Table III. Comparison of Total Ascorbic Acid Determination by Derivative Polarography and 2,4-DNPH Analysis

Product	Total Ascorbic Acid (Mg. %) Polarographic	2,4-DNPH
String beans (canned)	1.9	2.3
Corn (canned)	3.1	3.6
Whole fresh milk	1.5	1.5

polarogram obtained on a product estimated by the 2,4-DNPH method to contain less than 3 mg.% total ascorbic acid. The conventional polarogram (Figure 4 top) shows that the ascorbic wave is not easily defined under such conditions but rather is diffused into the wave of the supporting electrolyte. A derivative polarogram (Figure 4 bottom) for this same sample produces a maximum identical with ascorbic acid at a voltage close to the wave of the supporting electrolyte. Total ascorbic acid values obtained from the derivative curves are presented in Table III. These values agree with those determined by 2,4-DNPH analyses. Thus, the derivative technique provides means of extending the sensitivity of the present method to concentrations of total ascorbic acid which otherwise cannot be determined in the conventional manner.

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Literature Cited

(1) Assoc. of Vitamin Chemist, "Methods of Vitamin Assay," p. 76, Interscience, New York, 1951.
(2) *Ibid.*, p. 93.
(3) Blattna, J., Franger, J., Sanda, V., Tuman, P., *Ref. Zh. Khim.* **4,** 402 (1953).
(4) Delahay, P., *Anal. Chim. Acta* **1,** 19 (1947).
(5) Friedmann, E. D., Marrian, Simon, Reuss I., *Biochim. Biophys. Acta* **9,** 61 (1952).
(6) Gillham, W. S., *Ind. Eng. Chem. Anal. Ed.* **17,** 217 (1945).
(7) Hughes, R., *Biochem. J.* **64,** 203 (1956).
(8) Kolthoff, I. M., Lingane, J. J., "Polarography," p. 331, Interscience, New York, 1952.
(9) Krauze, S., Bosyk, Z., *Mitt. Gebiete Lebensm. u. Hyg.* **50,** 228 (1959).
(10) Müller, O. H., "The Polarographic Method of Analysis," p. 88, Mack Printing Co., Easton, Pa., 1941.
(11) Ono, S., Takagi, M., Wasa, T., *Bull. Chem. Soc. Japan* **31,** 356 (1958).
(12) Page, J. C., Waller, J. B., *Analyst* **71,** 65 (1946).
(13) Räken, K. O., *Z. Anal. Chem.* **173,** 1024 (1960).
(14) Reiss, R. J., *J. Sci. Food Agr.* **10,** ii, 189 (1959).
(15) Roe, J. H., Kuether, C. A., *J. Biol. Chem.* **147,** 399 (1948).
(16) Roe, J. H., Mills, M. B., Oesterling, M. J., Damron, C. M., *J. Biol. Chem.* **174,** 201 (1948).

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PROTEIN ISOLATION

Extraction and Precipitation of Nitrogenous Constituents of Dry Navy Beans *(Phaseolus vulgaris)*

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Methods of extraction and precipitation of proteins from dried beans were investigated. Maximum levels of nitrogen were extracted with HCl at pH 1.5, with NaOH at pH values above 7.0, or by sodium chloride solutions of 1 to 8%. Minimum levels were extracted in acid solutions of pH 3.8. Protein extracted at pH 1.5 did not precipitate well at pH 3.8, but all except 27% of the nitrogen was precipitated when the pH 7.0 extract was adjusted to pH 3.8. Protein precipitated at pH 3.8 apparently was contaminated by nonprotein material, as shown by its low nitrogen content of 11.09%. Protein extracted with 2% sodium chloride solution and precipitated by dialysis contained 14.63% nitrogen and appeared to be the most satisfactory for further study. It consisted of at least four proteins separable by filter paper electrophoresis or chromatography on DEAE-cellulose columns.

Ritthausen (*16*) and Osborne (*13*) used sodium chloride solutions to extract protein from dried beans, and part of the extracted protein was precipitated by dilution or by saturation of the solution with ammonium sulfate. Later, Waterman, Johns, and Jones (*22*) separated the sodium chloride extracted proteins by fractional precipitation with ammonium sulfate into three proteins—phaselin, phaseolin, and conphaseolin.

