Nutritional Value of Proteins in Powdered Infant Formula: In Vitro and in Vivo Methods

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The use of in vitro methods to predict the nutritive value of the protein in powdered soy-based and milk-based infant formulas was investigated. A comparison of the in vitro digestibility, as measured by a single pH equation, and the in vivo rat digestibility of several infant formulas indicated that the in vitro method underestimates the digestibility of formulas. Some humidity-related protein nutritional damage of both soy-based and milk-based formulas stored for 1 month was predicted by enzymatic apparent digestibility, dye-bound lysine, and relative digestion rate methods. The results obtained using the in vitro methods showed qualitative similarities in some cases; however, agreement among the in vitro assays was generally poor. The changes in dye-bound lysine and browning activity of the stored milk-based and soy-based formulas did not reflect changes in relative protein efficiency ratios measured by the in vivo method. The data suggest a lack of accuracy of the in vitro methods as predictors of protein nutritional quality for the rat.

Chemical changes can occur during storage and processing of protein foods because of the unique chemical and physical properties of the food. Some changes may be beneficial, while others may have a detrimental effect on nutritional quality. Many chemical reactions reduce the protein nutritive value of a food (Dworschak, 1980; Carpenter, 1981).

Animal bioassays are the most commonly used methods for measuring the nutritive value of protein foods. However, in recent years there has been considerable interest in developing rapid, inexpensive in vitro methods that may be used as alternative approaches (Altschul, 1981). The in vitro methods are generally single or combined measures or predictors of the amounts of essential amino acids in protein, the digestibility of the protein food, and/or the bioavailability of the amino acids, factors that are among the main determinants of the protein quality of a food. One of the main concerns about current in vitro methods is their adequacy as predictors of protein nutritive value. Information on the precision, reproducibility, and accuracy of existing in vitro methods is limited and incomplete.

Satterlee et al. (1981) indicated that one of the difficulties that has arisen in the development of in vitro methods to measure protein nutritive value is that a method may work well for one class of foods and poorly for others. Two factors that may limit an assay's ability to predict the protein nutritive value of a food are the limited data base used to develop the assay and the unique physical and chemical properties of the food (Satterlee et al., 1981).

The objectives of this study were to expand the data base on the use of in vitro methods and to test the applicability of selected in vitro methods for determining the digestibility and reactive lysine of complex food products such as infant formulas.

EXPERIMENTAL SECTION

Sample Preparation. Commercially available milk-based and soy-based powdered infant formulas were obtained from a retail store in Washington, DC. The expiration dates on the formulas were generally 2–3 years from the purchase date. The contents of an appropriate number of cans of each formula of the same lot were mixed before use. The formulas used as test samples were as follows: (1) milk-based I, nonfat milk, reduced minerals, whey, lactose, oleo, coconut, soy, and oleic oils, lecithin; (2) milk-based II, nonfat milk, lactose, coconut and corn oils; (3) milk-based III, nonfat milk, lactose, soy and coconut oils; (4) soy-based I, soy protein isolate, corn syrup solids, coconut and corn oils; (5) soy-based II, soy protein isolate, soy oil, lecithin, sucrose, tapioca dextrin.

Storage test samples were prepared by using a static method. Three of the formulas mentioned above were randomly selected for the first storage test. Weighed amounts of each test sample (milk-based I and II and soy-based I) were placed in open containers over sulfuric acid–water solutions that created relative humidities (RH) of 20, 40, 60, or 80% in closed containers at 25 °C. The test portions were maintained under these conditions for at least 1 month. The test portions were weighed to determine the increases in moisture at various time intervals. At the end of the test period, moisture was determined on each sample according to the AOAC (1984) method.

The stored test portions of milk-based II and soy-based I formulas used in the rat assay to determine protein efficiency ratios (PERs) were subjected to the same experimental storage conditions as previously described except that the test portions were stored over a 27% sulfuric acid–water solution. Because of the large volume of product and equipment required for storage, it was difficult to maintain a specified humidity. Therefore, the moisture content of each test portion was determined.

