower free starch index which results in a better quality product when rehydrated.

Given these facts, Counter (1969) produced a product by drum dehydration which was acceptable to taste panels. The product made in this manner reconstituted readily, had acceptable flavor, a light tan or brown color, a non-pasty texture, and a low free starch index. The production of bean powder by drum dehydration instead of spray dehydration provides a savings of 2500 BTU for each pound of beans because the product is not pureed (Bakker-Arkema et al., 1967).

Considering the reports by Watterman and Jones (1920), Kakade and Evans (1965), and Hackler et al. (1965) regarding the antinutritional factors present in navy beans, it is certainly important that adequate heat processing to inactivate these deleterious substances be a part of the production of instant legume powder (Altschul, 1966). Such heat processing is necessary to improve the protein quality of the product. Iriarte (1969) presented data indicating that the nutritional value of the protein in the retort cooked, drum dehydrated product was lower, when fed to meadow voles, than the atmospheric cooked drum dehydrated product. There was not a significant difference in the amino acid content of the bean powders processed by either method. This same study also showed that methionine supplementation enhanced the nutritive value. Miller et al. (1973) reported the PER for drum dried instant bean powder at 1.28 which was corrected to 0.92 based on 2.5 for casein. This value

agrees with the 1.31 figure reported by Bressani et al. (1963), which was determined on the black bean of Guatamala. Their product had been cooked, dried, and ground into a flour before it was fed in a standard, rat, PER assay.

Stability of Instant Dry Bean Powder

Burr et al. (1969) reported the results of a storage study involving instant dry bean powder. In this study, the product was stored at three different temperatures; -23°, 22°, and 38°C under two atmospheres, air and nitrogen. They also stored the product at three different moisture contents at each temperature, 10, 5, and 4% water. The product was evaluated subjectively through a trained taste panel and objectively through indices of lipid oxidation measured by gas chromatography. They found that the most favorable storage conditions were 4% moisture, nitrogen atmosphere, and 22°C temperature. This product was stable for eleven months before a flavor change was detected by the panel. Air packs were found to be unstable with the 38°C samples showing evidence of flavor changes within one month while the samples at 22°C did not show a flavor change for two months. A nitrogen environment protected the products at all moisture levels and temperatures. The 10% moisture product had its shelf life doubled by the use of a nitrogen environment, while the 5% moisture product had its shelf life extended longer than its air packed counterpart. Lastly, the study indicated that the product with 4% moisture was slightly more stable in all instances than the 5% moisture samples, and butylated hydroxy toluene (BHT, an antioxidant) was shown to extend shelf life when used at the 3 ppm level.

The maximum shelf life for instant navy bean powder was shown by Counter (1969) to be 90-120 days if the product was stored under nitrogen without an antioxidant present. The major changes observed in this study involved the lipid and protein fractions. He showed that the free fatty acid content increased on storage and the nitrogen solubility decreased. No trends were observed in color changes measured by the Hunter color instrument, nor in the total reducing sugar content. The purpose of his study had been to judge the effects of retort cooking or atmospheric cooking of the beans on their rates of deterioration while stored as an instant navy bean powder. His results indicated that the time in storage and the temperature of storage exerted more significance on the rates of deterioration in storage than the cooking method.

Water Activity, a_w

Foods stored in environments of differing relative humidity take on or give up water to that environment attaining a moisture content which represents an equilibrium condition. The water or moisture content reached under these conditions has been defined as the equilibrium relative moisture content (Heldman, 1972; Labuza, 1968; Labuza et al., 1970; and Rockland, 1969).

In a physical sense, the water content of the food can be stated by its vapor pressure (p, or partial vapor pressure of water in the food) as a fraction of the vapor pressure of pure water at the same temperature (p_o). This fractional relationship, p/p_o, for foods is a measure of its water activity, a_w(Ayerst, 1965). It has many important relationships to the properties and stability of the food. Ayerst (1965)

pointed out that the a_w is a measure of the ability of food to support biological processes (i.e., microbiological growth). Heldman (1972) stated that the a_w (p/p_o) may establish the structure of the material and the manner in which the water is found. Figure 1 shows a typical sorption isotherm for foods. It relates the a_w and equilibrium moisture content and is a Class II isotherm (Brunauer, 1945). Wolf et al. (1972) indicated that the shape of the isotherm reflects the manner in which the water is bound. Further, they stated that up to an a_w of about 0.3, where the first inflection appears, water is held on polar sites and is bound with relatively high energies. This region of the isotherm is commonly called the "monolayer region." Water in the food up to an a_w of 0.3 is thought to be distributed in a single layer over the surface of the food. Between a_w's of 0.3-0.7 the water is thought to exist in multiple layers with the water held by van der Waals forces (Stitt, 1958). Above an a_w of 0.7, the water approaches the condition of condensed water existing relatively free. The isotherm reflects this condition of water solution and capillary condensation by the steepness of the curve. Conceptually, this analysis fits the multimolecular adsorption theory of Brunauer et al. (1938).

Numerous procedures are commonly used for determining the sorption isotherms for products. Landrock and Proctor (1951) reviewed the four common methods in use at that time, while Stitt (1958), Taylor (1961), and Karel and Nickerson (1964) developed procedures for isotherm determination on dehydrated food samples. It is noted that isotherms for materials determined by desorption procedures do not coincide with the values.

generated by an adsorption procedure for attaining equilibrium moisture contents. As a result of this situation, a food can exhibit two different moisture contents at a single a_w, depending upon the method used for reaching equilibrium. The difference between the adsorption and desorption moistures has been defined as hysteresis. The technical implications of this difference and the relation of this process of hysteresis to changes in product quality has been reviewed (Rockland, 1969; Labuza, 1968; Labuza et al., 1970; and Wolf et al., 1972). The degree of hysteresis or distance between the two loops reflects changes in the properties of the food on processing and storage. Increases in hysteresis can be related to losses in product quality (Wolf et al., 1972).

A_w and Stability of Low Moisture Foods

Water content as it relates to water activity, a_w, has an important bearing on the stability of low moisture foods. The various deteriorative processes which occur in stored processed foods have a direct dependence upon a_w. The major processes and their rates have been shown to depend upon a_w as water is present to function as a solvent or as a reactant. Labuza (1968); Labuza et al. (1970); and Rockland (1969) reported on the relatedness of these processes to the a_w. The rates of lipid oxidation in food shows a bimodal activity curve. Rates are high at very low a_w's, reaching a minimum in the region of the monolayer. The rates begin to increase with increasing a_w to another peak rate at a_w = 0.65. Beyond this a_w the rate decreases again. Non-enzymatic browning rates exhibit a different pattern. The reactants in these processes are water soluble. The rates, therefore, increase

with increasing a_w until the water content is sufficient to dilute the reactants, slowing the rate. These two chemical processes of deterioration are important in the quality changes exhibited in processed and stored low moisture foods.

Most of the theoretical aspects of the relation between a_w and product deterioration have been worked out in model systems containing critical reacting components of their food analog. The results of some of these studies have been summarized in the review by Labuza et al. (1970). Few studies have been carried out involving foods which naturally are more complex. Wolf et al. (1972) reported observations of the changes in the degree of hysteresis relative to measured changes in organoleptic qualities of freeze-dried beef, haddock, potato, and carrots stored for six months at 37°C. Counter (1969) in his study of the stability of instant navy bean powder stored the product at the final moisture content with no variations during storage. He made no attempt to measure the a_w and only conjectured about the relation between water content and the state of water in his product.

Two other studies have been carried out on foods which show the significance of a_w in the rates of deterioration of the quality and nutritive value of a stored food product. Henry and Kon (1948) reported that high moisture content was an important factor in the deterioration of milk powder during storage at 37°C. The sample of highest moisture (7.6%) showed a progressive decrease in protein quality during storage. The biological value had decreased a statistically significant amount within two months. Samples stored at lower temperatures were six times

more stable than those stored at 37°C. Samples stored at 3% moisture and 37°C exhibited little chemical deterioration during storage. For this sample the atmosphere exerted the greatest influence. Samples stored in air deteriorated more than those in nitrogen atmospheres.

As a part of the same study, Lea and White (1948) reported that the powder with 7.6% moisture at 37°C discolored most rapidly and exhibited greatest insolubility. The changes were more pronounced in air than in nitrogen atmospheres. Their chemical indices indicated that the cause of deterioration was the tying up of ε-NH_2 groups of the amino acid, lysine, by reaction with the reducing sugar, lactose, present in the product. The reaction proceeded through a two-step process initially indicated by a decrease in the number of ε-NH_2 groups with no discoloration followed by a disappearance of a greater number of ε-NH_2 groups and discoloration. The combined effects of these processes were decreased powder solubility and decreased protein quality. The authors argued that the activity of the water in the powder (its moisture content) determined the rate of product deterioration.

Lipid Oxidation

Organic compounds react with oxygen from the air representing one of the most important of all chemical processes (Ingold, 1969). Many of these reactions are spontaneous, not involving carbon dioxide nor water, and occur under comparatively mild conditions. These processes are termed autoxidations. A majority of organic compounds, unsaturated lipids included, autoxidize by a free-radical chain process, involving

peroxy radicals. This chain sequence can be presented by the following schema:

Initiation

$$RH + \text{initiator} \rightarrow R\cdot \qquad (1)$$

Propagation

$$R\cdot + O_2 \rightarrow ROO\cdot \qquad (2)$$

$$ROO\cdot + RH \quad ROOH + R\cdot \qquad (3)$$

Termination

$$ROO\cdot + ROO\cdot \quad \text{non radical products} \qquad (4)$$

As related by Ingold (1969) the addition of oxygen to the radical, R· (Reaction 2), is an extremely fast process, and it is likely diffusion controlled in many instances. Further, peroxy radical products have been detected in oxidizing systems. They exhibit hyperfine electron spin resonance (esr) signals at 2.014-2.019 gauss (g). Signals of this character are distinct for ^{17}O in the R-O-O· structure. These products also exhibit an ultraviolet absorption maximum between 260nm and 320nm (Ingold, 1969). Chemically, they enter into a variety of reactions including hydrogen abstraction, important in the chain process (Reaction 3), addition to unsaturated systems, oxygen transfer, and self addition, the chain termination process (Reaction 4). Privett and Blank (1962) from studies on oxidizing methyl linoleate showed that the formation of the hydroperoxide (ROOH) involves a discrete reaction which is catalyzed by heavy metals, when present in sufficient concentrations. Ultimately, they were able to show that the stability of an oxidizing polyunsaturated system was related to the hydroperoxide content. If the level of ROOH

was kept low, the stability was extended and is an important factor in stabilizing any polyunsaturated system. If the formation of ROOH can be minimized, stability will be increased.

Other evidence of the reactivity of ROOH is found in the report by Rice and Beuk (1953). Their studies on autoxidizing lipids in model systems showed the destruction of the added amino acid cystine through oxidation by ROOH. Additional data showed that the reaction of ROOH from oxidizing lipid reacted with -SH in enzymes inhibiting their activity. Lewis and Wills (1962) postulated from evidence based on oxidizing model systems that the ROOH's were responsible for the destruction of the -SH groups. Further, they contended that the rate of disappearance was proportional to the ROO· content. The primary products of cystine oxidation in their study were disulfides (-S-S-) cysteic acid ($-SO_3$), and cystine disulfoxide (-S-S-). The peroxides were also destroyed in these processes.

In understanding these oxidation processes, it is necessary to be able to relate these reactions to changes manifest in processed and stored foods. Some insight can be gained by surveying the studies conducted in model systems. Roubal and Tappel (1966a) reported the occurrence of transient free-radicals in peroxidizing lipid-protein model systems. They observed that the damage done to the proteins occurred through destruction of amino acids. By their calculations, these free radicals were the major actors in these processes and were responsible for 90% of the protein damage while peroxides were responsible for only 10% of the amino acid losses.

Reactions of the hydroperoxides are also important in understanding deterioration of foods. The secondary products of lipid oxidation include numerous volatile monocarbonyls. Evidence of their occurrence has been shown by numerous researchers. Lea and Parr (1961) reported that the oxidation of lipids by atmospheric oxygen produced a variety of flavor defects. They stated that polyunsaturated fatty acids in glycolipids and phospholipids developed painty, grassy, and fishy odors and flavors. Autoxidation of phospholipids and enzymatic action were the major sources of these products. Ellis et al. (1961) surmised the existence of a plethora of carbonyl products in oxidized unsaturated oils.

Among the products present in mildly oxidized systems of esters of oleic, linoleic, and linolenic acids and fats were 7-n-alkanals, 8-n-alk-2-enals, and 4-alk-2,4-dienals (Yu et al., 1961). A complete listing of the volatile substances postulated as being present in oxidizing model compounds of unsaturated fatty acids was presented by Hoffman (1962). In this review were listed the products which had been actually present in oxidized unsaturated model compounds and oils. Included among these compounds were saturated and unsaturated aldehydes, unsaturated alcohols, methyl ketones, and dicarbonyls. These compounds arise from scission and dismutation of hydroperoxides (Hoffman, 1962). All of these products are volatile substances which contribute to the characteristics of an oxidized fat or food. Beyond the subjective influence of these products is the fact that many of them are extremely reactive compounds.

Crawford et al. (1967) established the role of malonaldehyde (a secondary product of lipid oxidation) in reactions with proteins. Their analyses indicated that in vitro systems showed sufficient evidence to state that the carbonyl was reacting with free amino acids in proteins by nucleophilic attack via an SN-2 mechanism to produce enamines. The amino groups involved included the ε-NH_2 group of lysine and the N-terminal γ-NH_2 of aspartic acid. Malonaldehyde reactivity in the process was enhanced by the resonance stabilization of its conjugated system.

Chio and Tappel (1969a) reported evidence of production of fluorescent products in the reaction of model systems, containing amino acids and malonaldehyde. The products produced were Schiff base compounds which possessed characteristic visible and ultraviolet absorption. They indicated that the electronic absorption and fluorescent properties were attributable to the chromophoric groups, N-C=C-C=N, which contain six mobile, π-electrons. The infrared spectra of these same products contained a peak of 1650 cm^{-1} which is characteristic of the C=N of the Schiff base. Other evidence indicated that the stoichiometry of the reaction was based on the condensation of 1 mole of malonaldehyde with two moles of amino acids resulting in the production of N-N'-disubstituted amino-3-imino propenes. This study was a confirmation of the report of Crawford et al. (1967) which was discussed above.

Having shown the production of fluorescent compounds in model systems involving amino acids and malonaldehyde, Chio and Tappel (1969b) investigated the reaction of malonaldehyde or oxidized ethyl arachidonate with selected proteins. Their findings indicated that the

oxidation products from lipids had the capability of reacting with enzymes to produce yellow fluorescent products and loss of enzyme catalysis. The compounds were the N-N'-disubstituted 1-amino-3-iminopropenes. Amino acid analyses of the proteins indicated that lipid products had destroyed lysine, tyrosine, and histidine and the -SH groups of the proteins. They also reported loss of methionine; however, it was not related to loss of enzyme activity.

Fletcher and Tappel (1971) contended that the peroxidation of fatty acids bound to horse serum albumin (HSA) resulted in the production of fluorescent chromophores in the protein whether the HSA was stored in liquid, powdered, or crystalline state. They indicated that the peroxidizing polyunsaturated lipid reacted with amino acids to give products which had fluorescent spectra similar to those characteristics of the Schiff base imine products. These kinds of products were also found in studies with serum albumin or ribonuclease A and peroxidizing lipids. They theorized that the process involved the reaction of secondary products of lipid oxidation, the carbonyls, with primary amines to produce the Schiff base imine products.

In response to these Tappel publications on carbonyl reactivity, Roubal (1971) reiterated that the process, responsible for the greatest amount of amino acid losses in oxidizing lipid-protein system results from the free radical attack on the proteins. It was not the result of aldehyde reactions. By correlating oxygen uptake data, esr signals for free radicals, fluorescence development, and loss of amino acids on the same time scale. His data indicated that the oxygen uptake precedes

free radical formation. The free radical production reached a maximum level at 14 hours, declining to a low, and yet, detectable level after 60 hours of storage. His amino acid data indicated that at 14 hours the greatest percentages of methionine, lysine, tryptophan, alanine, and tyrosine had been lost. His analyses further revealed that fluorescence did not occur until after radical production had declined to the low level. Losses of amino acids also occurred later in the process of storage, which he indicated could be attributed to the reactions of aldehydes or the small number of free radicals still present in the system. The conclusions of this study were that protein damage was caused predominantly by free radical attack and not by aldehyde condensation reactions. Antioxidants functioning as free radical trappers, effectively inhibited the free radical attack process.

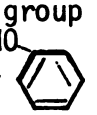
Gamage and Matsushita (1973) stated that no general rule can be made for a single mechanism to describe the reaction of autoxidized lipids with different proteins. They have shown that both radical and non-radical products of peroxidized lipids can polymerize proteins.