With the development of newer methods of protein separation, such as zone electrophoresis and chromatography on substituted cellulose columns, the need arises for a thorough study of the extraction and precipitation of the proteins of beans, such as has been done for the proteins of flaxseed (*14, 18*) peanuts (*3, 7, 10*), cottonseed (*8*), soybeans (*4, 17*), and peas (*6*). Stoikoff and Sweschtarowa-Dinewa (*21*), Smith, Earle, and Wolff (*20*), and Powrie (*15*) have conducted limited studies of solubility characteristics of the proteins of dried beans. The present investigation was made to determine suitable ways to extract the protein from dried Navy beans (*Phaseolus vulgaris*) and to precipitate the extracted protein for further study and isolation of the individual proteins.

Experimental

Navy beans of the Michelite variety were ground (1-mm. mesh) in a Wiley mill. Triplicate 2.0-gram portions of the bean flour were extracted in one experiment by the procedure of Lund and Sandstrom (*11*), which separated the protein into water-soluble, potassium

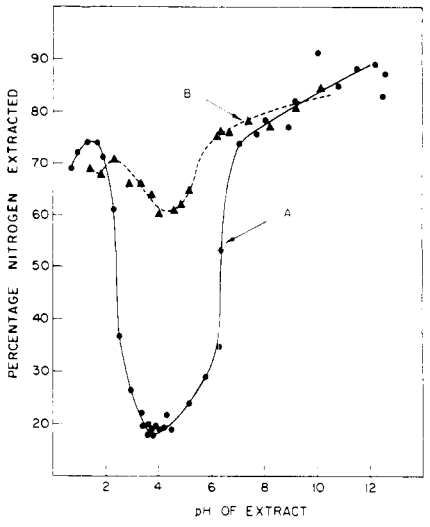

Figure 1. Percentage of total nitrogen extracted from dry beans by (A) hydrochloric acid and sodium hydroxide solutions or (B) 2% sodium chloride solution with pH adjusted with hydrochloric acid and sodium hydroxide

Table I. Extraction of Bean Proteins by Different Salt Solutions

Salt	0.25N		0.50N		1.00N		2.00N	
	pH[a]	%[b]	pH	%	pH	%	pH	%
$CuCl_2$	3.02	62.5	2.88	61.0	2.61	52.6	2.38	59.0
$CuSO_4$	3.30	50.7	3.19	56.2	3.14	52.1	3.13	55.2
$BaCl_2$	4.97	62.3	4.86	63.0	4.82	60.6	4.57	64.4
$CaCl_2$	4.93	61.8	4.83	64.6	4.73	67.0	4.62	58.7
NaCl	5.95	75.1	5.87	75.9	5.79	72.3	5.37	72.7
KCl	5.98	73.9	5.88	75.6	5.82	74.5	5.79	73.1
Na_2SO_4	5.95	74.4	5.92	78.2	5.84	74.1	5.76	52.1
$NaNO_2$	6.18	25.2	6.31	15.6	6.41	13.9	6.80	4.4
$Ca(C_2H_3O_2)_2$	6.22	69.8	6.08	69.7	6.55	61.9	6.97	61.3
$NaHCO_3$	8.31	85.6	8.32	85.5	8.13	76.3	..	..

[a] pH of extract. [b] % of total nitrogen extracted.

Table II. Properties of Different Bean Protein Preparations

Protein Preparation	%N	Electrophoresis[a] Main band	Electrophoresis[a] Others	Chromatography[b] Main peak	Chromatography[b] Others
Extd. pH 7.0, sol. pH 3.8	6.0	−	+, 0	10	37, 55, 60
Extd. pH 7.0, pptd. pH 3.8	11.1	+	0	60	10, 45, 70
Extd. 2% NaCl, sol. dialysis	10.1	−	+, 0	10	45, 55, 60, 70
Extd. 2% NaCl, pptd. dialysis	14.6	+	0, −	60	10, 45, 50, 55, 70
Conphaseolin		0			10, 50, 60
Phaseolin		+	0, −	60	10, 40, 70

[a] Band designations: 0 = did not migrate, − = migrated toward cathode, + = migrated toward anode.
[b] Numbers refer to tube at highest point of peak of protein eluted.

chloride solution-soluble, ethyl alcohol–soluble, potassium hydroxide–soluble, and residual protein fractions. Because very little protein was extracted by hot alcohol, this step was omitted in later extractions so that a high speed Servall centrifuge and cellulose nitrate tubes could be used.