In Vitro Tests. A multienzymatic technique was used to estimate protein digestibility (Satterlee et al., 1982). The pancreatic enzymes trypsin, L-chymotrypsin, and protease and the intestinal enzyme leucine amino peptidase were purchased from Sigma Chemical Co. (St. Louis, MO). A single pH equation was used to calculate the digestibility. An Orion 601A recording pH meter was used to measure the decrease in pH of the enzyme–protein substrate mix during the 20-min assay period. Milk-based I and II and soy-based I and II formulas were tested along with portions of the stored test samples milk-based I and II and soy-based I.

The relative digestion rate (RDR) was calculated from the pH decrease experiment cited above for the determination of apparent digestibility. The basis of the calculation of the RDR was described by Hung et al. (1984). RDR is defined as t(pH 7.3) untreated test sample/t(pH 7.3) corresponding stored sample, where t = the time re-

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quired for the untreated test sample or the corresponding stored test sample to reach pH 7.3 in the pH drop experiment.

Dye-bound lysine (DBL) and the dye-binding capacity (DBC) of the formulas were measured by the alternative procedure of Hurrell et al. (1979), using standard laboratory equipment and an acid azo dye. The mechanism of the dye–protein binding reaction is complex and not fully understood; however, the simplified hypothesis is that the binding of the dye is primarily associated with the basic amino acids lysine, arginine, and histidine. DBC of the basic groups in the proteins of the infant formulas for acid Orange 12 (85% purity) was measured before (A readings) and after (B readings) reaction with propionic anhydride, a blocker of the lysine groups. The difference between the A and B readings represents reactive lysine. Browning of the infant formula test portions was determined by using the modified method of Choi (Labuza and Saltmarch, 1981).

In Vivo Tests. All diets used in the in vivo studies were formulated to contain 10% protein (Mitchell and Jenkins, 1985). ANRC casein was used as the protein source in the control diets. Infant formula samples made up approximately 50–80% of the diets containing the formulas and contributed 16–24% fat and 30–42% carbohydrate. Therefore, matched ANRC casein control diets were formulated to contain the same fat and carbohydrate source and level contained in a corresponding infant formula diet. Control diets corresponding to the stored infant formula diets were also formulated to contain matched amounts of moisture.

To determine rat in vivo apparent digestibility, male weanling Sprague–Dawley (Harlan Sprague–Dawley, Madison, WI) rats, 27 days old and weighing 44–55 g, were housed in individual stainless-steel cages with suspended bottoms in a room maintained at 22 ± 2 °C, 50 ± 10% RH, with alternating 12-h periods of light and dark.

The rats were fed a commercial rat chow for 2 days. They were then weighed and the animals at the extremes of the weight distribution curve were discarded. The rats were allocated to groups (10 rats per group; average weight per group, 61.2 g), and the groups were randomly assigned the experimental infant formula diets milk-based I and II and soy-based I and II. Food and water were given ad libitum. On days 14–18, feces were collected and nitrogen intake was recorded. Five pooled fecal collections (rats were randomly paired) per formula were dried in an air oven and analyzed for nitrogen by the Kjeldahl method (AOAC, 1984). Apparent digestibility was calculated as [(nitrogen ingested − total fecal nitrogen)/nitrogen ingested] × 100.

To study the effect of storage on protein quality, PERs of stored and untreated milk-based and soy-based powdered formulas were determined. Sprague–Dawley weanling rats (35–49 g) were maintained under the experimental conditions described above with the following exceptions: (1) the average weight of each group of rats (five rats per group) assigned to the test diets was 58.0 g; (2) no feces were collected; (3) rats were fed their designated diets and water ad libitum for 28 days; (4) animals were weighed weekly, and weight gain and food consumption were recorded. At the end of the study, PERs (g of weight gain/g of protein consumed) were calculated.