Most recently (Matsushita, 1975) a study has been published to further support this argument. Using model systems of linolenic acid hydroperoxides (LAHPO) at low concentrations or secondary products of these hydroperoxides (SP), the carbonyls, and proteins, the researcher characterized the reaction products. The results showed that LAHPO reacts by a radical mechanism with damage to amino acids and potential enzyme inactivation. The lost amino acids included lysine, histidine, tyrosine, methionine, and cystine. Other evidence from this study

indicated that the secondary products were capable of reacting with proteins, however, to a lesser extent than LAHPO. Since it was not as reactive toward the proteins, fewer amino acids were damaged. It was concluded that LAHPO and SP were capable of inducing protein polymerization. LAHPO reacted in accordance with the radical sequence proposed by Roubal and Tappel (1966b). BHT, an antioxidant, was an effective inhibitor of the free radical reactions while ascorbic acid enhanced the polymerization reaction. SP took a longer period to produce polymers and its reaction products exhibited fluorescence, implying that the products of condensation were chromophoric conjugated Schiff base systems. This fact agreed with the theory of Chio and Tappel (1969b) and Malshet and Tappel (1973).

Tappel and co-workers have published a series of papers which further elaborate the nature of the production of fluorescent materials in protein, peroxidizing lipid systems. Bidlack and Tappel (1973a) reported the occurrence of fluorescent materials in systems of microsomal membranes and peroxidizing lipids. The data reported showed that the oxidation process produced fluorescent products with an excitation wavelength, λ, at 360 nm and an emission λ at 430 nm. These are wavelengths characteristic of 1-amino-3-imino propenes. They also observed that during the process there was a decrease in the number of reactive groups in the phosphatidyl ethanolamine (PE) fraction and a reduction in extractable PE content. Dillard and Tappel (1973) reported that Schiff base products, which fluoresced (if conjugated), were the products resulting from reaction of malonaldehyde and other carbonyls

from lipid oxidation in a Maillard type browning reaction with free amines. They surmised that this type of reaction was the mechanism responsible for the oxidation of phosphatidyl ethanolamine and browning reported by Corliss and Dugan (1970) and the report of Lea and Parr (1961) concerning the loss of phospholipids during oxidation by oxygen. Dillard and Tappel (1973) also reported that the fluorescent products produced had an excitation λ of 360 nm and an emission λ in the range of 430-460 nm. A similar finding was reported by Reiss and Tappel (1973) in a system involving oxidized polyunsaturated fatty acid and deoxyribonucleic acid. Bidlack and Tappel (1973b) studied the fluorescent chromophores which developed during oxidation of phosphatidyl ethanolanine and phosphatidyl serine. The products exhibited characteristic spectra (excitation λ of 365-470 nm, emission λ of 435-450 nm), implying the existance of a family of chromophores. Fluorescent intensity of these substances was proportional to the degree of peroxidation.

As has already been indicated, Schiff base compounds do form as the result of the reaction between carbonyls and free amino groups. In certain instances these compounds are fluorescent and in other instances they are not. Cognizant of this fact Malshet and Tappel (1973) undertook a study of the structural requirements for Schiff bases to fluoresce. Their study revealed that the Schiff base must be in conjugation with an electron donating group such as the -N-H in R-N=CH-CH=CH-NH-R or the -OH in $OH-CH_2-CH_2-N=C-$  . Products with these conformations exhibit fluorescence (excitation λ of 385 or 362 nm and emission λ of 436 or 446 respectively), ultraviolet absorption maximum at 215 nm, and

characteristic infra red stretches in the region 1620-1660 cm^{-1}. The electron donating substituent in conjugation with the Schiff base was an absolute requirement for fluorescence.

Dugan and Rao (1972) in an extensive study of the role of aldehydes from lipid oxidation in the development of flavors and browning pigments in freeze dried foods showed that non-enzymatic browning reactions producing deterioration in freeze-dried foods involved compounds containing carbonyls and free amino groups. Lipid browning reactions in their model systems proceeded readily at a low moisture level (2.5%) and ambient or elevated temperatures. Schiff base formation was the dominant feature of nearly all of the reaction systems with the formation to varying degrees of oxypolymers, other polymers, methyl phosphatidate, and other less well-defined scission products, according to experimental conditions. One phase of the study involved the storage of "lipid free" muscle fiber (a protein matrix) with phosphatidyl ethanolamine or aldehydes. Carbonyl condensation reactions proceeded with both amino groups from PE and protein. Certain amino acids such as lysine, alanine, phenylalanine, and tyrosine reacted with the carbonyls to reduce the content of amino acids in the protein.

Measures of Lipid Oxidation

Numerous methods are available to follow the autoxidation process in lipid samples and foods. Researchers have used direct methods of analysis to follow changes in reactants. Included among these techniques are measurement of diene conjugation (Lundberg and Chipault, 1947) and oxygen uptake (Karel and Labuza, 1968, and Love, 1972).

Measurement of the products of these reactions has also been used. Free radical formation was followed by Roubal (1971) and Roubal and Tappel (1966a, 1966b). The peroxide content has been used as an index of oxidation for decades (Wheeler, 1932). It has been modified to a colorimetric analysis for small sample sizes (Swoboda and Lea, 1958). Aldehyde production has also been measured (Henick et al., 1954 and Ellis et al., 1961). Other end products or physical properties which have been measured include: fluorescence (Dillard and Tappel, 1973; Chio and Tappel 1969a and 1969b; Braddock, 1970; Dugan and Rao, 1972; Love, 1972; and Matsushita, 1975), the production of lipid solvent soluble browning products (Fishwick and Zmarlicki, 1970 and Hendel et al., 1950) and the thiobarbituric acid reactive substances (Lea, 1962; Sinnhuber et al., 1958; Kwon et al., 1965; Love, 1972 and Bidlack and Tappel, 1973).

Indirect measures of the extent of lipid oxidation have also been developed. One of these was the change in the unsaturated fatty acid content as assessed by gas-liquid chromatography (GLC) (Buttery et al., 1961a,b). Changes in the content of extractable lipid fractions (Lea and Parr, 1961 and Betschart and Kinsella, 1975) is another. Sapers et al. (1970 and 1972), developed a technique for GLC analysis of head space gases of stored products, searching for products of lipid oxidation (hexanal) and Maillard browning. Coupled oxidation processes such as carotenoid destruction during lipid oxidation has also been used. Martinez and Labuza (1968) followed astacene bleaching while Arkcoll (1973) utilized β-carotene loss as an index of the extent of lipid oxidation.

Given this array of techniques the autoxidation of lipids can be measured from initiation through the reactions of the secondary products. Depending upon the technique chosen it is possible for the researcher to follow lipid oxidation and the consequences of these reactions.

Non-enzymatic Browning (Maillard Browning)

Non-enzymatic browning is a descriptive term for the sequence of reactions without enzyme catalysis which produce a host of desirable and undesirable changes in foods. Among these changes are alterations in color, texture, flavor, odor, and nutritive value. Within the general class of reactions there are two subclasses of reactions. The differences in these two classes lie in the origin of the reactants. The products are similar and the end effects are generally the same--browning. One subclass of reactions involves the sequence of processes in which the carbonyls are derived from water soluble reducing sugars. The other subclass involves the sequence of reactions in which the carbonyls arise from lipid oxidation.

L. C. Maillard (1912) characterized a sequence of reactions between the carbonyls of reducing sugars and free amino groups. These reactions are often referred to as the Maillard browning reactions. Since his reports (L. C. Maillard, 1912, 1913, 1916 and 1917) much research has been conducted to elaborate the chemistry of the browning processes from reducing sugar and free amine to the characteristic amorphous polymeric melanoidins (the browning pigments). Numerous complete and comprehensive reviews have been published detailing the chemistry of these reactions (Hodge, 1953, 1955; Ellis, 1959; and

Reynolds, 1963 and 1965). Prior to these reviews Danehy and Pigman (1953) and Rice and Beuk (1953) had documented the importance of these processes in the browning of dehydrated eggwhite, stored milk powder, and soybean flour. They also indicated that there was evidence to implicate polysaccharides in the overall food deterioration to which browning contributed. They questioned, however, that polysaccharides contributed to discoloration. Schroeder et al. (1951) indicated that the same browning reaction produced destruction of lactalbumin when heated.

In his review, Hodge (1953) surveyed the chemistry of the browning reactions with particular emphasis on the non-enzymatic processes. He characterized the overall non-enzymatic browning process as occurring in three stages: 1) the initial stage (colorless products) resulting from sugar amine condensation followed by Amadori rearrangement; 2) the second stage (colorless to yellow products which absorb strongly in the near ultraviolet) resulting from sugar dehydration, fragmentation of the sugar moiety, and amino degradation; and 3) the final stage (producing highly colored products) resulting from aldol condensation and aldehyde amine polymerization with the formation of amorphous polymeric melanoidin compounds. These reactions proceed spontaneously at ordinary temperatures. Hodge (1955) indicated that the glucosylamine compounds possessing Schiff base structures had been found in solid state reaction systems (i.e., dry model systems as opposed to aqueous ones). Hodge (1967) and Hodge et al. (1972) emphasized the role of these browning reactions in the production of the characteristic aromas,

flavors, and colors of browned, toasted, roasted, and cooked foods. Particular emphasis was given to the correlation of chemical structures, pathways of production, and aroma attributes.

Bujard et al. (1967) emphasized that the chemistry of the non-enzymatic browning process had been largely defined in model systems. Further, they noted that since the N-glucoside produced in non-enzymatic browning reactions may be stable it would be possible to isolate amino acid glucosides with Schiff base structures in browned foods. These compounds would be found in addition to the classical 1-amino-2-deoxy ketones from Amadori rearrangements which were described by Hodge (1955).

McCullum and Davis (1915) hypothesized that the loss of nutritive value for heated milk was due to changes "wrought in casein" during the heating process. As pointed out by Maillard (1912), the chief influence on the browning sequence was temperature. Miller (1956) indicated that the protein quality of foods is impaired to a variable extent during the drying. The impairment is due to the effect of heat in the presence of moisture producing browning in accordance with the Maillard reactions. Earlier support of this conclusion had been given by the work of Stevens and McGinnis (1944) which revealed loss of lysine during the heating of casein for 30 minutes at 140°C. The browning reactions had also been implicated in the browning processes of potato chip production (Stevens and McGinnis, 1947).

Other researchers (Patton et al., 1948a,b) had shown that heating casein at 96.5% for 24 hours produced loss of leucine, lysine, arginine and tryptophan by Maillard browning reaction in their model systems.

Friedman and Kline (1950) stated that the importance of the non-enzymatic browning reaction had not been fully appreciated at that time. They reported that the presence of a reducing sugar (e.g., glucose) and a protein under mild conditions would produce an extensive decrease in the biological value of the protein. They also indicated that a refined mixture of amino acids was also susceptible to the same reactions in the presence of glucose. Even under mild conditions histidine, threonine, tryptophan, phenylalanine, lysine, and methionine were rendered unavailable by the browning reactions. Indications were that some complex was formed which made these compounds unavailable for digestion. Burton et al. (1963) reported the development of chromophores on storage of glucose-amino acid model systems as a result of Maillard reactions. These processes, they stated, were iron catalyzed. The final product was a nitrogen containing polymer which exhibited fluorescense.

Much of the foundation for the understanding of the role of reducing sugar in protein interactions in dried foods comes from a series of studies on browning of glucose and casein. Lea and Hannan (1949) published the results of a study of the browning reaction in a dry model system of casein and glucose. Numerous chemical indices were measured, including free amino nitrogen, and physical measures were also taken, including gel formation, solubility, and color changes. Their data indicated that the loss of amino nitrogen is dependent upon the a_w of the sample. Rates of amino nitrogen loss were greatest at 65-70% relative humidity (RH) and decreased on either side of this region. This relationship was true at all temperatures, 37°, 70°, and 90°C. The rate

of loss increased with increasing pH from 3-8 and possibly to 10. Color development rates paralleled those of nitrogen loss relative to a_w, pH, and temperature. They indicated that the mechanism relating the effects of water and rates of deterioration was the multilayer adsorption theory of Brunauer et al. (1938). The maximum rates were attained in the "bimolecular" layer region. Rates declined at higher a_w's, they theorized, due to simple dilution of the reactants. They also reported marked insolubilization of the casein and impaired gel formation as a consequence of the chemical changes which they had recorded.

In a companion paper on the same system, Lea and Hannan (1950a) reported that the rate of the browning reaction was dependent upon the hydration of the protein surface. The rate was a maximum at 70% RH because sufficient hydration of the surface of the protein had occurred to act as a solvent for the reactants, promoting the process. Later (Lea and Rhodes, 1952) it was shown that other reducing sugars (e.g., galactose) had the same reactivity as glucose. In 30 days storage studies at 37°C, these researchers showed that galactose had an equivalent condensation rate to that of glucose, while deoxy-glucose or deoxy-galactose reacted more slowly.

Current research interests in the non-enzymatic browning reactions and deterioration in stored foods has concerned changes in the nutritive value. Hurrell and Carpenter (1973) reported the isolation of 2-N-formyl-ε-N-(deoxyfructosyl) lysine (FFL). This compound forms from a reaction of glucose and lysine when browning occurs and is a model of one of the Maillard compounds. Nutritionally, it is unavailable to rats

as a source of lysine. Hurrell and Carpenter (1973) have also isolated this compound from hydrolyzates of mildly heated protein-sugar systems.

The studies by Clark and Tannenbaum (1970 and 1974) have taken a similar orientation. These workers have characterized the browning pigments found in model systems and foods, including non-fat dry milk solids (NFDMS), glucose-casein, and insulin-glucose. From the first two systems, they concluded that the pigments formed by reaction with sugar and protein increased in amount as the length of storage and the temperature increased. In both systems, non-homogeneous non-hydrolyzable limit pigment peptides (LPP's) were formed. These compounds were not single chemical entities but a family of compounds. In the third system, the protein selected had a known amino acid sequence. Use of this protein aided in the elucidation of the LPP's structure. Their results indicated that the LPP's arise from cross-linking between insulin peptides in association with the condensed sugar residues. In some instances the sugars had reacted with amino groups producing aggregates of up to 31 amino acids in the highest molecular weight LPP's. Condensed sugar residues and cross-linked peptides likely would sterically hinder digestive enzymes from hydrolyzing peptide linkages. In this way they suggest a mechanism to account for the loss of nutritive value for the protein, beyond the lysine, histidine, and arginine which are accountable directly. This mechanism also is in line with the reports of Boctor and Harper (1968) and Ford and Slater (1966) which had shown greater loss in nutritive value of their browned proteins than could be

accounted for by amino acid analyses.

The other pathway to non-enzymatic browning derives its carbonyl reactants from autoxidizing lipids. From their work on stored wheat flours and in model systems, Jones and Gersdorff (1941) concluded that aldehydes arising from oxidized lipids were capable of interacting with proteins to produce browning products. The amino acids which were involved in these reactions were lysine, its ϵ-NH_2 group, phenylalanine, and glycine. N-terminal amino groups and the guanidino groups were not involved. As a result of these reactions, proteins became crosslinked with the production of insoluble polymers. Tappel (1955) recounted that the final oxidation products of fat were aldehydes which readily reacted with amino acids in proteins to form aldimines. Ultimately nitrogenous polymers, co-polymers, and melanoidins were produced in a sequence of reactions called browning. He indicated that the processes were predominant in dried meat, milk and eggs. Their effects were changes in color, texture, and functional properties. These browned products were not susceptible to enzymatic hydrolysis by proteases such as papain. This indigestibility makes the amino acids unavailable for use by organisms.

Lea (1958) reported on a study with stored herring meal. This product browned extensively when stored in air. There was a loss in the nutritive value of the protein. Storage under nitrogen delayed the process as did antioxidants, implying a role for lipid oxidation. The meals of lowest moisture content had the highest rates of lipid oxidation and related browning. Burton et al. (1962) reported that

α,β-unsaturated carbonyls accelerated the development of chromophores in model systems of amino acids and aldehydes. Unsaturated aldehydes of this type are plausible from oxidized unsaturated fatty acids (Hoffman, 1962). Koch (1962) reported on the participation of oxidized lipids in browning reactions in freeze dried foods. The amino acids lost through these reactions were lysine, phenylalanine, and glycine. In contrast, Narayan and Kummerow (1958) indicated that the complexes formed between oxidized linoleic acid and egg albumin were the result of hydrogen bonded aggregations and not from covalent bond formation reactions.

Crawford et al. (1967) reported (as previously discussed) the reaction of malonaldehyde with ε-NH_2 of lysine and N-terminal NH_2 groups of aspartic acid. The reaction proceeded via an SN-2 mechanism to produce Schiff base fluorescent compounds which are characteristic browning intermediates. A more definite connection of malonaldehyde interaction with proteins in browning processes was documented by Chio and Tappel (1969b). Tannenbaum et al. (1969) reported that in dehydrated foods proteins were destroyed as a consequence of lipid hydroproperoxide decomposition. The carbonyls formed proceeded to react to produce browning in the products studied. The end products were browning pigments and methionine sulfoxides.

Buchanan (1969a) implied that carbonyls from lipid oxidation reacted in a Maillard sequence to cause a decrease in leaf protein concentrate. The browning reactions were induced as a consequence of the dehydration temperatures during processing. Braddock (1970) showed

evidence that the browning which occurred in frozen Coho salmon correlated with oxidation of the highly unsaturated lipids in the tissue. He also reported changes in myosin as a consequence of interaction with oxidizing methyl linoleate. Dugan and Rao (1972) extensively documented the role of aldehydes from lipid oxidation in the browning of stored dried foods. Dillard and Tappel (1973) and Arkcoll (1973) further documented the role of carbonyls from lipid oxidation as reactants in Maillard browning reactions. As a consequence of these reactions, Arkcoll concluded that the binding of proteins through carbonyl condensations makes these proteins unavailable for digestion. In this manner, their nutritive values are decreased.