For other extraction studies, 4.0-gram samples of the ground dry beans were weighed into 250-ml. Erlenmeyer flasks or into 250-ml. centrifuge bottles. One hundred milliliters of the extracting agent were added, and the mixture was shaken mechanically for 1 hour and then centrifuged at 1400 r.p.m. Twenty-five-milliliter aliquots of the supernatant liquid were transferred to Kjeldahl flasks, and nitrogen determinations made by the Kjeldahl-Wilfarth-Gunning method (*1*). The pH values were determined with the Beckman Model G pH meter on aliquots of the centrifuged solutions. Single extractions were carried out with each solvent since this appeared satisfactory for comparative purposes, but the extractions were replicated two or three times to obtain extraction and precipitation data presented in Figures 1 and 2.

Electrophoretic separations of protein fractions were made on Whatman No. 3 MM filter paper or cellulose acetate strips in a Spinco Model R electrophoresis cell using a diethylbarbiturate buffer of pH 8.6. Chromatographic separations were made on columns of DEAE-cellulose using a sodium chloride gradient elution system. The column was eluted with 0.01*M* phosphate buffer of pH 7.6 until the first protein peak was eluted, then a gradient was applied by allowing the buffer containing 0.3*M* sodium chloride to replace buffer flowing from the reservoir.

Conphaseolin was isolated from the 2% sodium chloride extract of dried beans by precipitation from a 35% saturated solution of ammonium sulfate (*22*). Phaseolin was precipitated between 55 and 80% of saturation with ammonium sulfate.

Results and Discussion

Fractional Extraction of Bean Proteins. The dried beans contained 23.1% crude protein (N × 6.25). Distilled water extracted 70.7% of the total nitrogen from finely ground dried bean seeds in the procedure of Lund and Sandstrom (*11*), and 5% potassium chloride solution extracted an additional 9.4%. Eight per cent of the nitrogen was not extracted from the beans, and 11.9% was soluble in alkali. Approximately 80% of the nitrogen was extracted with neutral solvents. According to Osborne (*13*), 8.5% of bean protein is albumin, and 63% is globulin. Therefore, water extracted part of the bean globulins, as well as the albumins and the nonprotein nitrogen, which according to Powrie (*15*) comprises 10.4% of the total nitrogen of the beans.

Extraction of Bean Proteins by Salt Solutions. The effect of various concentrations of sodium chloride on the extraction of protein from ground dried beans was investigated. Salt concentrations between 1 and 8% (0.19 to 1.52*M*) extracted similar amounts of nitrogen (74 to 76%).

The nature of the salt used had more effect on the percentage of nitrogen extracted from dried beans than did the salt concentration (Table I). Ten different salts were studied at normalities between 0.25 and 2.0. As the salt concentration increased, the pH decreased except for sodium nitrite, calcium acetate, and sodium bicarbonate. The 1.0*N* salt solutions had pH values between 2.61 for copper sulfate and 8.13 for sodium bicarbonate, and except for calcium acetate and sodium nitrite, salt solutions with the higher pH values extracted more nitrogen.

To study whether the difference in amount of nitrogen extracted by different salts was due to pH, beans were extracted with 2% solutions of sodium chloride containing different amounts of hydrochloric acid or sodium hydroxide. The pH of the extracts ranged from 1.4 to 10.2 (Figure 1). The least nitrogen was extracted at pH 4.0 (60%) and, as acidity increased or decreased, the amount of nitrogen extracted increased until at pH 10.2, 85% of the total nitrogen was extracted, and at pH 2.3, 72% was extracted.

Effect of pH of Suspension on Extraction. Extractions were made with solutions of various concentrations of hydrochloric acid and sodium hydroxide. Final pH values ranged from 0.7 to 12.6 (Figure 1). The least nitrogen was extracted from beans at pH 3.8, where 18% of the total nitrogen was extracted.