RESULTS AND DISCUSSION

Apparent Digestibility. The apparent digestibility values of four powdered infant formulas are shown in Table I. The protein digestibility values for the formulas ranged from 85 to 94%. The in vitro assay tended to underestimate the digestibility of the soy-based formulas when compared with the in vivo values, 88–89 and 91–94%, respectively. The in vivo apparent digestibility values for milk-based formulas I and II were 86 and 85%, respectively, and the in vitro values were 88 and 85%. However, it is well-known that the amounts of lactose in the milk-based infant formula diets exceed the rats digestive and absorptive capacity, resulting in increased fecal excretion of nitrogen. Unpublished results from this laboratory indicate that the apparent digestibility (96%) of the milk protein casein is reduced approximately 11% when an amount of lactose equivalent to that found in the infant formula diets is added to the diet. Based on this, the in vitro method also underestimates the digestibility of the milk-based formulas. In addition, some of the differences found using the two methods support the suggestion made by others (Marshall et al., 1979; Bodwell et al., 1980; Satterlee et al., 1981) that food class may affect the rate of digestion of proteins in in vitro assays. Satterlee et al. (1981) reported that in vitro assays predict apparent digestibility values moderately well in all foods. However, in vitro assays could estimate the digestibility of a protein in humans or rats much more accurately if specific equations, related to food class, were used.

Even though there were some numerical differences in the digestibility values of the formulas, the data indicate that the digestibilities are quite high from a nutritional standpoint regardless of the method used. The coefficients of variation (CV) of the in vivo digestibility values of the various formulas were consistent, 1.2–1.9%. The CVs for in vitro values ranged from 0.5 to 3.0%. There did not appear to be a clear indication of an effect of formula class (soy vs. milk) on variation. In a collaborative study (Satterlee et al., 1982), the range of CVs when six food products were tested using an in vitro digestibility technique was 1.4–2.4%.

Application of Dye-Binding Procedure. Most dye-binding procedures that have been used to evaluate protein quality originated from a method devised by Frankel-Conrat and Cooper for the determination of basic groups in proteins (Lakin, 1973). The DBC can be closely related to the total basic amino acids (Lawrence et al., 1970; Hurrell and Carpenter, 1975; Hurrell et al., 1979). The DBL values ranged from 5.9 to 7.7 g per 16 g of nitrogen (Table II). With the exception of the DBL value for milk-based I formula, the values compared favorably with

Table I. In Vivo and in Vitro Measures of the Apparent Digestibility of Infant Formulas

formula	in vivo,[a] %	CV,[b] %	in vitro,[c] %	CV, %
milk-based I	86	1.8	88	0.5
milk-based II	85	1.2	85	3.0
soy-based I	94	1.5	88	1.6
soy-based II	91	1.9	89	0.8

[a] Rats were randomly paired to provide five pooled fecal collections from 10 rats. [b] Coefficient of variation. [c] Triplicate test portions per formula.

Table II. Dye-Binding Values of the Infant Formulas

formula	DBL,[a] g/16 g of N	total lysine,[b] g/16 g of N
milk-based I	6.9 (46.9)[c]	9.9
milk-based II	6.8 (46.8)	7.5
milk-based III	7.7 (52.7)	8.2
soy-based I	5.9 (40.1)	5.4
soy-based II	6.2 (42.4)	6.3

[a] DBL = dye-bound lysine. [b] Manufacturers' values. [c] Millimoles per 16 g of nitrogen in parentheses.

Table III. Changes in Dye-Bound Lysine and Digestibility with Storage[a]

formula	humidity, %	moisture, %	DBL,[b] g/16 g of N	app digestibility,[c] % no treatment	rel digestibility rate[d]
milk-based I	no treatment	1.7	6.9 ± 0.19[a]		1.00
	20	3.4	6.2 ± 0.04[c]	98	1.00
	40	3.4	6.7 ± 0.29[a,b]	98	1.00
	60	5.8	6.3 ± 0.9[b,c]	98	1.00
	80	7.2	7.0 ± 0.14[a]	99	1.00
milk-based II	no treatment	1.8	6.8 ± 0.21[a]		1.00
	20	3.6	6.3 ± 0.10[b]	98	0.67[b]
	40	5.4	6.1 ± 0.03[b]	98	0.50
	60	6.1	6.1 ± 0.13[b]	95	0.13
	80	8.1	6.2 ± 0.2[b]	95	0.18
soy-based I	no treatment	3.1	5.9 ± 0.81[a]		1.00
	20	5.3	5.7 ± 0.18[a]	103	1.00
	40	6.8	5.5 ± 0.40[a]	103	1.00
	60	10.0	6.2 ± 0.0[a]	102	1.00
	80	15.7	4.0 ± 0.25[b]	91	0.33