Measures of Protein Quality

In Vivo

Two time honored methods for the biological assessment of protein quality are the biological value (BV) and the protein efficiency ratio (PER). The procedures for the BV technique were established by Mitchell (1924) and represent an assay technique which, over an arbitrary period of time, measures nitrogen retention by the test animal on a zero protein diet compared to its nitrogen retention while fed the test protein for the same period. In this manner, a numerical ranking of the protein quality is obtained. Osborne et al. (1919) proposed an animal assay method for determining the relative value of the test protein for growth. This method also produced a numerical rank for the protein quality. Later this method became known as the PER. It relates the grams of

weight gained (growth) to the grams of protein consumed. Most of these procedures have been standardized by international convention (FAO or national organizations, AOAC). The test animal has conventionally been the rat. Elliot (1963) proposed a new assay for biological assessment of protein quality. Utilizing the meadow vole (Microtus pennsylvanicus) as the assay animal, he proposed a six day specific growth response test. Iriarte (1969) utilized this procedure to measure the quality of instant navy bean powder. Her results ranked the protein similarly to the PER analysis of Bressani et al. (1963) and Miller et al. (1973).

Other methods of biological assessment of protein quality have been developed. Micro-organisms were proposed as reliable test organisms by Ford (1960). S. zymogenes (NCDO-592) was the strain selected because it has less of an exaggerated lysine requirement than S. fecalis. Overall the amino acid requirements of NCDO-592 are the same as for the growing rat. His method correlated well with other biological measures (Henry and Ford, 1965).

Ford (1962) stressed an important consideration related to the use of any data on protein quality measured with laboratory test organisms. He stated that careful quantification is of utmost importance, particularly in terms of specie, strain, and age of the test organism. He emphasized that these estimates are for individual proteins and cannot be extended to predictions of their value in mixed diets. Lastly, he stressed that since lysine and/or methionine are frequently the limiting amino acid in a food, foods should be evaluated as sources of these amino acids. Table values are unreliable for this purpose.

Chen et al. (1962) reported on the importance of protein digestibility in assessing protein quality by biological techniques. Their data indicated that insoluble protein sources (e.g., Zein) resulted in the accumulation of nitrogen in the intestine of the test animal contributing to lowered nitrogen retention and a lowered nutritional value score for the test protein.

In Vitro

Biological methods for the assessment of protein quality have their limitations. They are at best time consuming and at worst complicated by error due to specie variability. Extrapolations of the results from such measures are limited by the fact that most laboratory studies are performed on rats or mice and must be judged from these limitations. Direct chemical methods of protein value have been developed: amino acid analyses (Moore et al., 1958), chemical score (Mitchell and Block, 1946), and essential amino acid index (Oser, 1951). Application of these techniques is circumscribed by the fact that each is based on the chemically measured amino acid content. They give no indication of the availability of the amino acids or the effects of processing, storage or cooking unless direct destruction of the amino acid has occurred. Said et al. (1974) pointed out that these indices are based on the assumption that an equivalent degree of deficiency of any essential amino acid (EAA) will impair protein utilization. A zero value for any EAA assumes that utilization will be prevented. This assumption is valid for most EAA's (i.e., threonine); however, it does not apply to lysine.

Melnick and Oser (1949) pointed out the need for a precise objective test for the extent of protein damage during processing or storage. Clandinin (1949) published results of a study which indicated that *in vitro* enzymatic hydrolysis and measurement of the amino acids released was a reliable index of the nutritive value of different herring meals. This type of technique was not a particularly new suggestion. Numerous researchers had employed enzymatic digestibility as an index of protein quality for a number of years (Jones and Gersdorff, 1941; Jones *et al.* 1942; Mitchell and Beadles, 1949; Eldred and Rodney, 1945; Evans and St. Johns, 1945; Melnick *et al.* 1946; and Evans and Butts, 1948). Clandinin *et al.* (1951) developed a more elaborate technique. Carpenter (1958) commented that the *in vitro* techniques for the measurement of protein solubility or digestibility were valuable. Nevertheless, he cautioned that normal figures (controls) and multiple enzyme digestions must be used to prevent misleading results.

As an alternate to the enzymatic digestibility techniques, Carpenter (1958, 1960) and Carpenter *et al.* (1957) advocated the use of chemically measured available lysine. They reported correlations of this value with the nutritive value of proteins assessed by biological assays. This technique has proven to be reliable for the assessment of the quality of proteins from animal sources. Due to interference from carbohydrates it is unreliable for the measurement of protein quality from cereal sources. Buttersworth and Fox (1963) showed a correlation between chemically assessed available lysine and protein solubility. This same index correlated highly with their measures of net protein

utilization (NPU). The NPU could be predicted from a regression equation, $y = 10.1 + 10.4x$ (when x = available lysine and y = NPU).

Akeson and Stahman (1964) contended that although the chemical procedures which assessed protein quality based on total amino acid content were rapid and accurate, they made no allowance for variance in digestibility or bioavailability. They proposed a new index for evaluation of protein quality based on the digestibility of the protein in a double enzyme system, pepsin followed by pancreatin. This procedure was an extension of the pepsin digest residue index (PDRI) (Sheffner et al., 1956). The method of Akeson and Stahmann reduced the labor and sample size for each assay by use of automated amino acid analysis. Their procedure has been widely used although often the digestion procedure is as far as the test is followed. The index in many cases is not calculated (Mattu and Mauron, 1967; Saunders et al., 1973; and Sgarbieri et al., 1973). Other enzymatic digestibility tests have been devised and used for in vitro assessment of protein nutritive value (Ford and Slater, 1966; Boctor and Harper, 1968; Buchanan, 1969b; and Sgarbieri et al., 1973).

In vitro techniques for the measurement of protein quality are rapid and less complicated and costly than animal techniques. There are, however, some limitations. Byers (1967) indicated some problems regarding the specificity of proteases, and Saunders et al. (1973) discussed the ionic environment necessary for papain activity. With proper controls and enzyme selection Mattu and Mauron (1967) established the near identity between results obtained from in vitro assessment of

protein quality and those values from *in vivo* evaluation. They established a corrected method for chemical assessment of available lysine which agreed with enzyme digestibility and *in vivo* quality measurements of available lysine. The *in vitro* and *in vivo* assays had an r of 0.98. The biologically available lysine could be calculated from the equation: W = 1.02Y - 0.23 (Y is lysine in hydrolyzates of *in vitro* enzyme digested proteins).

Changes in Protein Quality on Storage

Melnick and Oser (1949) pointed out that storage of foods over extended periods of time appear to cause impairment of the nutritive value of the protein. These changes, they iterated, were similar to those noted following excessive heat processing. Most of these changes resulted through the formation of new resistant peptide linkages and not from direct destruction of amino acids. These new linkages function to slow the rate of enzymatic digestion and not the extent. Further, they argued that by slowing the rate of hydrolysis the amino acids are not released for absorption. Henry and Kon (1948) also argued a similar contention. They stated that the value of food as a source of protein is determined by the concentration of protein it contains, the digestibility of the protein, the availability of its amino acids, and its essential amino acid pattern. The digestibility and amino acid pattern presented to the organism for absorption determine the value of the protein to the organism. Processes which alter this digestibility (i.e., heating, storage-browning, etc.) have a pronounced effect on the nutritive value of a protein. They pointed out that in some instances

these changes can be beneficial, i.e., heating to improve the quality of legume protein. Mitchell and Beadles (1949) reported changes in stored grain (wheat and corn) as shown by decreased protein solubility which resulted in decreased *in vitro* digestibility and nutritive value of these grains. Stored ground grain was less stable than the whole kernel counterpart. This work also indicated the susceptibility of soybean globulin to a reaction with glucose, when held in a model system. Browning reactions were proposed as the mechanisms at work to decrease the nutritive value of the proteins.

Jones and Gersdorff (1941) reported that there were three types of deteriorative processes which occurred in stored wheat flour. These processes included: decreased protein solubility, partial breakdown of proteins with a decrease in true protein content, and lastly, a decrease in digestibility of the soluble nitrogen in peptic digests. They found that this indigestibility increased with the length of storage. Lipid oxidation in the flours and steric hindrance to enzyme action were cited as reasons for the reduced digestibility. Jones *et al.* (1942) indicated that the whole kernel products were also more stable under these conditions.

Hodson and Kruger (1947) reported changes in the EAA of casein during storage. The storage atmosphere (greater losses in air than nitrogen) and moisture were key factors in EAA losses. Discolored samples were reported as having suffered marked losses of arginine, histidine, lysine, and methionine. Small losses of tryptophan were reported. Samples which had not discolored exhibited only minor to

insignificant changes. A similar finding was reported (Hodson and Miller, 1957) in which it was found that the samples of milk protein exhibiting severe browning had marked losses of lysine. Earlier, Stevens and McGinnis (1947) had reported that, even though lysine was chemically present in a heated product, there was no assurance that it would be released by the digestive enzymes. Schultz and Thomas (1949) reported similar findings for samples of stored corn.

Mitchell and Block (1946), Evans and Butts (1949), and Henry and Kon (1950) all argued that indeed it was possible to alter the nutritive value of a protein without amino acid destruction. This fact was one source of variance between chemical and biological quality evaluation which must be reconciled. They reasoned from their data that linkages between reducing sugars and cross-linkages between proteins or peptide chains were formed which contributed to resistance to proteolytic action. Altered enzyme action produced an array of atypical peptides which were not absorbed and eliminated in the feces or absorbed and excreted in the urine (Mitchell and Block, 1946 and Seegers and Matill, 1935). Lea and Hannan (1950b) stressed the limitations in the use of strong acid or alkali at refluxing temperatures. These agents result in the regeneration of the amino acids condensed with carbonyls or the destruction of amino acids not previously reacted with the carbonyls. Ford (1962) reiterated this same point when he stated that protein foods subjected to unfavorable storage conditions may be highly resistant to <u>in vitro</u> enzymatic digestion though susceptible to chemical hydrolysis. The information obtained from the analysis of chemical hydrolysates may

be wholly misleading for these materials.

Henry and Kon (1948) and Mauron et al. (1955) indicated that the loss in the biological value of stored milk powder proteins was through the formation of enzymatically resistant Maillard reaction products. Since that time, Clark and Tannenbaum (1970) have produced and isolated such products. Similar phenomena have been observed in maize, wheat, barley and soybean meal. Melnick and Oser (1949) contended that the slowed rate of enzymatic hydrolysis of stored, browned foods was the factor contributing to lowered biological value. The greatest difference occurred in the early stages of the hydrolysis process. They reported that the amount of lysine released in browned samples was one-fourth of that released from unbrowned foods. This difference was measured during the early stages of hydrolysis. Greaves et al. (1938) alluded to the same phenomena. Evans and St. Johns (1945) related the length of cooking received by soybean meal to its digestibility by enzymes. Extended periods of heating at 121°C (longer than 15 min) resulted in decreased digestibility. McInroy et al. (1945) reported the formation of browning products including enzyme and acid resistant linkages which contributed to decreased protein value.

Maillard reaction products have been cited on numerous occasions as being the cause of decreased protein nutritive value (Hill and Patton, 1947; Patton and Hill, 1948; Patton et al., 1948a,b; Sgarbieri et al., 1973; Buchanan, 1969a,b; and Betschart and Kinsella, 1974a,b). Hill and Patton (1947) indicated that the processes involved reduction in the amounts of lysine, arginine, tryptophane, and histidine. ε-NH_2

in lysine, the indole group in tryptophan and the imidazole group in histidine were the side chains most involved in these reactions. Duckworth and Woodham (1961) observed the same processes in heated leaf protein concentrate (LPC). The lysine content was most susceptible in their study. Buchanan (1969a) reported that lipid oxidation products resulted in decreased digestibility of LPC due to formation of complexes with the proteins. This process was reported as being water dependent with the greater losses of protein value occurring at higher moisture contents. The length of storage also had a major effect on the extent of damage in the study. Rao et al. (1963) showed that samples of casein and glucose showed greatest losses over a 10 day period when stored at 15% moisture and 37°C.

Oxidized foods have been implicated in the loss of the nutritive value of these foods in numerous studies. Olley and Duncan (1965) reported a correlation of the rate of protein denaturation with increases in the amount of free fatty acid released from frozen stored fish muscle. There was no obvious trend of protein loss with increases in any particular free fatty acid except that the C_{22} polyene losses correlated with the greatest amount of protein denaturation. The neutral lipid fraction appeared to offer some protection against the process. Earlier (Lea et al. 1960) lipid oxidation had been related to the changes in nutritive value measured in stored herring meal at 6.1% moisture and 20°C for 12 months. Nitrogen and antioxidants were effective in retarding these processes. Carpenter et al. (1962) indicated that at less severe storage conditions (i.e., not heated) browning

reactions between the carbonylic, secondary products of lipid oxidation and ε-NH_2 of lysine were significant contributors to the deterioration of fish meal.

EXPERIMENTAL

Materials and Equipment

Beans and Bean Powder

Michigan Navy Beans, Michelite (Phaseolus vulgaris) were supplied for this study by The Michigan Dry Bean Shippers Association. The beans were from the 1968 and 1970 harvests. They were processed into instant dry bean powder in the pilot plant in the Food Science Building at Michigan State University.

Chemicals

All chemicals used during this study were Analytical Reagent Grade with the following exceptions:

Bacitracin A	Mann-Schwartz, Orangeburg, NJ
Boron trifluoride-Methanol (Lot 1057)	Applied Science Laboratories, Inc. State College, PA
Celite #545 (Filter Aid-Johns Manville Co.)	Fisher Chemical, Pittsburgh, PA
Citruline, D-6 (Lot #R4844)	Cyclo Chemicals, Los Angeles, CA
Cytochrome C	Calbiochem, LaJolla, CA
Dextran 2000	Pharmacia Fine Chemicals Piscataway, NJ
2,4-Dinitrophenyl hydrazine	Eastman Organic Chemicals, Distillation Products Industries, Rochester, NY

Fatty Acid Methyl Ester Standards	Applied Science Laboratories, Inc. State College, PA
Glycine (Control #5983)	Nutritional Biochemicals Corp. Cleveland, OH
Insulin	Calbiochem, LaJolla, CA
Leucine	Nutritional Biochemicals Corp. Cleveland, OH
Lysine, NRC (Lot #80745)	General Biochemicals Chagrin Falls, OH
Ninhydrin (Lot #6835)	Pierce Chemical Company Rockford, IL
Norleucine Standard (0.1 μM/0.1 ml)	Lab W. G. Bergen Michigan State University East Lansing, MI
Pancreatin (3X, Hog Pancreas)	Nutritional Biochemicals Corp. Cleveland, OH
Pepsin (1x, Hog Stomach, 20,000)	Nutritional Biochemicals Corp. Cleveland, OH
Phenylalanine	General Biochemicals Chagrin Falls, OH
Phosphorus Standard	Hartman-Leddon Company Philadelphia, PA
Sephadex G-15 (medium)	Pharmacia Fine Chemicals, Inc. Piscataway, NJ
Silica Gel G (acc. Stahl)	E. Merk Darmstadt, W. Germany
Sugar Standard	(Solution of Sucrose, Raffinose, Fructose and Stachyose Fruit and Vegetable Processing Lab. Food Science Bldg., Michigan State University East Lansing, MI
Sulfosalicylic Acid (Lot #73055)	Fisher Certified, Fisher Chemical Pittsburgh, PA
Trihydroxymethylaminomethane (THAM)	Sigma Chemical Co. St. Louis, MO

Trinitrobenzenesulfonic Acid (TNBS)	Nutritional Biochemicals Corp. Cleveland, OH
Trypsin (Hog Pancreas, 4x, Pancreatin)	Nutritional Biochemicals Corp. Cleveland, OH
Zephan Chloride (Antimicrobial Agent)	Pierce Chemical Company Rockford, IL

Solvents

All solvents with one exception were freshly redistilled before use. Methanol was made carbonyl free by refluxing with 2,4-dinitrophenyl hydrazine and trichloroacetic acid before distillation. Ethanol was made carbonyl free according to the procedure of Henick et al. (1954). Methyl cellosolve (2-methoxyethanol), Pierce Chemical, Rockford, IL was used as purchased.

Gases

All compressed gases used during this study were obtained from central stores, Michigan State University, East Lansing, MI. A prepurified grade of nitrogen was used throughout the study.

Thin Layer Chromatography

All thin layer chromatography (TLC) was carried out on 20 x 20 cm glass plates coated with silica gel G. The TLC layers were prepared with a Desaga Stahl TLC-spreader, Desaga C., Heidelburg, W. Germany.

Gas Liquid Chromatography

A Varian Aerograph Model 200 gas chromatograph equipped with a flame ionization detector, Varian Aerograph, Walnut Creek, CA was used for all fatty acid methyl ester analyses. A stainless steel

0.32 cm x 210 cm column packed with chromosorb W, acid washed (80-100 mesh) and coated with 15% diethylene glycolsuccinate (DEGS) was obtained from Applied Science Laboratories, Inc., State College, PA for use in the FAME analyses. A Beckman 25 cm, 0.1 millivolt recorder (Beckman Instruments, Fullerton, CA) was used to record the chromatograms.

Gel Filtration

Sephadex G-15 (medium-grain) was obtained from Pharmacia Fine Chemicals Inc., Piscataway, NJ. The columns used were Pharmacia glass chromatography columns, K-25, Pharmacia Fine Chemicals, Inc., Piscataway, NJ, and R-25 Marriott flasks from the same supplier were used for eluant reservoirs.