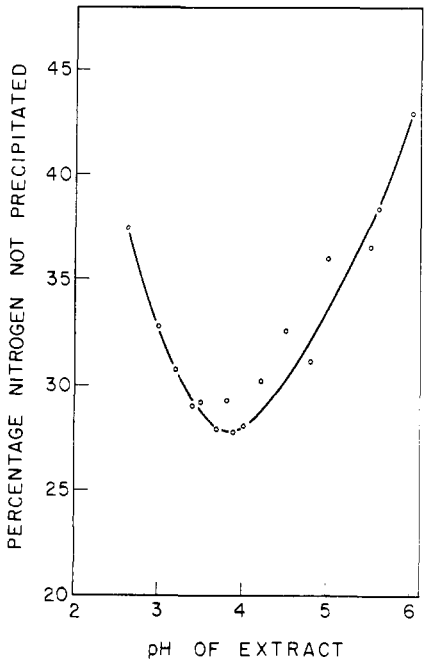

Figure 2. Percentage of total nitrogen not precipitated by hydrochloric acid solution after extraction of dry beans with water at pH 7.0

According to data of Powrie (*15*), only about 8% of this would have been protein nitrogen, an amount very near the 8.5% that Osborne (*13*) obtained as albumin. Proteins extracted at pH 3.8 are probably bean albumins because they gave similar paper electrophoresis and DEAE-cellulose column chromatographic patterns. More protein was extracted by solutions that were either more acidic or more alkaline than pH 3.8. One point of maximum extraction was at pH 1.3 to 1.7 where 74% of the nitrogen was taken into solution. A similar percentage of nitrogen was extracted at pH 7.1, and the percentage increased to 89% at pH 12.2.

Minimum extraction occurred at pH 3.8 no matter what acid was used to adjust the acidity, but different acids extracted differing levels of protein at higher acidities. Sulfuric acid did not show a point of maximum extraction as hydrochloric acid did, but it extracted nearly 70% of the nitrogen at pH 1.0, differing in that regard from the way it extracted the nitrogen from dry peas (*6*). Trichloroacetic acid extracted a maximum of 40% of the nitrogen at pH 1.3 and a minimum of 16% at pH 0.8. Phosphoric acid extracted similar amounts of nitrogen to hydrochloric acid, but formic acid and monochloroacetic acid did not extract as much at pH 2 or lower. Potassium hydroxide extractions were similar to those with sodium hydroxide.

Isolation of Dry Bean Protein. Reference to Figure 1 suggests three possible methods for isolating protein fractions from dried beans. The first is to extract with a hydrochloric acid solution at pH 1.5, and to precipitate the protein by adjusting the pH to 3.8. The second method is to extract at pH 7.0 and precipitate by adjusting the pH to 3.8, which should give a protein with least change because of the more neutral conditions used. The third is to extract with sodium or potassium hydroxide at pH 10 to 12 and to precipitate by adjusting the pH to 3.8. Erratic results were obtained when protein extracted at pH 1.5 was precipitated by adjusting the pH to 2.6 to 6.0, and the data indicate that this method would be unsatisfactory for the production of a protein of uniform composition or as a preliminary step in the isolation of the individual bean proteins.

All but 27% of the total bean protein extracted at pH 7.0 was precipitated at pH 3.8 (Figure 2), and less was precipitated at either higher or lower acidities. Similar results were obtained with protein extracted at pH 10.0. Although either of the above procedures would appear to be satisfactory for the isolation of bean proteins, the pH 7.0 extraction followed by extraction at pH 10.0 would be a method for obtaining greater fractionation of the proteins. Extraction at pH 10.0 would appear to be more satisfactory for preparing a representative total bean protein, because it extracts more of the total protein.

Another method of extracting the protein from dried beans is with a salt solution. Only a small amount of the protein extracted by 1.5% sodium chloride solution was precipitated by adjusting the pH to 3.5 to 4.5, the range at which the protein was least soluble; 70% of the protein was still soluble at this point. The best procedure for precipitating proteins dissolved in salt solution is by dialysis.