[a] Stored 1 month at 25 °C. [b] DBL = dye-bound lysine. Mean ± SD. For each formula, means with the same superscript are not significantly different ($P \leq 0.05$). Significance was determined by the Duncan's multiple-range test (Steele and Torrie, 1960). [c] In vitro digestibility. [d] Values were calculated according to Hung et al. (1984).

lysine values reported by the manufacturers of the selected infant formulas. The reason for the large difference between the in vivo and in vitro values of milk-based I formula is unclear.

Measurement of Storage Damage. Humidity conditions during storage affect chemical reactions that may occur in foods, especially between reducing sugars and susceptible amino acids such as lysine (Ben-Gera and Zimmerman, 1972). Three in vitro methods were examined to determine their sensitivity in detecting humidity-related nutritional damage that can occur in stored foods. Table III summarizes the results obtained with infant formulas. During storage, the moisture content of the formulas increased with increasing RH. The largest increase occurred with the soy-based formula. The changes in the in vitro DBL values, digestibility, and RDRs varied among the infant formulas and were not related to changes in the moisture content. The largest reductions in DBL values for the milk-based I formula were at 20 and 60% RH. The DBL values of milk-based II were significantly decreased at all humidities. The DBL value for the soy-based formula was significantly lower at 80% RH. Factors that may contribute to the differences among the formulas are protein source and other ingredients in the formulation.

Saltmarch and Labuza (1980) suggested that the physicochemical state of the sugar lactose might affect the rate of chemical reactions (Maillard reaction) known to affect quality. Their data indicated that the maximum reaction rate at low water activity for whey powder is related to the translation of lactose from the amorphous to the crystalline form. The crystallization of amorphous lactose has been associated with discontinuities in the isotherms of milk products. Data from the present study (not shown) indicated that the discontinuity in the isotherm for the two milk (lactose-containing) products occurred between 40 and 60% RH. Reductions in DBL values for the two milk-based formulas were not always largest in this humidity range. The isotherm for the soy-based formula did not show discontinuity. The soy-based formula contained corn syrup solids as the carbohydrate source.

The in vitro apparent digestibility values of the formulas generally remained high regardless of the storage conditions. The largest reduction in apparent digestibility was with the soy-based formula, at 80% RH. One of the factors affecting the value of the soy-based formula stored at 80% RH was a decreased solubility of the formula.

The effect of storage on the RDRs also varied among the infant formulas and with humidity (Table III). Storage of formulas at 80% RH caused a significant reduction in RDRs of milk-based II, 0.18, and soy-based formula, 0.33. As the moisture content of milk-based II increased, the rate of digestion decreased. No changes occurred in the RDR value of milk-based I with increasing moisture content. The enzymatic digestion data may indicate possible structural changes in the protein components of milk-based I. All of the methods were able to identify some change due to the storage conditions; however, in some cases the apparent digestibility and DBL and RDR values showed no relationship to each other. The RDR of milk-based II (Table III) changed from 0.67 to 0.13, and apparent digestibility only varied from 98 to 95.