Constant Temperature Storage

The 37°C walk-in incubator in room 232 Food Science was used for constant temperature storage.

Desiccators

Pyrex vacuum Desiccators (250 mm), with venting sleeves were purchased from Corning Glass Co., Corning, NY for use in the storage studies.

Spectrophotometers

A Beckman DU-2, Beckman Instruments, Fullerton, CA was used for all of the absorbancy measurements for this project. When recorded spectra were needed, a Beckman DK-2 spectrophotometer from the same manufacturer was used.

An Agtron M-500-A, Magnuson Engineers Inc., San Jose, CA reflectance spectrophotometer was used during this study to measure objectively the color of the instant bean powder.

Spectropnotofluorometer

All fluorescence measurements were made on an Aminco-Bowman spectrophotofluorometer (SPF) with a Houston X-Y recorder (American Instruments Company, Silver Springs, MD).

Infrared Spectrophotometer

A Beckman IR-12, double beam infrared spectrophotometer (Beckman Instruments, Fullerton, CA) was used for all of the infrared analyses performed during the study.

Water

Distilled deionized water was used in all aqueous solutions for this study unless specified otherwise.

Centrifuges

All centrifugations during this study were carried out on an IEC model HR 1 Equipped with a #856 head. This instrument is manufactured by International Equipment Company, Boston, MA.

Vacuum Oven

A vacuum oven from Precison Scientific Instruments, Chicago, IL equipped with a Cenco Hyvac 7, vacuum pump, Cenco Instrument Corporation, Chicago, IL, was used for all moisture determinations and product desiccation.

Shakers

A Burrell Wrist-Action Shaker, Burrell Corporation, Pittsburgh, PA was used for mixing samples during extractions and to facilitate solution of various chemicals. A temperature regulated, NBS Gyrotory Shaker, manufactured by New Brunswick Scientific Company, New Brunswick, NJ was used during the enzymatic digestions in the *in vitro* protein quality studies.

Amino Acid Analyses

All amino acid patterns analyzed in this study were produced on a model TSM Amino Acid Analyzer, Technicon Instruments, Inc., Tarrytown, NY.

Methods

Instant Bean Powder Preparation

All bean powder used in this study was prepared from Michelite, Michigan-Navy Beans (*Phaseolus vulgaris*) according to the procedure of Counter (1969). This procedure produced a powder which met all of the criteria set forth by Bakker-Arkema *et al.* (1966) for a commercially acceptable instant bean powder.

Moisture Determination

Following the procedure outlined by the AOAC, 13.003, 9th ed. (1960), a method was established for measuring the moisture content of the instant bean powder. The procedure, as developed, involved weighing accurately, to the nearest 0.1 mg, a 2.0 g sample of powder into a

previously dried and tared glass weighing bottle. The samples were dried at 70°C for 5 hours at a vacuum less than 100 mm. Sulphuric acid dried air was bled into the oven at the rate of six bubbles/minute. At the end of the drying time the vacuum was released and the samples removed, capped and cooled to room temperature in a desiccator. The bottles were reweighed and the weight loss was assumed to be water. The per cent moisture was calculated as follows: (Initial weight) - (Final weight)/Initial weight X 100 = % Moisture.

Isotherms

Moisture vapor isotherms were established for the instant bean powder under adsorption and desorption conditions. The procedures of Karel and Nickerson (1964) and Stitt (1958) were modified to provide the information for this study.

All samples for the adsorption isotherm were desiccated in a vacuum oven for three days under vacuum at ambient temperature until the sample reached 3% moisture. Once this moisture content had been achieved throughout the lot, the sample was removed from the oven and 5.0 g portions (weighed to the nearest 0.1 mg) were placed into uncovered plastic petri dishes. Three samples were placed into each of nine vacuum desiccators equilibrated to different relative humidity levels. The relative humidity levels were achieved by employing saturated salt solutions according to the method of Rockland (1960). The desiccators containing the three dishes were then evacuated, sealed, and stored for four days at 37°C. At the end of this initial period the vacuums were released and the dishes were removed, covered, and

weighed to establish their weight gain. The dishes were then returned to their appropriate relative humidity chamber. The chambers were sealed, and returned to storage at 37°C. After another storage period of two days and at successive two day intervals until a constant weight was achieved, the dishes were removed from storage, covered, weighed, and returned to storage.

The final moisture content of the product was established by taking the weight gained, when a constant weight was achieved after storage, and adding to it the weight of water in the product before storage was initiated. This amount of water was divided by the total weight of the product at the end of storage. From these data an isotherm of moisture content vs. a_w was plotted for the adsorption loop of the study.

The desorption isotherm was derived in a similar manner; however, these samples were first humidified to a moisture content of 10% and then stored in the same nine relative humidity environments. The sampling schedule was maintained as it had been for the adsorption samples.

The final moisture content of the product was determined by subtracting the weight lost during desorption from the weight of water in the product before storage. The weight of water determined in this manner was divided by the final weight of the product to determine the moisture content for the plot of the desorption isotherm.

BET transformations by the method of Brunauer et al. (1938) were performed. These data provided graphical and mathematical solutions for the water activity monolayers for the instant bean powder.

Extraction and Measurement of Soluble Protein in Instant Bean Powder

Instant bean powder in 5.0 g amounts (weighed to the nearest 0.1 gm) was dispersed into 10 ml of 8 M urea: 1 N sodium hydroxide (1:1, v/v) which was contained in 50 ml centrifuge tubes. After 30 minutes, the extracts were clarified by centrifugation at 10,000 g for 15 min. in the model HR-1, IEC centrifuge. An aliquot (0.50 ml) of the supernatant was analyzed by the Biuret procedure (Leggett-Bailey, 1969 and Gornall 1949) for the protein content.

Extraction of Lipid from Instant Bean Powder

Lipid extractions of instant bean powder were made using the procedure of Takayama et al. (1965). This method utilized a chloroform methanol solvent system (2:1; v/v) and produced a total lipid extract which served as the source material for all lipid related analyses. Gravimetric estimates of lipid contents were performed on these extracts to provide a measure of total lipid content and as a check for completeness of extraction. The total lipid content was compared to crude lipid analyses made on instant bean powder using an eighteen hour extraction with the benzene-ethanol solvent system of Takayama et al. (1965).

Extraction of Sugars from Instant Bean Powder

Instant bean powder sugars were extracted according to the AOAC (1960) procedure 22.041. A 5.0 g sample was used in these analyses, and the reagent quantities were reduced by one-tenth. This extract was subjected to TLC for a qualitative analysis of the sugars present.

During the subsequent storage study, this extraction technique was utilized to provide samples for quantitative analysis of reducing sugar content.

Thin Layer Chromatography

All TLC separations were carried out on 20 X 20 cm glass plates which had been coated with silica gel G using a Desaga spreader and air dried. All plates were activated for one hr. at 100°C prior to use and cooled to room temperature in a desiccated cabinet.

Separation of the Components in the Total Lipid Extracts

The lipid content of the chloroform methanol extracts were adjusted to 25-30 mg/ml before TLC separation. One ml amounts of the concentrated extracts were then streaked on the thin-layer plate under an atmosphere of nitrogen. The plates utilized in these separations were 0.5 mm layers of silica gel G. The solvent system used in the development of the chromatograms was chloroform: methanol: water: ammonium hydroxide (65:55:4:0.25, v/v/v/v; Del Rosario, 1970). When the solvent front reached a height 2.5 cm. from the top of the plate, the plates were removed from the developing tank. An ultraviolet light was utilized to visualize the location of the neutral lipids on the plate. The other classes of lipids were located by partially covering the plate and spraying the uncovered portions with freshly prepared Dragendorff's reagent (Spray #97, Stahl, 1969) to locate PC and LPC bands and ninhydrin spray (Spray #178, Stahl, 1969) to visualize PE, LPE and PS bands.

The Rf values of these lipid classes were compared to Rf values for standards to confirm the location of PC and PE.

Bands containing the NL, PC, and PE were scraped from the plates onto fritted funnels where the lipids were eluted from the solid support with $CHCl_3$:MeOH (4:1; v/v).

Fluorescent materials were extracted from stored bean powder using chloroform: methanol, as previously described. One ml volumes of the concentrated extracts were applied to preparative TLC plates which were developed in the chloroform: methanol: water: ammonium hydroxide solvent system, previously described. The neutral lipid fraction which also included fluorescent materials was scraped from these plates. The fraction was eluted from the solid support with chloroform: methanol (4:1, v/v) and rechromatographed twice. The first system in which it was developed was hexane: diethyl ether (60:40; v/v). The carbonyls and related materials migrated with the solvent which was flashed from the plate under a stream of N_2, and the plate was chromatographed a second time. This second solvent system was chloroform: methanol: water (65:25:4; v/v/v). The fluorescent products separated into two bands under these conditions. Each fraction was scraped from the plate into a fritted funnel and eluted from the solid support with a $CHCl_3$:MeOH (4:1; v/v) solvent mixture. Once separated, the unconcentrated eluted material was stored at -20°C, under a N_2 headspace.

Separation of Sugars

Sugar extracts of instant bean powder and a standard solution of sugars were spotted on TLC plates coated with borate-impregnated silica

gel G, prepared according to Stahl (1969). The developing solvent was methanol: chloroform; acetone: conc. ammonium hydroxide (42:16.5:25:16.5; v/v/v/v). The sugars were visualized and identified by using a naphthoresorcinol spray (Spray #175, Stahl (1969)). R_f values for the extract spots and the standard were determined. The colors of the spots after reaction with the spray were also recorded and compared to literature values.

Preparation of Fatty Acid Methyl Esters

Fatty acid methyl esters (FAME) were prepared for all three major lipid classes NL, PC, and PE. The methylations were carried out using the boron trifluoride (BF_3) method of Morrison and Smith (1964). Pyrex test tubes with teflon lined screw caps (Pyrex #9826) were used for reaction vessels and pentane was the solvent for extraction of the esters. After separation and drying of the pentane layer with sodium sulfate (powdered anhydrous), the pentane was driven off and the samples diluted with petroleum ether, which served as the injection solvent.

Gas Chromatographic Analysis of Fatty Acid Methyl Esters

GLC separations of the FAME's were carried out isothermally under the following conditions: Temperatures: injector-220°C, column--180°C, and detector--195°C; Gas flows: air--250 ml/min, hydrogen 30 ml/min, and nitrogen--35 ml/min; Chart speed 0.5 cm/min; Flame setting 1. The peaks in chromatograms from instant bean powder lipid fractions were identified by comparison of retention times with those from known FAME mixtures chromatographed under identical conditions. The percentage

composition of fatty acids was calculated by manually measuring the area under each peak (height X width at ½ height) and expressing this area as a percentage of the total area for the peaks on the chromatogram.

Non-enzymatic, Non-lipid Browning Index

Water soluble browning products were assessed in the stored samples by using the method of Choi et al. (1949) as modified by Fishwick and Zmarlicki (1970). The trypsin utilized in this study was found to release the maximum levels of colored products at a concentration of 100 mg/ml. At the conclusion of the digestion, particular care had to be taken when adding the Celite to insure that a spectrophotometrically clear filtrate was obtained. The Browning Index was derived by multiplying the absorbance of the clarified filtrate measured at 390 mm by 100.

Fluorescence Measurement

Two types of fluorescence measurements were made during this storage study. Routinely, an aliquot of the total lipid extract was analyzed for relative fluorescence in the Aminco Bowman SPF. The excitation λ_{max} was 350 mm and the emission λ_{max} was 425 mm for these analyses.

The other type of fluorescence analysis involved characterization of the fluorescence spectral properties of the two fluorescing bands which had been separated by TLC. Using the same instrument, excitation and emission spectra were recorded on these fractions.

Protein Determinations

The micro-Kjeldahl method, AOAC, Sec. 42.014 (1970) was used to determine the protein content of whole beans, fresh instant bean powder, and various protein isolates. In this instance, the standard hydrochloric acid solution was standardized with TRIS primary standard according to the Sigma Technical Bulletin 106B (Anonymous, 1972).

A colorimetric estimation of soluble protein was also used. The Biuret method as modified by Gornall (1949) and Leggett-Bailey (1969) was used to assess the protein content of the 8M Urea: 1 N sodium hydroxide extracts of soluble protein.

Reducing Sugar Determinations

Analyses of reducing sugar content were performed on fresh instant bean powder samples and stored bean powder samples according to the method of Furuholman *et al*. (1964) These analyses were performed on extracts which were prepared according to AOAC 22.041 (1960).

Phosphorus Determinations

Phosphorus (P) determinations were made on the PC and PE fractions. Following separation by TLC and elution from the solid support, the fractions were clarified by filtration and made to a standard volume (25 ml) in a volumetric flask. A 0.9 ml aliquot was analyzed according to the procedure of Rouser *et al*. (1966). [*Warning* Perchloric acid and MeOH are *explosive*! Care must be taken to *completely* evaporate the solvent before the addition of the perchloric acid for the digestion.] The color complex between P and molybdenum was developed in a boiling

water bath and read at 820 mm. The absorbance was converted to µg P using the factor which was 11.22 µg P/0.1 A unit for this study.

Color Measurement of Instant Bean Powder

An Agtron Relative Reflectance Spectrophotometer was used to assess changes in the color of the bean powder during storage. Following the procedure outlined by Johnson (1966): 5.0 gram samples were spread over the bottom of a sample holder, and their relative reflectances were measured on the Agtron M-500-A, in the blue mode. The standards used in setting the instrument were the 44 and 56 disks.

Infrared Analyses for Schiff Base Containing Compounds

Infrared analyses were performed using the IR-12 spectrophotometer. Aliquots of the TLC separated fluorescent fractions were pipetted on to previously ground and dried potassium bromide (KBr). The solvent was evaporated under a stream of nitrogen. The KBr with fluorescing material adsorbed on it was placed in a pellet press and formed into 5 mm disks. The pellet was transferred to the sample holder, and an infrared spectrum was scanned from 25 to 3 µ. The operating conditions for the scans were as follows: SB/DB Ratio 1:1; Speed 8; Gain 430; Period 2; Ordinate Scale % T:0-100.

Gel Filtration

Two columns of Sephadex G-15 were prepared for use in the filtration of protein hydrolysates. The following procedure outlines the requirements which proved necessary to insure proper separations on the columns.

Hydration of the Gel

The gel was prepared for chromatography by dispersing gently the requisite amount of dry Sephadex into one liter of boiling 0.01 M phosphate buffer at pH 7.0. Table II in Sephadex Theory Practice (Anonymous, 1970) prescribes the quantity of dry Sephadex necessary to fill a column of a particular volume. The dispersion was allowed to hydrate 24 hr. before the fine particles and excess buffer were suctioned off through a Pasteur pipet connected to a vacuum aspirator. The thick slurry was suspended three more times in 0.5 l. of boiling buffer. These washings were aspirated as soon as the fine particles floated to the surface. Finally, the slurry was suspended in the amount of buffer equal to two times the bed volume.

Pouring the Column

Regular ends for the K-25 columns were fastened to the bottom and eluant reservoirs (R-25) were positioned at the upper ends as column extensions. The columns must be made level. A level, which was 20-30 cm long, worked well to erect the column and check its vertical alignment. Every precaution was taken to insure that the alignments of the columns were true. The bed heights (40 cm for this study) were carefully measured on the outside of the column. Buffer was forced through the bottom of the column to eliminate air bubbles under the column support net and then added through the outlet to fill the column to a height of 30 cm. A glass rod of sufficient length to reach from the top of the reservoir to the bottom of the column was put into place inside the column. The slurry was agitated sufficiently to suspend the

gel. Care was taken during this process not to incorporate air bubbles in the suspension. All of the slurry was poured at one time. It was poured down the glass rod taking care to prevent the entrapment of air bubbles during the pouring operation. Immediately after the column was poured, flow through the outlet was initiated. The glass rod was removed slowly by carefully stirring it through the column while the rod was withdrawn.

The column was allowed to settle during which time the flow of buffer through the column was continuous; however, it was never permitted to run dry. Once the bed had settled below the top of the column, it was stabilized by converting the column extensions to Marriott flasks. These eluant reservoirs were positioned at the proper height to provide sufficient hydrostatic pressure to obtain a flow rate of 1.5 ml/min (Anonymous, Table IV, 1970). Stabilization of the column was completed by washing the new bed with three bed volumes of buffer. At the end of this process, the headspace was adjusted to the exact height and the sample applicator was inserted, taking care to exclude air bubbles and to not disturb the top of the stabilized bed.

Calibration of the Column

After pouring, the columns were calibrated using the procedure of Ford (1965). The void volume was determined by visually monitoring the passage of 1% blue dextran in a 5% sucrose solution. Cytochrome C and Bacitracin A were used to determine the volume required to elute substances with a molecular weight greater than 1500 (the exclusion limit of G-15). Cytochrome C was located in eluant fractions by reading the

A for the fractions at 408 nm, and Bacitracin A was located by reading the A for the fractions at 215 nm. The volume of eluant required to filter amino acids through the column was measured by chromatographing leucine, phenylalanine, lysine, and glycine. The fractions containing these amino acids were determined by adding ninhydrin, according to the procedure of Moore and Stein (1954).