Protein prepared by extraction of dried beans with sodium chloride solution and precipitation by dialysis appeared to contain less nonprotein material than one prepared by extraction with a water solution at pH 7.0 and precipitation at pH 3.8. A sample of lyophilized protein prepared by extraction at pH 7.0 and precipitation at pH 3.8 contained 11.09% nitrogen, and the lyophilized filtrate contained 5.97% nitrogen. Protein prepared by extraction with 2% sodium chloride solution and precipitation by dialysis (dried by lyophilization) contained 14.63% nitrogen, and the lyophilized filtrate contained 10.07% nitrogen. Fontaine, Pons, and Irving (*9*) observed that phytic acid was precipitated with the proteins of peanuts and cottonseed at the point of minimum solubility. Phytic acid was partially removed by dialysis (*12*, *19*). Bourdillon (*2*) isolated a protein-phytic acid complex from bean seeds. Part of the nonprotein nitrogen in the protein extracted at pH 7.0 and precipitated at pH 3.8 is probably phytin. Bean seeds contain 0.4 to 0.5% phosphorus (*5*). If all of the phosphorus was present as phytin and all of the phytin extracted and precipitated with the protein, the 11.3 grams of protein obtained from 100 grams of bean seed would contain at the most 1.8 grams of phytin (*12*). Correcting for phytin, the nitrogen content of the remaining portion would be 13.1% rather than 11.09%, and this is still lower than the 14.63% in the salt extracted protein precipitated by dialysis or of 16% in phaseolin preparations of Ritthausen (*14*), Osborne (13), or Waterman, Johns, and Jones (*22*). Other impurities must also be present. Stoikoff and Sweschtarowa-Dinewa (*21*) showed the presence of carbohydrate in bean protein extracted with water.

Properties of Protein Fractions. Both bean protein soluble at pH 3.5 and that soluble after dialysis of the salt extract were further separated into four or five fractions by chromatography on DEAE-cellulose columns. The largest fraction was eluted by buffer before sodium chloride was added to the buffer, and this fraction migrated to the cathode on cellulose acetate electrophoresis. Bean protein soluble at pH 3.5 and that soluble in salt-free solutions is mostly albumin (phaselin) (Table II).

Bean protein extracted at pH 7.0 and precipitated at pH 3.8 and that extracted with 2% sodium chloride solution and precipitated by dialysis (globulin fraction) were essentially the same when paper electrophoresis and DEAE-cellulose column chromatography were used as criteria. The principal protein of each was eluted by the sodium chloride gradient when the sodium chloride concentration was about 0.1*M*. Most of a preparation of phaseolin was also eluted at about the same sodium chloride gradient. The principal protein migrated as a single band toward the anode when subjected to electrophoresis on cellulose acetate strips. This electrophoretic band was also the principal one in phaseolin and in the total proteins precipitated by adjusting the pH to 3.8 or precipitated by dialysis of the salt extract. Conphaseolin appeared to be mostly a mixture of almost equal parts of three proteins, which were separated by chromatography on DEAE-cellulose. Phaseolin is the principal protein in the globulin fractions prepared either by pH 7.0 or salt extraction.

Acknowledgment

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Literature Cited

(1) Assoc. Offic. Agr. Chemists, Wash-

ington, D. C., "Official Methods of Analysis," 7th ed., p. 13, 1950.
(2) Bourdillon, J., *J. Biol. Chem.* **189,** 65 (1951).
(3) Burnett, R. S., Fontaine, T. D., *Ind. Eng. Chem.* **36,** 284 (1944).
(4) Circle, S. J., "Studies on Soybean Protein," Doctoral Dissertation, University of Chicago, 1941.
(5) Deschamps, I., in "Processed Plant Protein Foodstuffs," Altschule, A. M., Ed., p. 725, Academic Press, New York, 1958.
(6) Evans, R. J., Henry, J. L., St. John, J. L., *Ind. Eng. Chem.* **40,** 458 (1948).
(7) Fontaine, T. D., Burnett, R. S., *Ibid.*, **36,** 164 (1944).
(8) Fontaine, T. D., Irving, G. W., Jr., Markley, K. S., *Ibid.*, **38,** 658 (1946).
(9) Fontaine, T. D., Pons, W. A., Jr., Irving, G. W., Jr., *J. Biol. Chem.* **164,** 487 (1946).
(10) Fontaine, T. D., Samuels, C., Irving, G. W., Jr., *Ind. Eng. Chem.* **36,** 625 (1944).
(11) Lund, A. P., Sandstrom, W. M., *J. Agr. Res.* **66,** 349 (1943).
(12) McKinney, L. L., Sollars, W. F., Setzhorn, E. A., *J. Biol. Chem.* **178,** 117 (1949).
(13) Osborne, T. B., *J. Am. Chem. Soc.* **16,** 633, 703, 757 (1894).
(14) Painter, E. P., Nesbitt, L. L., *Ind. Eng. Chem.* **38,** 95 (1946).
(15) Powrie, W. D., J. AGR. FOOD CHEM. **9,** 67 (1961).
(16) Ritthausen, H., *J. Pract. Chem.* **103,** 204 (1883).
(17) Smith, A. K., Circle, S. J., *Ind. Eng. Chem.* **30,** 1414 (1938).
(18) Smith, A. K., Johnsen, V. L., Beckel, A. C., *Ibid.*, **38,** 353 (1946).
(19) Smith, A. K., Rackis, J. J., *J. Am. Chem. Soc.* **79,** 633 (1957).
(20) Smith, C. R., Jr., Earle, F. R., Wolff, I. A., J. AGR. FOOD CHEM. **7,** 133 (1959).
(21) Stoikoff, S., Sweschtarowa-Dinewa, M., *Nahrung* **3,** 193 (1959).
(22) Waterman, H. D., Johns, C. O., Jones, D. B., *J. Biol. Chem.* **55,** 93 (1923).