Table IV. Influence of Storage on Protein Quality Measures and Browning

formula	moisture, %	PER[a]	DBL,[b] g/16 g of N	incr browning[c]
milk-based II (untreated)	1.1	93 ± 8.7	6.81 ± 0.04	
milk-based II (stored)	3.6	97 ± 10.1	6.07 ± 0.0[d]	222 ± 48.2
soy-based I (untreated)	2.1	80 ± 7.2	5.75 ± 0.07	
soy-based I (stored)	12.1	72 ± 10.7	5.50 ± 0.0[d]	18 ± 5.9

[a] Mean ± SD; five rats per group. PERs were calculated as percent of each matched casein control. [b] DBL = dye-bound lysine; duplicate determinations. [c] Percent of untreated formula. [d] Significantly different from untreated milk-based formula ($P \leq 0.05$).

Comparison of in Vitro and in Vivo Methods. The diet containing the stored milk-based II formula caused a significant reduction in the weight gain and food intake (percent of control) of the test animals (data not shown). These changes compared favorably with the in vitro value for browning, which indicated a 222% increase in the browning activity of the stored formula and a significant reduction in DBL value (Table IV). However, there were no significant differences in the PER (percent of control) of the stored and untreated milk-based II formula, even though the browning activity had increased and the DBL (89% of control) decreased in the stored formula (Table IV). This was probably due to the fact that the PER method measures the first limiting amino acid, and the damage to the protein caused by storage was not severe enough to cause other essential amino acids such as lysine to become first limiting, even though the levels may have been reduced.

The in vivo measures of protein quality indicated that storage had no significant effect on soy-based I formula. The in vitro measures indicated smaller changes in DBL and browning activity than were seen with the milk-based formula. Several factors may have contributed to this finding: (1) the storage conditions chosen did not give a maximum reaction; (2) corn syrup solids served as the carbohydrate source instead of the more reactive simple sugars; (3) the methods used were not sensitive enough to detect any difference.

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Identification and Quantitative Estimation of Oxidized Cholesterol Derivatives in Heated Tallow

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Oxidized cholesterol derivatives (OCDs) in tallow were isolated by saponification overnight at room temperature and quantified by a newly developed capillary gas chromatography method that includes trimethylsilylation. 7-Ketocholesterol and cholesterol α-epoxide added to tallow survived saponification as well as the other OCDs tested, displaying recoveries of at least 95%. Continuous heating of tallow at 155 °C resulted in the formation of at least four OCDs as detected by capillary GC. OCDs were identified by capillary GC–mass spectrometry as 7α-hydroxy-, 7β-hydroxy-, and 7-ketocholesterol and α-epoxide. 7-Ketocholesterol was the predominant species formed. Its net formation was proportional to heating time, reaching up to ca. 10% of the initial content of cholesterol (in unheated tallow) after 376 h of heating. Capillary GC–MS also revealed the apparent formation of cholesterol β-epoxide in heated tallow.

Deep fat frying is a common method of food preparation in restaurants and in processing plants. During frying, the heating medium may experience abusive conditions due to repeated exposure to oxygen at elevated temperatures. The changes occurring in heated oils have been studied quite thoroughly with respect to fatty acids. The thermal oxidation and polymerization of fatty acids, including toxicological implications and loss of nutritional value have been reported (Kaunitz, 1967; Perkins, 1967; Chang et al., 1978; Thompson and Aust, 1983). However, concerning the changes induced in sterols, minor components of frying oils, only limited studies have been made (Larsen and Morris, 1943; Ryan et al., 1981).

In light of the potential deleterious impact on human health of cholesterol oxides, including interruption of sterol metabolism, cytotoxicity, atherogenicity, mutagenicity, and carcinogenicity (Bischoff, 1969; Black and Douglas, 1972; Kandutsch et al., 1978; Peng et al., 1978, 1979, 1982; Smith et al., 1979; Imai et al., 1976, 1980; Chan and Chan, 1980; Ansari et al., 1982; Addis et al., 1983), there is an urgent need to investigate thermally induced changes in sterols of fats and oils.

The present study was initiated to investigate whether cholesterol in tallow will undergo oxidation when it is heated at elevated temperatures similar to deep fat frying and specifically concerned the disappearance of cholesterol and appearance of oxidized cholesterol derivatives (OCDs). Findings were confirmed by combined capillary GC–mass

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