Sample Separation

Samples to be separated by gel filtration were applied to the column in 5.0 ml of 0.01 M phosphate buffer, pH 7.0, in a descending flow procedure. A glass syringe with a blunt 10 cm stainless steel needle was used to layer the samples onto the top of the column. After the samples had entered the bed, they were washed into the column with two 5.0 ml portions of eluting solvent. The headspace was carefully filled with eluting buffer, and the column end was connected to the Marriott flask containing the eluant buffer. After 120 ml of elution at room temperature, the filtration process was complete and the column was ready for regeneration.

Regeneration of the Column

Regeneration of the column was initiated with a 0.5 l. wash with DDI-H_2O, followed by recharging with 0.5 l. wash of 0.01 M phosphate buffer, pH 7.0. Checks were made periodically during the use of the column to insure that void volume and other operating characteristics had not changed through continuous use. Although two different columns were used to speed the separations, they had essentially the same

operating characteristics. Duplicate samples of any single treatment were always chromatographed over the same column to insure uniform separation. Throughout all chromatographic operations (hydration of the gel to regeneration of the column), Zephran chloride, an antimicotic agent, was added to all buffers and wash water.

In Vitro Protein Analyses

An in vitro measure of protein quality was performed on fresh and stored instant bean powder samples. Based on Kjeldahl analyses, the amount of instant bean powder containing 100 mg protein was subjected to enzymatic hydrolysis according to a modification of the procedure of Akeson and Stahmman (1964).

Fifteen ml of 0.1 N HCl containing 1.5 mg of pepsin were added to the 125 ml Erlenmeyer flasks containing the instant bean powder. The samples were hydrolyzed for three hr at which time the acid was neutralized with 7.5 ml of 0.2 N NaOH. Four mg of pancreatin contained in 7.5 ml of pH 8.0 phosphate buffer and 1 drop of toluene were added to the flasks. The pancreatic digestion was carried out for 24 hr at 37°C in the same constant temperature shaker. Each sample digestion was done in duplicate.

Following digestion, the flasks were removed from the shaker and the contents were transferred to 50 ml plastic centrifuge tubes. A 3.0 ml subsample was taken from the supernatant to measure the free amino acid pattern. These subsamples were placed in Pyrex test tubes (#9826) equipped with Teflon lined screw caps. The subsamples were depeptidized by adding 0.1 ml of 50% sulfosalicylic acid (SSA)/ml of sample.

Three-tenths μM of norleucine standard (0.1 μM/0.1 ml) was also added to each sample. These tubes were then centrifuged at 2500 RPM in the model HR-1 centrifuge. Subsequently, the free amino acid sample was flushed with N_2, frozen, and stored at -20°C until they were analyzed for free amino acids released during the hydrolysis.

The remaining hydrolyzates and the non-digested residues were washed from the digestion flasks into the centrifuge tubes with DDI-H_2O. The samples were centrifuged in the model HR-1 centrifuge at 25,000 g for 20 min. The supernatants were decanted into a filtration set up. The filtrates were passed through Whatman No. 1 paper and collected in 250 ml roundbottom (RB) flasks. The pellets were washed twice with DDI-H_2O and recentrifuged at 25,000 g for 20 min. after each washing. The pellets were resuspended in DDI-H_2O, and the entire contents of the tubes were transferred to the filtration set up. The supernatants from the hydrolysis and all of the washing filtrates were collected in the same RB flask, and shell frozen in a dry ice acetone bath.

The washed, non-digested residues were air dried on the filter papers on which they had been collected. When dry, they were scraped from the filter paper and collected in #9826 test tubes with Teflon lined caps. The residues were held in these tubes under N_2 at -20°C until acid hydrolysis and amino acid analyses could be performed. The shell frozen filtrates were freeze dried in a Stokes Pilot Plant freeze drier for 58.5 hr.

Once the soluble fraction had been lyophilized, the samples were scraped from the RB flasks into 50 ml Erlenmeyer flasks. Ten mg was

taken from each sample for nitrogen analysis. The remainder of the dried soluble fraction was dissolved in 0.01 M phosphate buffer, pH 7.0, and subjected to gel filtration chromatography in the manner described in the previous section. Based on the column calibration data, the column effluent was subdivided into a polypeptide grouping and the shorter peptide grouping. The effluent fractions corresponding to these groupings were combined and shell frozen in 50 or 100 ml RB flasks. After freeze drying, they were scraped into #9826 test tubes flushed with N_2 and capped with Teflon lined caps. These fractions were frozen at -20°C and held for acid hydrolysis and amino acid analyses.

Acid hydrolyses of the non-digested residues and the two peptide fractions were carried out in 6 ml of 6 N HCl in the #9826 test tubes with the caps on at 121°C for 12 hr in an autoclave. Upon completion of the hydrolysis, each sample was flash evaporated to remove the HCl. The remaining amino acids were dissolved in 2.0 ml of DDI-H_2O and analyzed for alpha-amino nitrogen content according to the procedure of Palmer and Peterson (1969). Based on these analyses, each fraction was diluted to a level of amino nitrogen such that when the internal norleucine standard was added at a level of 0.25 μM norleucine/0.5 μM amino acid, not more than 0.2 ml of standard was added.

When all four fractions; free amino acids, two peptide fractions, and the non-digested residue; had received the appropriate amount of internal standard, they were chromatographed on a model TSM Amino Acid Analyzer employing a divinylbenzene cross-linked sulfonated polystyrene resin and a buffer gradient from pH 3.5 to pH 5.97 (1 N lithium, 0.05 M

citrate) on the basic column and a buffer gradient from pH 2.7 to pH 4.6 (0.3 N lithium, 0.05 M citrate) on the acid neutral column.

Experimental Design

An accelerated storage study was performed with freshly prepared instant navy bean powder to assess the effect of temperature, water activity, a_w, and storage atmosphere on physicochemical measures of product quality. The powder was prepared in the usual manner. It was divided into four, 900 g lots and spread to a depth of 2 cm in 22.5 cm Pyrex plates. Each lot required two plates to hold the 900 g. Once the product had been distributed over the plates, each lot was placed in different a_w and atmospheric environments for storage.

Desiccators were used as storage chambers. Each one contained a saturated salt solution which had been equilibrated at 37°C. Two desiccators contained lithium chloride, which produced an a_w of 0.11 and the other two contained potassium acetate, which produced an a_w of 0.23, according to Rockland (1960). At each a_w the samples were stored in an atmosphere of nitrogen (N_2) or air. The air samples were placed directly into storage, after the samples were sealed into the desiccators. The N_2 atmosphere samples were sealed into the desiccators which were evacuated with a Cenco vacuum pump to the lowest constant manometric pressure reading. They were purged back to atmospheric pressure with prepurified N_2. The evacuation and purge were repeated once. All samples were placed into storage in the walk-in incubator. After ten minutes at 37°C, all desiccators were vented to release expanding gases. This precaution prevented the lids from being forced from the desiccators. A control

sample of fresh instant bean powder was stored under N_2 at -20°C during the study.

Initial product quality values and nutritional indices were assessed chemically by analyzing a subsample of the fresh instant bean powder for moisture, total lipids, relative fluorescence, phospholipid and neutral lipid fatty acid patterns (U/S ratios), soluble protein, reducing sugar content, relative reflectance, browning index, and *in vitro* protein quality index. The accelerated storage study was carried out for 30 days during which period each treatment was analyzed every four days. The analyses which the treatments received routinely included moisture, soluble protein, total lipid content, relative fluorescence, browning index, and relative reflectance. Sampling for each analysis was carried out to avoid bias. The product on each storage plate was thoroughly mixed before and after sampling and a random sample from both plates was chosen. At the conclusion of the storage test, each sample and the control received the same analyses which had been performed on the initial sample.

The data supplied from these analyses before, during, and after storage provided a basis for assessing the effects of storage a_w and atmosphere on these chemically measured indices of quality and nutritional value.

RESULTS AND DISCUSSION

Isotherms for Instant Navy Bean Powder

The final moisture content achieved by the isotherm bean powder samples at equilibrium relative humidity are presented in Table 1. Also included in the table are the BET transformation values for each moisture content. These values were calculated from the BET transformation equation:

$$a/(1-a)V = 1/V_mC + \frac{(C-1)}{V_mC} a$$

in which

$a = a_w = pv/ps$

V is the moisture content expressed as cc^3

V_m = volume of the monolayer moisture content, and

C is a constant related to the heat of adsorption.

As it is presented here it is in the standard form for the equation of a straight line with $a/(1-a)V$ the ordinate value; $1/V_mC$ the ordinate intercept, $\frac{C-1}{V_mC}$ the slope; and a the abscissa.

The same data are shown graphically in Figures 1 and 2. Slope values from the lines in Figure 2 were calculated to be 22.2 for the adsorption values and 19.3 for the plot of the desorption values. The intercepts were 1.05 and 1.0 respectively. From these data and graphical solutions monolayer moisture values were calculated. These values were 4.71% moisture for the adsorption product and 5.46% moisture for

Table 1. The equilibrium relative humidity, a_w, moisture contents and BET transformation values for bean powder samples prepared by adsorption and desorption methods.

	Adsorption		Desorption	
a_w	Moisture[a]	$a/(1-a)V$[b]	Moisture[a]	$a/(1-a)V$[b]
0.11	0.0355	3.48	0.0395	3.13
0.23	0.0480	6.22	0.0547	5.46
0.32	0.0576	8.17	0.0661	7.12
0.41	0.0648	10.16	0.0779	8.92
0.51	0.0794	13.11	0.0821	12.60
0.57	0.0916	14.77	0.0978	13.55

[a] Moisture as g H_2O/g powder.

[b] BET transformation in which V = volume of moisture adsorbed and a = pv/ps or the water activity, a_w.

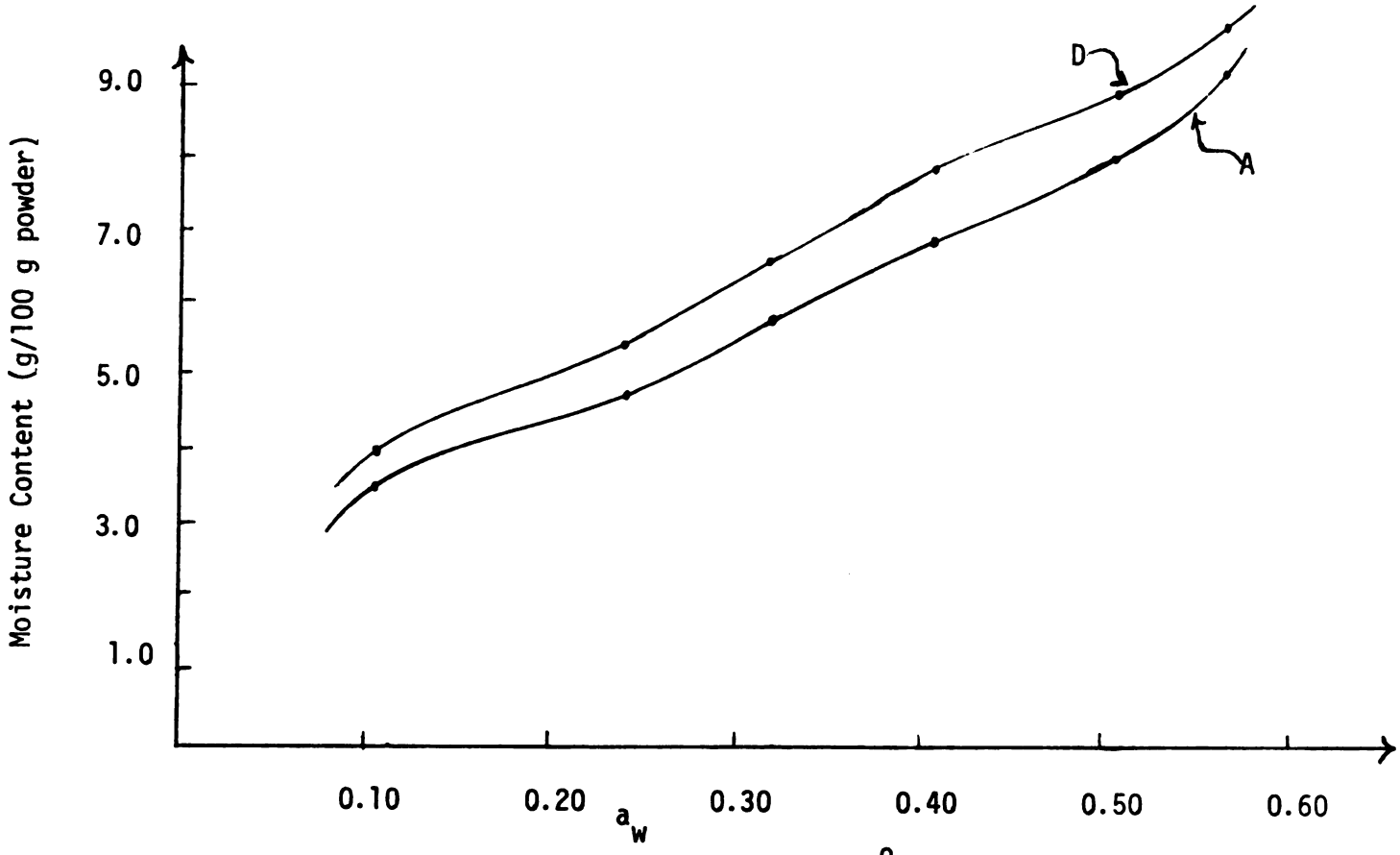


Figure 1. Sorption isotherms for navy bean powder at 37°C.

A = adsorption condition for determination of equilibrium relative humidity (a_w) moisture contents.
D = desorption conditions for the determination of a_w moisture contents.

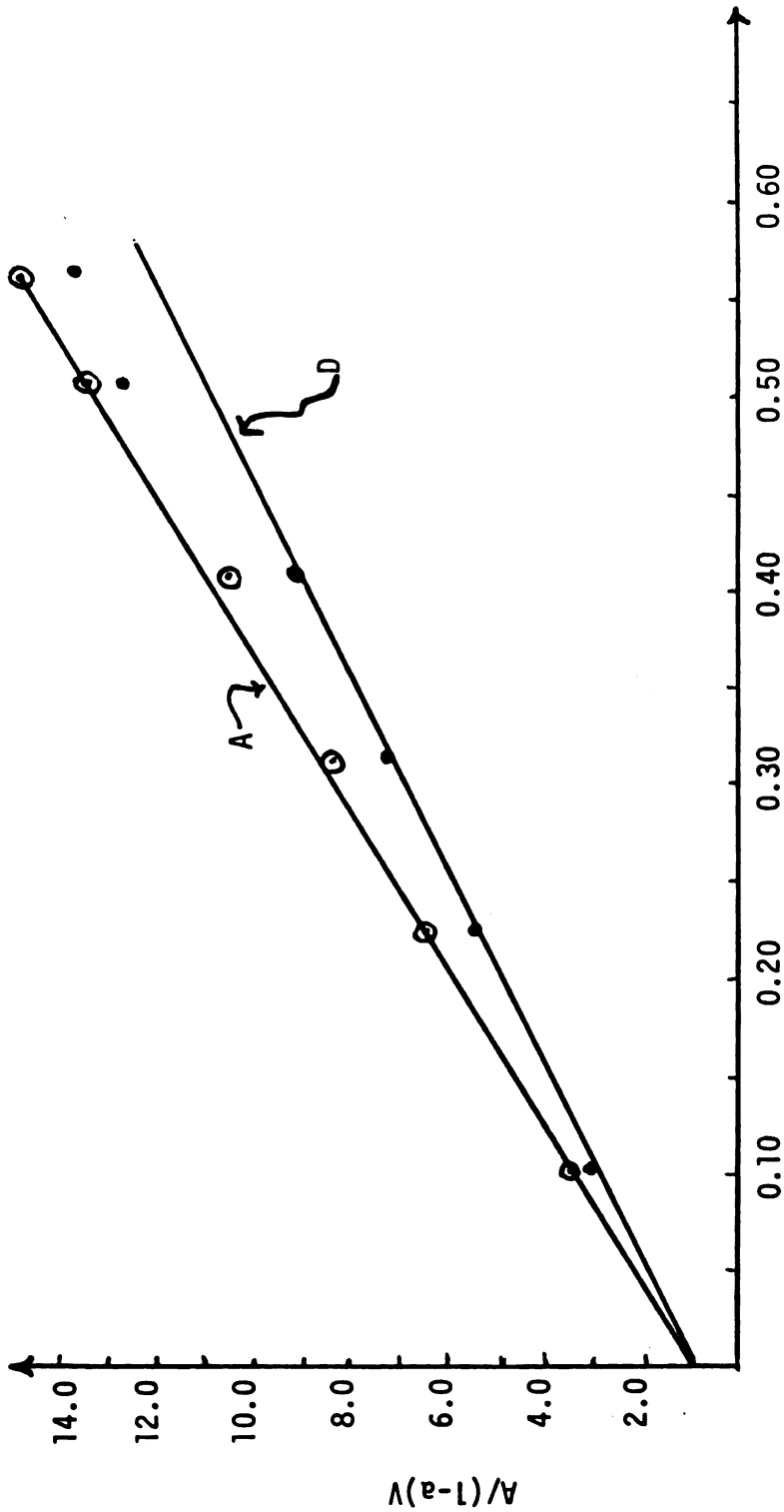


Figure 2. BET transformation for instant navy bean powder, 37°C.
A = adsorption product
D = desorption product

the desorption product. The a_w's which correspond to these monolayer moisture values are 0.16 for the adsorption product and 0.23 for the desorption samples.