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The Nutritional Evaluation of Processed Whole Corn Flours

The authors of this article, Ricardo Bressani, Sonia V. Castillo, and Miguel A. Guzman [(J. AGR. FOOD CHEM. **10,** 308 (1962)], have submitted the accompanying table as a clarification of the data which appeared in the original Table VI. ▶

Table VI. Mean Squares from the Analyses of Variance of the Results in Two Biological Trials

	Growth Trial				Protein Depletion-Repletion Trial	
Source of Variation	*Degrees of freedom*	*Weight gain, grams*	*Feed efficiency ratio*	*Protein efficiency ratio*	*Degrees of freedom*	*Weight gain, grams*
Replications	5	...	1.5676	0.03357	5	...
Groups	11	430.0441[a]	8.9909[a]	0.19881[a]	12	248.9572[b]
Controls[c]	2	169.3889[d]	5.8998[a]	0.14867[a]	3	325.6111[b]
Treatments	8	548.9166[a]	10.6065[a]	0.21612[a]	8	235.6250[b]
Control *vs.* treatments	1	0.3749[d]	2.2489[d]	0.16060[a]	1	125.6538[d]
Pressure	2	750.1667[a]	14.8346[a]	0.57579[a]	2	499.5000[b]
Times	2	634.7222[a]	13.3461[a]	0.13645[a]	2	187.0555[d]
Pressure × times	4	405.3888[a]	7.1226[a]	0.07613[b]	4	127.9722[d]
Experimental error	55	100.5805	0.8771	0.02118	59	107.1655
Total	71				76	

[a] Significant at the 1% level.
[b] Significant at the 5% level.
[c] M2, M3, and raw corn for growth trial; M1, M2, M3, and raw corn flour for protein depletion-repletion trial.
[d] No statistical significance.

NUTRITIVE VALUE OF PUMPKIN SEED

Essential Amino Acid Content and Protein Value of Pumpkin Seed

(Cucurbita farinosa)

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IN CENTRAL AMERICA, as in most tropical and subtropical areas, many agricultural products of potential use for human and animal food are utilized only to a limited extent or not at all. A major reason for the shortage of protein-rich products of vegetable origin is the lack of basic chemical and biological knowledge of their values. This article reports the results of chemical and biological studies of the *Cucurbita farinosa* seed, taxonomically described by Rojas (*21*) and commonly known as pepitoria or pumpkin seed. Calderón and Standley (*5*) described it under the name of *Cucurbita pepo* L. It is reported to contain 48.4% crude fat and 31.0% protein (*8*).

Liebscher (*13*) demonstrated in sheep that the organic matter of this seed was highly digestible. Recent trials by Zucker *et al.* (*27*) with rats and swine indicated that the nutritive value of pumpkin seed meal protein was inferior to that of soybean for both experimental animals, and concluded that the protein was of low biological value. In contrast, King (*10*), who also studied the biological value and digestibility coefficient of the protein of the pumpkin seed and of watermelon seed, reported the digestibility of both proteins as 92% and the biological value as 63% and 73%, respectively. Studies carried out by Masurowsky (*17*) have indicated that, contrary to common belief, pumpkin kernel meal is not toxic.

Materials and Methods

Pepitoria Kernel Samples. Because the seed is not clearly classified botanically, nine different 3-pound samples from several localities in Guatemala were studied. All seeds were of different sizes, although their general appearance was the same. The samples were stored at 4° C. until ready to be analyzed.

Pepitoria Kernel Flour. Besides the nine samples, 100 pounds of seed were