Because of the facility with which the desorption product could be made from product that was initially 6.8% moisture, this method was the one chosen to produce the product for the storage studies. The samples were desorbed to the desired, target moisture levels using relative humidities of 11% and 23% at 37°C. Comparison of changes in the chemical indices of quality at different moisture contents would offer insight into the role of water in storage deterioration of the product. According to Burr et al. (1969), the 0.11 product would have the greatest stability. In their study, product stored at moisture contents greater than 5% water showed greater deterioration.

Regular analyses of the stored product revealed that at 0.11 a_w, samples reached the target moisture content (~4.0%) by the fourth day of storage. The product stored at 0.22 a_w took longer to achieve its target moisture content (5.45%) attaining a moisture content of 5.5% on the twelfth day and maintaining this value through the remainder of the study. The mixture content of the product at a_w 0.11 was 4.0 from the fourth day through the remainder of the study. This latter value represents a moisture content 2.8% below that of the finished product and ~1.5% below the monolayer value for the desorption samples.

Instant navy bean powder, like most food products, exhibited Type II or S-shaped isotherm (Brunauer, 1945). Irreversible processes which occurred during the drying of the product for the adsorption

study are reasoned to be the source of the hysteresis shown in Figure 1 (Labuza et al., 1970; Labuza, 1968; Rockland, 1969; and Wolf et al., 1972).

Composition of Instant Navy Bean Powder

Base line values for the chemical and nutritional indices of quality were measured before initiating the storage study. These analyses were carried out using the techniques that were to be employed for analyses during the storage study. The values obtained are compiled in Table 2. Literature values were not readily available for comparison with these data. In many instances value comparisons for the instant bean powder were only found for unprocessed whole beans. The total lipid value is lower than that one reported by Takayama et al. (1965) and Takayama (1961). Those data were from samples which were only the cotyledons of dry beans and not for a processed instant bean powder. Counter (1969) did not analyze for total lipid content. The values for protein content agree with those of Iriarte (1969) for the Kjeldahl procedure. The Biuret value for protein content reported here were slightly higher than those reported by Counter (1969), The reducing sugar content was less than the one reported by Counter (1969). His extract for the analysis, however, had been made with boiling water while the extracts for the analysis in this study were made with 80% EtOH. A different, more specific analytical technique for reducing sugars was used in this study. The Agtron RR value was not measured by Counter (1969) nor was the BI value. Some of the product produced prior to this study and stored at -20°C had lower BI values (9.95) than those

Table 2. Instant navy bean powder quality indices and composition data. The sample was freshly prepared powder from the 1970 bean harvest.

Test or Index	Value
Moisture Content (%)	6.83
Lipid Analyses:	
Total Lipid (g/100 g)	2.99[a,d]/2.89[a,e]
NL (U/S)[f]	7.73
PC (U/S)	2.06
PE (U/S)	2.52
Relative Fluorescence Intensity	25.7
Sugars, Total Reducing	0.20%
Protein:	
Kjeldahl (%)	23.18[a,c]
Biuret (%)	23.9[a]
Agrton RR[b]	45.3[b]
Browning Index (BI)	11.07[a,c]

[a]Dry Weight Basis.

[b]Relative Reflectance Agtron M-500-A in Blue Mode, average of five determinations.

[c]For 35.0 mg sample by micro-kjeldahl.

[d]$CHCl_3$: MEOH (2:1, v/v).

[e]Continuous Extraction Benzene: Ethanol (68:32; v/v).

[f]Unsaturate to Saturate Ratio.

for the sample used in this study (11.07). This difference indicates that the product prepared for this study had been overheated during the drying process.

Thin Layer Chromatographic Analyses

Lipid Classes in the Extracts from Instant Bean Powder

Separation of the total lipid extracts was carried out by preparative TLC techniques. Characteristically, the hR_f values for the major classes of concern in this study were as follows: NL-99, PE-45, PC-20, LPE-5.0, and LPC-1.0. Figure 3 documents, through a schematic of a typical chromatogram, the location of these classes on a plate. When the plates were run to within 2.5 cm of the top of the plate, the PE and PC bands were sufficiently separated, permitting easy removal of these compounds without contamination. Ultraviolet light and sprays were used to detect these compounds, and standards run during the preliminary work confirmed that the compounds were correctly identified and located. This separation was similar to that one obtained by Del Rosario (1970) with the same solvent system.

Separation and Identification of Sugars in Instant Bean Powder

Chromatographic separations of the extracts showed the presence of five distinct compounds. Based on a comparison of colors on reaction with naphthoresocinol, data on standards run in the same chromatographic system, hR_f values, and literature values on the R_f's of the standard and the color of the standards, the five sugars identified in

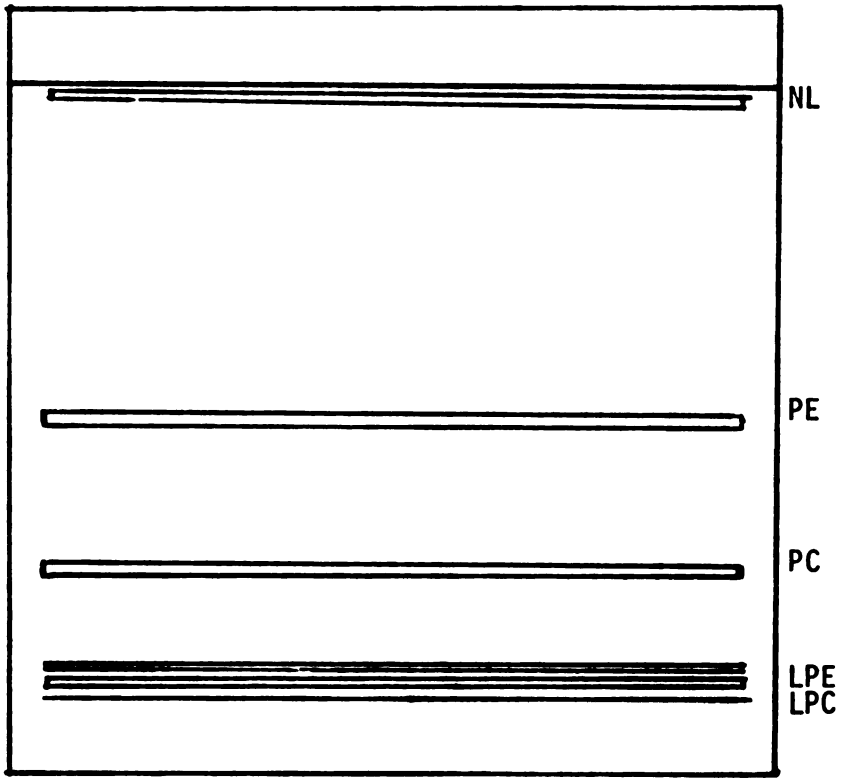


Figure 3. Schematic diagram preparative scale total lipid TLC separation. Neutral lipids (NL), phosphatidyl ethanol amine (PE), phosphatidyl choline (PC), lysophosphatidyl ethanolamine (LPE), lysophosphatidyl choline (LPC). Solvent systems $CHCl_3$: MEOH: H_2O: NH_4OH (65:35:4:0.25; v/v/v/v).

the ethanol extract of instant navy bean powder were sucrose, glucose, fructose, raffinose and stachyose. These sugars correspond to the spots numbered 4, 4, 3, 2, and 1 in the unknown extract (see Table 3).

Table 3. Data from the separation and identification of compounds in the 80% ethanol extracts of instant navy bean powder. Solvent system Benzene: Acetic Acid: MEOH (20:20:20; v/v/v). Silica gel G impregnated with 0.2 M boric acid.

Spot	hR_f		Color with Spray Naphthoresorcinol	
	Plate	Literature[a]/Standard	Plate	Literature[b]
Sucrose	29.2	29.2	Red	Red
Glucose	29.2	29.2	Blue-Violet	Blue-Violet
Fructose	20.8	20.8	Red-Black	Red-Black
Raffinose	12.5	12.5	Tan	NG[d]
Stachyose	6.6	6.6	Tan	NG
Sample				
Spot #1	6.6	NA[c]	Tan	NA
Spot #2	12.5	NA	Tan	NA
Spot #3	20.8	NA	Red-Black	NA
Spot #4	29.2	NA	Blue-Violet	NA

[a]Stahl, E (1969).
[b]Stahl, E (1969).
[c]Not applicable, NA
[d]Not given, NG

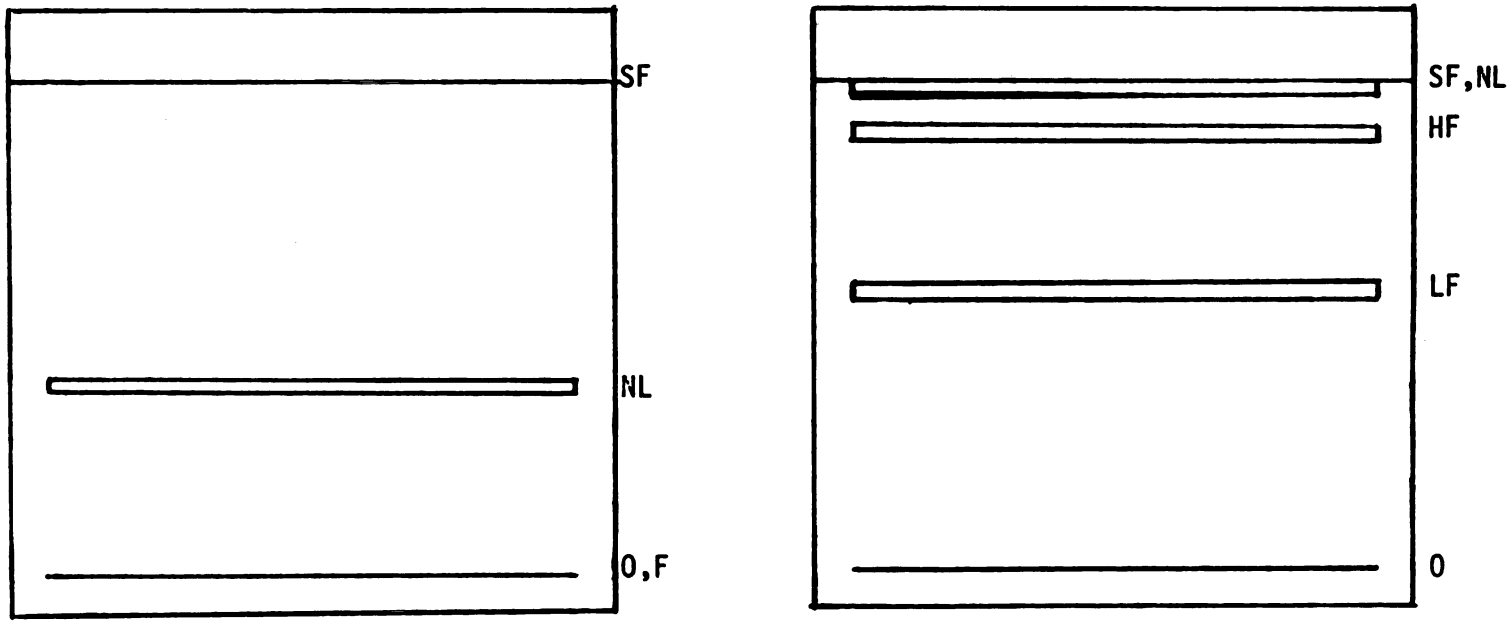
Chromatography of Fluorescing Compounds

Observations of the total lipid extracts from stored bean powder samples revealed the presence of fluorescing substances. When these extracts were chromatographed using the procedure of Braddock (1970), two fluorescing bands were separated. These bands were labelled HF for the one having a R_f of 0.91 and LF for the one with a R_f of 0.58. Separations of this magnitude made isolation of these distinctly different materials easy. Schematic diagrams of these chromatograms are shown in Figure 4 on the following page.

Analyses of Fluorescing Compounds

Fluorescence spectra were run on the LF and HF bands. The data revealed that they had characteristic fluorescence spectra for Schiff base compounds (Dillard and Tappel, 1973; Bidlack and Tappel, 1973; Malshet and Tappel, 1973; Dugan and Rao, 1972; and Braddock, 1970). The excitation and emission λ for the LF were 350 nm and 450 nm, and for HF they were 350 nm and 445 nm. These data indicate that the products were possibly conjugated Schiff base compounds. There was no significance placed on the emission peak recorded at 700 nm in the fluorescing samples. This peak is a harmonic of the excitation λ.

Infrared spectra of the bands were difficult to obtain due to the low concentrations of products in the extracts. Only minor peaks could be found. These corresponded to 1748 cm^{-1}, 1710 cm^{-1}, 1565 cm^{-1}, 1465 cm^{-1}, and 1385 cm^{-1} which Braddock (1970) and Dugan and Rao (1972) published as part of the spectra of fluorescing Schiff base compounds. The 1710 cm^{-1} peak and the 1565 cm^{-1} were cited as part of the condensed



Chromatogram A

Chromatogram B

Figure 4. Chromatographic separations of fluorescing materials in the NL fraction of TL extract from stored instant navy bean powder. 4A solvent system: Hexane: diethyl ether (60: 40; v/v). 4B solvent system $CHCl_3$:MeOH:H_2O (65:25:4). Neutral lipids (NL), fluorescing (F), fluorescing bands high on plate, HF:R_f-.91, and lower on the plate, LF; R_f-0.58, solvent front (SF), and origin (O). Chromatogram A was run, solvent flashed off under N_2 and B was run on the same plate.

Schiff base system. The 1748 cm^{-1} is for carbonyl stretching. These data are compatible with the structural features reported by Malshet and Tappel (1973).

Physical Changes During Storage

Development of Relative Fluorescence Intensity

Table 4 relates the relative fluorescence intensity (RFI) data for stored product. There do not appear to be any trends similar to those developed in the other indices. Most of the samples had lower levels of RFI at the conclusion of the storage study than they had at the beginning.

Table 4. Relative fluorescence intensity (RFI) for fresh, stored, and control instant navy bean powder samples.

	RFI[a]	
Sample	Highest (Day)	Final (Day)
Initial	25.5 (0)	25.5 (0)
0.11 A[b]	31.5 (4)	23.5 (28)
0.11 N[b]	30.0 (4)	24.0 (28)
0.23 A[b]	50.0 (23)	23.0 (29)
0.23 N[b]	42.5 (23)	27.0 (29)
Control[c]	25.7 (28)	25.7 (28)

[a]Excitation λ-360 mm, Emission λ-425 mm.

[b]Stored at 37°C, one month.

[c]Stored at -20°C, under N_2 atmosphere, one month.

It is of interest to note the highest levels of RFI recorded during the study, and the days on which these values occurred. The monolayer stored samples achieved their maximum RFI levels 20 days later than the samples stored below the monolayer. Maximum values for these samples were as much as 1.5 times greater than those recorded for the samples stored below the monolayer. Patterns of this type are not uncommon in processes related to lipid oxidation, when the method of analysis measures intermediate products of lipid oxidation. Dugan and Rao (1972) showed similar data for fluorescence development in dry model systems. Counter (1969) observed similar patterns of carbonyl production in instant navy bean powder. His retort cooked beans, when processed and stored, showed similar patterns of increases and then declines. Their maximum content occurred at approximately 30 days of storage at 40°C. This pattern implies that the conjugated fluorescing Schiff base system underwent further condensation and polymerization to non-fluorescing products (Dugan and Rao, 1972).

Changes in Instant Bean Powder Color

Johnson (1966) showed a correlation between the loss of whiteness in flour milling fractions and a decrease in Agtron reflectance in the blue mode. Fresh navy bean powder has a very light tan (white) color witn a high blue mode reflectance of ~60 (0 and 100 standardization disks). This reflectance was shown to decrease rapidly during preliminary studies on browning of instant navy bean powder. Samples which had an initial relative reflectance of 60.0 were browned at 120°C. for 8 3/4 hr. under a vacuum. The samples had a relative reflectance of

40.0 when removed from the vacuum oven.

In order to improve the sensitivity of this measure, the expanded scale technique was utilized. Expansion of the scale enabled distinctions between samples showing only slight differences on the broader 0-100 scale range. The standard disks used were 44 and 56 as the 0 and 100% reflectance points. On this scale the initial product used in the storage study had a relative reflectance value of 45.3. This reading indicated that the product was darker than some other products made by the process. In spite of this fact, the data in Table 4 shows that perceptible changes occurred during storage.

Table 5. Changes in Agtron M-500-A blue mode relative reflectance for instant navy bean powder stored at 37°C for one month. Standardization disks 44 and 56.

Treatment a_w/Atmosphere	Days in Storage	Relative Reflective Reading[b]
Fresh (Initial)	0	45.30
0.11/Air	28	33.80
0.11/N_2	28	29.80
0.23/Air	29	35.00
0.23/N_2	29	32.50
Control[a]	28	45.10

[a]Fresh product placed in sealed container with N_2 atmosphere and held at -20°C during storage study.

[b]Blue mode reflectance. Lower reflectance implies decreases in whiteness.

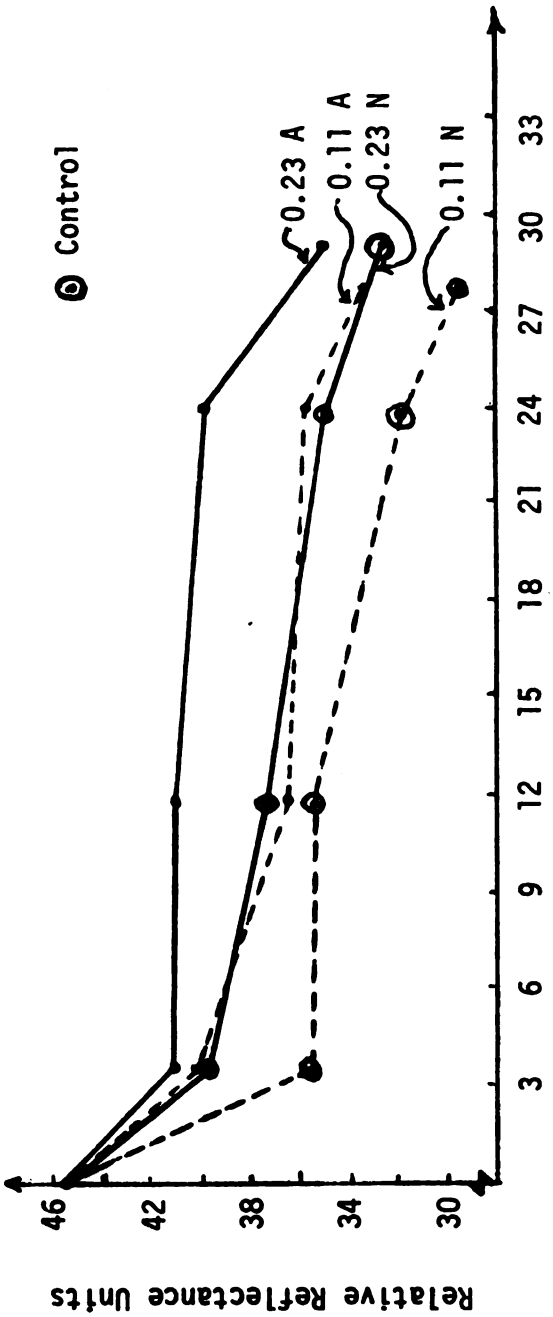


Figure 5. Changes in Agtron M-500-A blue mode relative reflectance for instant navy bean powder during one month storage at 37°C. The samples were stored at two a_w's (0.11 or 0.23) and in air or nitrogen.

From this plot of relative reflectance vs. time (Figure 5, on the preceding page) it is obvious that measurable browning occurred sooner in the samples stored below the monolayer (0.11A or 0.11N). Although all samples decreased at least ten reflectance units during the study, the samples stored at the monolayer took a longer period of time to reach the final values as shown by the displacement of the 0.23N curve 7 days to the right of the 0.11N curve and the 0.23A curve to the right by 14 days.

Chemical Changes During the Storage Study

During the accelerated storage at 37°C changes were noted in several chemical indices of product quality. Of these indices, protein content, browning index (BI), and relative fluorescence (RFI) were measured every four days, while the U/S ratio of fatty acids, total reducing sugar content, and protein quality (*in vitro* digestibility) were measured at the beginning on the fresh product and at the conclusion of the study on each of the four treatments and the control.

Changes in Soluble Protein

Detectable losses in soluble protein were registered during the storage period. The data in Table 6 indicate that the samples stored at the monolayer experienced greater losses of soluble protein than those stored below the monolayer. The greatest loss was in the sample stored in an air atmosphere at the monolayer. Those samples stored below the monolayer exhibited a slightly greater loss of protein for the samples stored in a nitrogen atmosphere.

Table 6. Changes in soluble protein content of instant navy bean powder stored at 37°C for a month.

a_w/Atmosphere	Days in Storage	Soluble Protein,[a] %
Fresh	0	23.90[c]
0.11/Air	28	21.80[d]
0.11/N_2	28	21.40[d]
0.23/Air	29	19.90[e]
0.23/N_2	29	22.10[e]
Control[b]	28	24.00[d]

[a]Biuret Method, DWB.
[b]-20°C, N_2 Atmosphere.
[c]Average 5 readings.
[d]Average 4 readings.
[e]Average 3 readings.

The rates of protein loss for this study (see Figure 6, on the following page) were compatable with those of Counter (1969). The rates of loss were even a little more rapid in certain cases. His product was stored for 120 days and at 30 days one of his samples (the retort cooked product at 6.9% moisture) had similar losses of protein. The rise in his percent protein above initial value for the other sample can likely be explained by the fact that this procedure is plagued by cloudy extracts if care is not taken during the centrifugation and pipetting of the aliquot for the analysis. Cloudy aliquots would artificially elevate the A_{545} nm for the Biuret test. The most rapid rate of loss of protein was in the samples stored in air at the

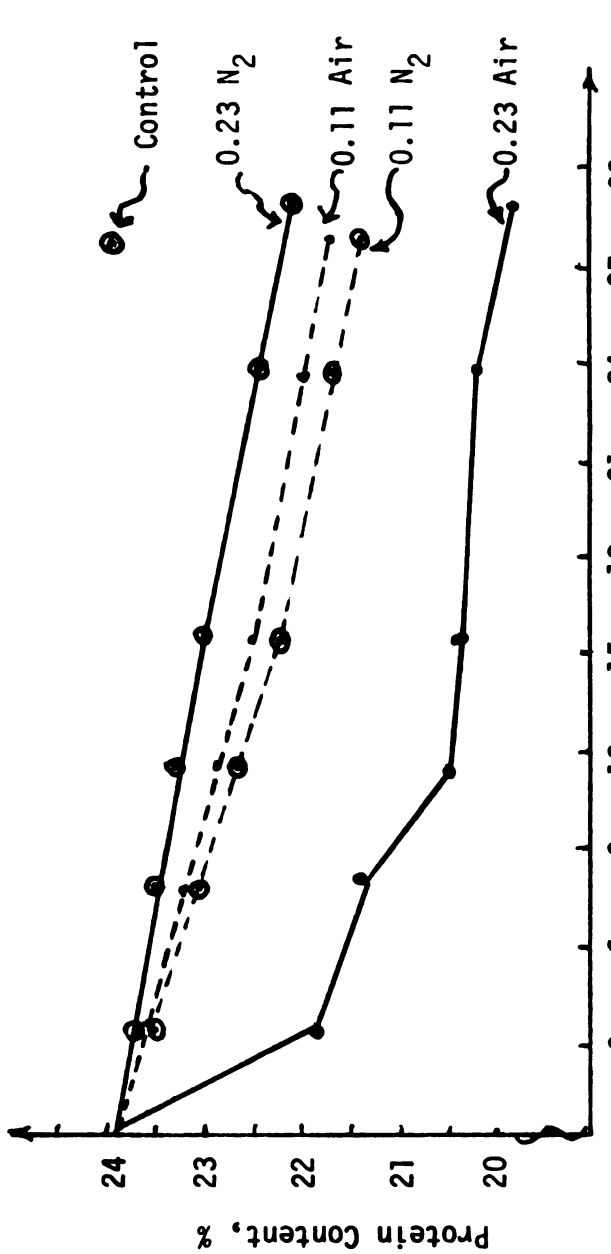


Figure 6. Changes in instant navy bean powder soluble protein content during storage at 37°C for one month.

monolayer, and the least loss occurred in a sample stored in nitrogen at the monolayer. Intermediate losses were recorded by the sample stored in a nitrogen atmosphere below the monolayer moisture level.

Changes in Lipid Composition

The fatty acid composition of the three major lipid classes, NL, PC, and PE are given in Table 7. The saturated fatty acids are hexadecanoic acid (16:0) and octadecanoic acid (18:0) while the unsaturated fatty acids shown by the analyses included octadecenoic acid (18:1), octadecadienoic acid (18:2), and octadecatrienoic acid (18:3). Peaks with retention times greater than the 18:3 fatty acids peak appeared in the chromatograms for all classes of lipids from the lipid extracts of freshly prepared instant bean powder. These longer chain compounds were possibly unsaturated fatty acids. There were no peaks for these compounds in any of the chromatograms of stored product lipid extracts. Quantitatively, these FA's of chain length greater than 18:3 ranged from 0.55-1.5% of the FA distribution depending upon the class of lipids.

The data indicated that the neutral lipids were more highly unsaturated than phospholipids. The literature values do not show the presence of 18:3 in the phosphatide fraction. This fact was likely the result of improper handling of the beans before extraction which would have permitted destruction by the action of lipoxygenase enzyme (Holden, 1970). Takayama (1961) theorized that phospholipases were responsible for the absence of 18:3 in their samples. In this study, there was no question concerning the presence of 18:3 in these chromatograms. The presence of this fatty acid was confirmed by a comparison

of retention times to those for a standard containing octadecatrienoic acid under the identical chromatographic conditions (see Table 7).

Table 7. Composition of fatty acids in NL, PC, and PE fractions of a $CHCl_3$:MeOH (2:1, v/v) extract of fresh instant navy bean powder.

	Percentage Composition				
Fatty Acid	GLC Analysis[a]			Literature[b]	
	NL	PC	PE	NL	Phosphatides
14:0	--	--	--	0.1	--
16:0	9.81	25.70	29.84	15.8	53.6
16:1	--	--	--	--	--
18:0	1.93	2.70	2.43	1.0	3.4
18:1	11.71	15.70	12.76	13.3	8.1
18:2	26.15	30.04	33.54	26.9	35.0
18:3	50.92	25.34	21.43	42.6	--
> 18:3	0.55	0.86	1.23	--	--

[a]Average of three determinations.
[b]Takayama (1961) and Takayama et al. (1965).

Changes in the U/S ratio of the fatty acids patterns for the three classes of lipids were used as a measure of changes in lipid composition of stored product. These U/S ratios were calculated from the GLC analyses of NL, PC, and PE fractions of fresh, stored, and control samples. They are shown in Table 8. These data indicated that lipid oxidation occurred in the neutral lipid fraction of all stored product. The samples stored at the monolayer oxidized to a lesser extent than those

stored below with the exception of the 0.11A PC fraction. For the samples stored below the monolayer, the nitrogen atmosphere protected the lipids from oxidation. At the monolayer there was no protection of the lipids by the nitrogen atmosphere as indicated by similar U/S ratios.

Table 8. Changes in the U/S ratio of NL, PC, and PE fractions for fresh and stored instant navy bean powder and in the μg P/g powder.

Sample	Days in Storage	Unsaturate/Saturate Ratio[c]			μg P/g powder[d]
		NL	PC	PE	
Fresh[a]	0	8.55	2.11	2.52	298.0
0.11 A[b]	28	7.49	2.03	2.25	274.0
0.11 N[b]	28	8.09	2.09	2.37	284.0
0.23 A[b]	29	7.59	1.91	2.46	266.0
0.23 N[b]	29	7.50	2.07	2.48	287.0
Control[a]	28	8.57	2.11	2.50	288.0

[a]Contained > 18:3 FA's in all fractions.
[b]Contained no FA's > 18:3.
[c]Ratio of unsaturated FA's to saturated FA's ($C_{18:1} + C_{18:2} + C_{18:3}$/ $C_{16:0} + C_{18:0}$).
[d]Present in total lipid (TL) extract.

The data in the extreme right hand column is for phospholipid content in the total lipid extract. These values of μg of phosphorus/ g of powder are of the same magnitude as those of Takayama (1961) for the same $CHCl_3$:MEOH 2:1 (v/v) extraction technique. Variance from his

values can be attributed to a different type of starting material (only the cotyledons of whole beans). These data indicate that phospholipid extractability followed the same trends as the U/S ratios. These data would indicate that during storage the extractability of the phospholipids decreased due to oxidation.

Changes in Reducing Sugar Content

The qualitative TLC analysis revealed the presence of two reducing sugars, glucose and fructose in extracts from instant navy bean powder. Quantitative analysis of the same extracts for total reducing sugar showed that fresh instant bean powder contained 2.03 mg of total reducing sugar per gram of powder. As with the U/S ratio, the reducing sugar content was measured on fresh product and at the end of the storage study. The changes in this index for the study are shown in Table 9.

Table 9. Summary of total reducing sugar content in fresh and stored instant navy bean powder.

Sample	Days in Storage[a]	Total Reducing Sugar Content (g/100 g)[b]
Fresh	0	0.2020
0.11 A	28	0.1532
0.11 N	28	0.1406
0.23 A	29	0.1364
0.23 N	29	0.0691
Control	28	0.2026

[a]37°C.

[b]Average of two determinations.

The reducing sugars, glucose and fructose, are not present in appreciable amounts in whole navy beans. Lee et al. (1970) indicate that the sucrose content of whole beans is 2.4%. The glucose and fructose are likely formed during the processing to instant bean powder. Counter (1969) measured the reducing sugar content of instant navy bean powder and found the content to be greater than the value reported here. Different extracting solvents were used in the two procedures. The 80% EtOH is a more specific solvent for sugars than hot boiling water and is also the solvent specified in the AOAC procedure for sugar extraction. The method employed by Counter (1969) involved reaction of the reducing sugars and other carbohydrates with phenol. The product which formed was measured colorimetrically. DuBois et al. (1956) reported that this method was sensitive but not specific for reducing sugars. The phenol, they reported, reacts with mono-, di- and oligosaccharides and some polysaccharides. The procedure used in this study was based on the oxidation of the reducing sugars with an alkaline solution of metal salts (ferricyanide), and the colorimetric estimation of the reduced metal (ferrocyanide). One advantage of this method over a copper salt reagent arises from the fact that the ferrocyanide is less susceptible to oxidation by dissolved oxygen, and therefore, less chance for error (Ting, 1956).

The greatest losses of reducing sugars occurred at the monolayer in nitrogen, where 66% was lost, while the sample stored in air showed a loss of 33%. The samples stored below the monolayer showed greater retention of reducing sugars, losing only 25% in air and 31% in nitrogen.

In samples held below the monolayer and at the monolayer, the greater amount of reducing sugar was lost when the samples were stored in a nitrogen environment.

Finding a greater loss of reducing sugars at the higher a_w agrees with the general concept that sugar destruction through reaction with ε-NH_2's and other components occurs when more water is present in the product to act as a solvent (Labuza et al., 1970; Lea and Hannan, 1949; and Lea and White, 1948).

Changes in the Browning Index

The Browning Index (BI) was checked for reliability as an index to changes in product color. Samples which had been stored for varying periods of time were assessed for their color score on the Agtron M-500-A and for their Browning Index. The results of this comparison are shown in Table 10 on the following page. From those results it is apparent that samples which had high blue mode reflectance readings (i.e., appeared whiter) also had low BI values.

Changes in the BI for the four storage treatments ranged from 4 BI units at the greatest to 1.2 for the least. These data are shown in Table 11 on the following page, and they indicate that the samples stored at the monolayer in nitrogen produced the greatest amount of water soluble browning pigment as measured by this method. The sample showing the next greatest amount of pigment formation was the product stored at an a_w below the monolayer in an air atmosphere.

Table 10. Analyses of instant navy bean powder for Agtron Color Score and Browning Index.

Sample	Agtron Blue Mode Relative Reflectance[a]	Browning Index
Retort Cooked 1970 Beans[b]	54.0	9.95
Retort Cooked 1970 Beans[c]	48.7	11.00
Retort Cooked 1970 Beans[d]	45.1	11.10

[a]Standardization disks 44-56.

[b]Prepared and stored 18 months 4°C.

[c]Prepared and stored -20°C, 12 months.

[d]Prepared for the study and stored at -20°C for 28 days.

Table 11. Browning Indices for fresh, control, and stored instant navy bean powder samples.

Sample	Browning Index[c,d]	
	Initial	Final
Fresh Powder	11.10	---
0.11 A[a]	11.10	13.00
0.11 N[a]	11.10	12.30
0.23 A[a]	11.10	12.60
0.23 N[a]	11.10	15.20
Control[b]	11.10	11.10

[a]Stored at 37°C, 28 days.

[b]Stored -20°C in N atmosphere for 28 days.

[c]$A_{390\ nm} \times 100$.

[d]Average of 5 replicates.

Changes in Protein Quality

Calculation of a PPDRI for each of the storage treatments was precluded due to the absence of one or more essential amino acids from some of the digest fractions. Alternatively, a comparison of the digestibility of each protein source (0.11 A and N, 0.23 A and N) was done. These data are presented in Table 12. The comparisons which appear there were made from the concentrations of these eight amino acids in the four fractions from the pepsin-pancreatin digests of the storage samples. These comparisons represent relative percentages of the amono acid content in the samples calculated from the amount in the control sample of instant navy bean powder, which had been subjected to the same digestion procedure. Values greater than 100 imply that there were greater amounts of a particular amino acid in that fraction than were present in the same fraction of the control. Upon gel filtration and acid hydrolysis the amino acid was found to have accumulated in different fractions than it had been found in the control, due to less than complete hydrolysis on digestion with the enzymes. Values less than 100 imply that there was less of this amino acid present than had been found in the control fraction. Since less had been released on digestion, less was found in certain fractions. Checks were made to assure that the shifts in amino acid distribution were relative and did not represent "synthesis" of "new" amino acid residues. A check of the absolute amounts of μmoles of an amino acids for each treatment also substantiated this fact.

Table 12. Comparison of the elution pattern for eight amino acids from pepsin-pancreatin digested, stored (37°C, 1 month) samples of instant navy bean powder.

Treatment	Fraction	Amino Acids[e]							
		LYS	HIS	ARG	THR	½CYS	MET	TYR	ø-Ala
0.11 A	FAA[a]	100.0[f]	83.7	45.5	146.0	91.1	61.4	98.2	94.4
	1-7[b]	24.7	13.6	64.7	55.5	NP[g]	NP	13.3	21.2
	8-12[c]	103.0	119.0	105.0	89.5	P[h]	27.0	128.0	121.0
	ND[d]	94.5	99.6	83.0	95.7	NP	51.6	84.0	103.0
0.11 N	FAA	103.0	71.2	92.5	91.7	127.0	85.6	87.7	108.0
	1-7	77.4	25.3	72.3	145.0	NP	70.5	76.2	62.1
	8-12	94.3	147.0	119.0	81.0	P[i]	192.0	143.0	163.0
	ND	100.0	95.2	83.3	72.5	NP	64.5	105.0	91.5
0.23 A	FAA	86.1	43.9	84.0	98.1	151.0	143.0	97.9	95.1
	1-7	110.0	35.8	76.5	30.9	NP	76.2	40.0	85.5
	8-12	114.0	136.0	129.0	163.0	P[i]	172.0	152.0	134.0
	ND	97.4	96.1	75.9	72.3	NP	104.0	109.0	112.0
0.23 N	FAA	83.8	44.5	90.8	77.1	84.7	50.2	75.0	81.7
	1-7	39.4	16.8	15.2	39.1	NP	43.9	17.9	19.2
	8-9	102.0	90.4	89.1	88.0	NP	153.0	152.0	135.0
	ND	174.0	162.0	132.0	160.0	NP	38.3	160.0	167.0

[a]Free amino acid fraction. [b]Polypeptide fraction, m wgt > 1500. [c]Peptide fraction, m wgt < 1500 and > 200. [d]Non-digested residue fraction. [e]Lysine, histidine, arginine, threonine, cysteine, methionine, tyrosine, and phenylalanine. [f]Relative percent compared to aa content in control. [g]Not present at the concentration of analysis. [h]AA present in sample but not the control. [i]Content 2 x that in the 0.11A fraction.

Careful attention was paid to the digestibility of the sample. The extent of digestion was similar to values in the literature, and there was no evidence of incomplete heat processing which would have inhibited digestibility as Riesen et al. (1947) had shown with soybean oil meal.

From a comparison of the distribution pattern of an amino acid between treatments and between fractions within one treatment, additional insight was gained into the deteriorative processes, which occurred in the samples. Such a comparison revealed that the lysine content in the free amino acid (FAA) fractions for samples stored below the monolayer a_w were the same as the content in the FAA of the fresh control. Although other data indicated that lipid oxidation had occurred in these samples, reaction between the products of lipid oxidation and the protein had not affected the availability of lysine in the FAA or peptide fractions (8-12). There were marked decreases in the lysine and histidine content of the polypeptide fraction (1-7). The lysine content for the 0.11 a_w samples stored in air was down 75% and it was down 25% for the 0.11 a_w sample stored in nitrogen. The decrease could be attributed to a direct destruction of the lysine. Such destruction could occur through reactions of carbonyls or free radicals from lipid oxidation and the ε-NH_2 group of lysine (Bujard et al., 1967; Block et al., 1946; Roubal, 1971; Clandinin et al., 1951; and Eldred and Rodney, 1945). Such products evidently are released or regenerated to the free amino group upon acid hydrolysis (Lea et al., 1958, 1960).

The product apparently was protected from oxidation by the nitrogen atmosphere. The contents of cysteine and methionine were greater in the polypeptide and FAA fractions for 0.11 N sample than in 0.11 A sample. Methionine was also lost as a consequence of sugar amine browning (Lea and Hannan, 1950b). The levels of methionine in the 0.23 N fractions were evidence of this type of destruction. The question certainly can be raised as to whether these were real losses or only an artifact of hydrolysis and analysis. Miller and Carpenter (1964) showed that losses of only 10% of cysteine and methionine occurred when proteins were hydrolyzed in acid in the presence of glucose. The destruction of cysteine under proper conditions produces dehydroalanine, which adds to the ε-NH_2 group of lysine,when they are in close proximity, to produce N-δ-(2 amino-2-carboxymethyl)ornithine(lysino alanine). This product is resistant to hydrolysis by acid, and its formation could also be responsible for losses of lysine (Ziegler et al., 1967; Bohak, 1964; and Bjarnason and Carpenter, 1970).

The amino acid profile for the samples stored at the monolayer suggested a different pattern of deterioration. The FAA patterns were decreased for all amino acids measured. Of particular note were the decreases in lysine and histidine. A survey of the other fractions for their content of these amino acids revealed that in the 0.23 A sample the lysine accumulated in the polypeptide fraction and peptide (8-12) fraction. A part of the products of sugar amine reaction, which decreased digestion of the protein, were apparently destroyed by acid hydrolysis with regeneration of the amino acid (Lea and Hannan, 1950b).

In the nitrogen atmosphere sample, the lysine content was reduced in the FAA and polypeptide fraction with accumulation in the non-digested residue (ND). These data implied that the protein from the 0.23N sample was less digestible than the other three samples. The patterns for distribution of the other amino acids (except methionine) exhibited similar distributions.

The data in these comparisons indicated that there was altered digestibility of the protein in the monolayer sample (0.23 A and N) as shown by this *in vitro* method of analysis. The reducing sugar data (Table 9) indicated greatest losses in the 0.23 N fraction. Lea *et al.* (1951) reported changes greater than the magnitude of changes reported here; however, their samples were purified protein and glucose model systems, and they were stored at higher relative humidities than were used in this study. Changes in samples of spray dried skim milk at 7.6% moisture were more rapid than those reported here (Henry and Kon, 1948 and Lea and White, 1948).

The patterns for all of these amino acids followed similar distributions exhibiting the same general magnitude of change. Exceptions to this generalization can be attributed to direct destruction of the amino acid or to being held in an indigestible complex, lowering the amount of amino acid released into a particular fraction. In some cases the atmosphere or higher moisture content lessened lipid oxidation and protected sensitive amino acids such as cysteine and methionine (0.23 A and 0.11 N treatments). Dugan and Rao (1972) reported greater losses of these critical amino acids which can largely be attributed to their

different experimental design. Their study was carried out in model systems with unsaturated lipids and/or aldehydes and protein. Their storage conditions were 22°C and 14% RH. The same argument would hold to explain the variance between the magnitude of these changes and the amino acid losses reported by Chio and Tappel (1969b). In their study, they used a purified protein and ethylarachidonate or malonaldehyde to study the effects of these compounds in oxidized model systems and on amino acid retention.

SUMMARY AND CONCLUSIONS

Chemical and physical indices of instant navy bean powder quality have indicated that the storage conditions (37°C in air or nitrogen at two different a_w's) afforded the opportunity for deteriorative processes to occur. Table 13 arrays the data for changes in all of the indices except those for the *in vitro* measures of protein quality and fluorescence, which are presented in Tables 12 and 4 respectively. The data presented here represent percentage changes from initial values for fresh instant navy bean powder. Depiction of these data in this manner facilitates comparison of the effects of storage conditions on the powder.

In all treatments, browning of the product occurred as is shown by the trends in the Agtron RR and the BI columns. A comparison of the two values for any one treatment indicates that all of the browning, which had occurred, was not attributable to water soluble products. There was a certain portion of the browning which resulted from lipid oxidation. Indirect evidence of such contributions were seen in changes in the U/S ratios, the losses in extractable phospholipid phosphorus, and the fluorescence data in Table 4. All of these are processes which were indicative of lipid oxidation, particularly of the NL fraction, which presumably contributed reactants which produced browning in these samples. There was a protection of the lipids by storage in a

Table 13. Percentage changes in physical and chemical quality indices for instant navy bean powder stored one month at 37°C.

Sample	Percent Changes in Indices							
	Soluble Protein	U/S			µg P in Total Lipid Extract	Reducing Sugars	Browning Index (BI)	Agtron RR[a] (Blue Mode)
		NL	PC	PE				
0.11 A	-8.8	-12.4	-3.8	-11.8	-8.1	-24.2	+17.0	-26.0
0.11 N	-10.6	-5.4	-0.9	-6.0	-4.7	-30.5	+10.8	-34.2
0.23 A	-16.7	-11.2	-9.5	-2.4	-10.7	-33.4	+13.5	-22.7
0.23 N	-7.5	-12.3	-1.9	-1.6	-3.7	-66.8	+36.9	-28.2

[a]Relative Reflectance, decreases in blue mode RR indicates browning.

nitrogen atmosphere (0.11 A indices vs. 0.11 N indices). Greater color changes were recorded in samples stored in nitrogen than those stored in air. Fishwick and Zmarlicki (1970) observed the same relationship in freeze-dried turkey at 37°C. The trends which were observed here in the BI data were also observed by Fishwick and Zmarlicki (1970) and Lea et al. (1958). The trends seen in the lipid data are compatible with those for products of related composition and type. Buchanan (1969a) showed similar losses in the extractability of phosphorus which could be prevented by the use of an antioxidant. Buttery et al. (1961a) changes in potato granule lipid content for samples stored in air at 24°C for 29 days, which were the same magnitude as the ones observed here. The losses were 10% in a P/S ratio ($C_{18:2} + C_{18:3}/C_{16:0} + C_{18:0}$) and increased with the length of time in storage up to four months. As in the case of these bean powder samples, a nitrogen environment partially protected the potato granule lipids from autoxidation. Walter et al. (1972) reported a similar magnitude of loss in a lipid component in stored sweet potato flakes. In their sweet potato flake study, hexane extracted flakes stored in air at ambient temperature for 41 days lost 8% of their carotene. This change was comparable to the magnitude of lipid losses in this study with the navy bean powder. Walter et al. (1972) also contended that the phospholipids (bound lipids) oxidized more slowly than the rate which would be theoretically expected from their degree of unsaturation. They theorized and supported with histological data a concept that the bound lipids were protected from oxidative deterioration by the carbohydrate matrix in which they

were held. It is possible that such micro-regions are impervious to oxygen penetration, therefore, preventing autoxidation. This argument is attractive as an explanation of the slight amount of oxidation which occurred in the bound lipids, particularly the phosphatidyl ethanolamine fraction. Miller et al. (1965) had slower rates of lipid oxidation in stored herring meal, however, the meal had been stored at 25°C which could account for their slower rate of oxidation.

The changes in reducing sugar content indicate that greater amounts of sugars were lost in samples stored at higher a_w's. These trends were similar to the ones reported by Labuza et al. (1970); Lea and Hannan (1950b); Danehy and Pigman (1953); and Karel and Labuza (1968).

Insolubilization of the protein was recorded in all storage treatments. The magnitude of these changes were comparable to the ones recorded by Counter (1969) and Fishwick and Zmarlicki (1970). The development of protein insolubility as the result of inter- and intramolecular cross-linking is common when browning has occurred (Ford and Sharrock, 1971).

The most consistent trends which appeared in these data were the changes in the monolayer samples and their altered protein digestibility, which was shown in Table 12. These samples developed oxidized neutral lipids, the greatest losses of reducing sugar, increases in browning index, and insolubilization of protein. They also had the greatest alterations in the protein digestibility with the greatest amounts of amino acid increases in the non-digested residue fractions and decreases in amino acids in the FAA fractions, particularly the amino acids lysine

and histidine. The 0.23 N treatment which had the greatest losses of neutral lipids also had the greatest destruction of reducing sugar for all treatments, which likely contributed to the greatest amount of lysine, histidine, and associated amino acids shifted to the non-digested residue fraction. This sample also had sizeable destruction of cysteine and methionine which was consistent with the data on browning of dry skim milk studied by Lea and White (1948), the stability of herring meal studied by Carpenter et al. (1962); and the studies of glucose protein browning by Clark and Tannanbaum (1970, 1974). The 0.23 A treatment which had less destruction of reducing sugars showed a different pattern of protein digestion. In this treatment lysine accumulated in the polypeptide fraction. There was also an accumulation cysteine and methionine in the FAA fraction.

From these data it appears that during one month storage at 37°C, changes occurred in the in vitro digestibility of the proteins in instant navy bean powder. These changes were particularly evident in the samples stored at a monolayer a_w. The consequence of such changes from a nutritional standpoint were minimal, and it is invalid to try to extend the implication of the changes measured here. They do not have an impact upon the mixed diet of which the product would be a part (Ford, 1962). These findings, however, do support the arguments of Melnick and Oser (1949). These data provide valuable insight into the consequences of storage browning and lipid oxidation on important essential amino acids and the digestibility of the protein. Certainly, losses of limiting amino acids such as cysteine and methionine during storage are critical

information related to changes in protein quality for any product (Bjarnason and Carpenter, 1970). Slower and less complete digestion of a protein is also detrimental to the utilization of the protein source, particularly if a protein source represents a disproportionate part of the diet (Boctor and Harper, 1968 and Ford, 1964). Pader et al. (1948) and Porter and Rolls (1971) indicated that such altered or slowed digestion would decrease absorption and prevent amino acid supplementation necessary for protein synthesis (Sgarbieri et al., 1973).

From recent studies it could be argued that the peptides which would remain after digestion of the stored product are not detrimental to the nutritional value. As early as 1936, Verzár and McDongall (Mathews, 1972) suggested that peptones are absorbed as rapidly from the small intestine as are the amino acids. Evidence is mounting (Smyth, 1972 and Mathews, 1972) to indicate that this hypothesis is true. However, even if some of the complexes or peptides left after hydrolysis of the browned samples are absorbed, there are serious doubts about their utilization by the organism (Sgarbieri et al., 1973 and Ford, 1964). It would be possible for these peptides to saturate the transport sites preventing amino acid absorption and thus decreased protein utilization (Porter and Rolls, 1971).

Numerous methods have been employed in this study to assess physical and chemical quality characteristics of instant navy bean powder during storage. Of these methods a few readily lend themselves to quality control or facile assessment of changes in a particular index. Other techniques were either too sophisticated or befrought by

physical and technical problems to permit ready adoption as quality control measures. The first class would include Agtron RR, P analysis of the TL extract, reducing sugar content, RFI, and soluble protein, if done with a different solvent. Correlations could be run between Agtron RR and reducing sugar, which if correlated to another parameter (i.e., days of off flavor and odor panel data or soluble protein), might provide reliable sensitive indices of changes occurring in the processed and stored product. Of the second class of methods the BI, which was originally proposed to measure scorch in dried milk (Choi et al., 1949), and soluble protein, as performed in this study, are befrought by physical clarification problems which can artificially elevate the results. The U/S ratio and in vitro digestibility studies were tedious and take a protracted period of time plus sophisticated equipment the operation of which is technically demanding. The data which these techniques provided were valuable in that it substantiated trends indicated by other tests. Neither of these techniques lends itself to quality control checks. Some modification of the in vitro digestibility technique which would make it a more routine analysis is suggested in the Proposed Research section of this dissertation.

Ultimately, the concern of this study is the consequences of the measured changes on the quality of stored instant bean powder. These data substantiate the taste panel data of Burr et al. (1969). They chemically confirm the changes which Burr reported. Their data showed air packed samples to be unstable. Their product browned appreciably in the same time period as did these instant navy bean powder samples.

Their taste panel data revealed off flavors at the end of one month for samples stored at 38°C in air. They indicated that packing the product in nitrogen with an antioxidant (BHT) at a maximum temperature of 22°C provided stability for up to one year from an organoliptic standpoint. From this navy bean powder study, it was concluded that a nitrogen atmosphere for product stored at 37°C did not provide complete protection from lipid oxidation and related deterioration. A maximum storage temperature of 20°C seems to be an appropriate recommendation since Burr et al. (1969) and Counter (1969) found no major enhancement in product shelf life by storage of the product at -23°C and 0°C, respectively.

The water activity, a_w, of the product had a pronounced effect on the deteriorative process which the product underwent. Below the monolayer at 0.11 a_w and 4.0% moisture the product underwent lipid oxidation and browning with destruction of lysine, histidine, cysteine, and methionine. In the monolayer, a_w environment (0.23 a_w and 5.23% moisture) sugar amine browning, protein insolubilization, neutral lipid oxidation, discoloration and lessened protein digestibility predominated. It is the concluding recommendation of this study that instant navy bean powder be processed to a final moisture content of 4.0% and packaged with an antioxidant (Burr et al., 1969) in an inert atmosphere. The storage temperature for the packaged product should be maintained at 20°C or below without refrigeration. These recommendations are consistent with the data from this study and the findings of Counter (1969) and the taste panel work of Burr et al. (1969).

RECOMMENDATIONS

Numerous facets of this study hold promise as fertile ground for future exploration and study:

1. Esoterically, it would be interesting to isolate and characterize the browning products from stored bean powder. Clark and Tannenbaum (1974) reported the occurrence of limit pigments of 1500 m wgt in browned model systems. Further, separation and analysis of the 1-7 fraction in the digests from stored product might indicate the presence of the limit pigments.

2. It would also be a valuable search to attempt to isolate compounds such as lysinoalanine (Bjarnason and Carpenter, 1970 and Bohak, 1964) from browned stored bean powder. Isolation of such species would assist in the documentation and elucidation of the role of lipid oxidation products and sugar amine browning in foods.

3. Extended storage studies combined with organoleptic analyses and the appraisal of the role of antioxidants on stability of the powder would provide more complete documentation of the deterioration of stored bean powder. Such a study should provide more exact criteria for judging when physical and chemical indices have changed to the extent that the product would be rejected by the consumer.

4. Correlate indices of change with the critical amino acids in the FAA fraction to monitor changes in protein digestibility.

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