Characterization of Thiamine in the Raw Peanut (*Arachis hypogaea* L.)

Richard H. Dougherty[1] and William Y. Cobb[2]

The various forms of thiamine in the dormant peanut cotyledon were characterized. The ratio of unphosphorylated to phosphorylated forms by thiochrome analysis was 64 to 36. Utilizing gel filtration, paper chromatography, and bioautography, the presence of three forms was demonstrated: thiamine (TCH), thiamine monophosphate (TMP), and thiamine pyrophosphate (TPP). The three compounds were estimated to occur in a ratio of roughly 33:10:1, respectively, with these techniques. There were no indications that either hydroxyethylthiamine or thiamine disulfides were present. Dialysis experiments indicated very low levels of thiamine bound irreversibly to the peanut proteins. Model system studies demonstrated no reversible binding of thiamine with peanut protein or starch in dilute aqueous environments. Weak association of the vitamin with peanut proteins during curing of freshly-harvested nuts was indirectly indicated by: association with a protein fraction which precipitates from buffered extracts during high speed centrifugation and appearance of inordinately high concentrations of thiamine with protein fractions "salted out" from peanut extracts.

Peanuts are known to be an excellent source of certain vitamins, notably B, E, and K. The peanut is, in fact, one of the richest known sources of thiamine (B_1) in the higher plant kingdom. Unlike certain of the other B vitamins, thiamine decomposes quite readily in most nonacidic environments, particularly under application of heat. Several investigators (Fournier *et al.*, 1949; Pickett, 1941; Willich *et al.*, 1952) have reported losses up to 90% of the original thiamine during roasting of peanuts. In order to establish a basis for more effective study of the mechanisms of thiamine degradation, derivatives of the vitamin and their possible associations with other constituents in the raw peanut were investigated.

EXPERIMENTAL METHODS

Freshly-harvested peanuts (var. NC-5) were cured, shelled, vacuum packaged in cans, and stored at −20° C until used. Those shelled nuts which were retained on an 18/64 × 1-in. slot screen—medium and extra large size grades (Woodroof, 1966)—were utilized. Prior to analysis, the testa were removed by hand after immersion in liquid nitrogen.

The general forms of thiamine were determined by the thiochrome procedure (Association of Official Analytical Chemists, 1965). Slight modifications included cysteine reduction of extracts to include any disulfide forms and protein digestion with pepsin or papain (Strohecker and Henning, 1965). Sensitivity of the method for concentrations as low as 4×10^{-4} μg per ml has been demonstrated in the peanut system (Dougherty and Cobb, 1970a). All quantities of thiamine derivatives herein are expressed on the basis of thiamine hydrochloride equivalents.

Peanut Extracts. Peanut extracts were obtained by homogenization (1 to 3, w/v) in the selected medium with an Omni-Mixer, maintaining the temperature at about 15° C. The homogenate was centrifuged at 1610 × G for 15 min, yielding a supernatant liquid overlaid with fat pad. The supernatant liquid was carefully removed with a pipet and filtered through Whatman No. 40 filter paper under vacuum. This aqueous mixture was designated the nonhigh-speed centrifuged (NHSC) extract. When necessary, further clarification of the extract was effected by centrifugation of the filtrate at 27,000 × G. Again the aqueous layer was carefully removed, then filtered through Whatman No. 40 paper *without* vacuum. This filtrate was designated as the high-speed centrifuged (HSC) extract. Such extracts were freshly prepared for each experiment to avoid, insofar as possible, enzymatic or microbiological deterioration.

Separation of Derivatives. Peanut extracts were submitted to a prefractionation step before paper chromatography, as thiamine appeared in exceedingly small concentrations in relation to other material present. An HSC extract was prepared in 0.1*N* HCl, adjusting the final pH to 2.5. Macromolecular material in the supernatant was then removed by filtering 5-ml aliquots through a column of Sephadex G-25 in 1*N* acetic acid (pH 2.5). Gel filtration columns were prepared as directed by the manufacturer (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). Fractions (4.0 ml) were collected from these columns while monitoring the absorbance at 280 nm. The fractions containing the lower molecular weight material were combined and reduced to approximately 2 ml *in vacuo* at 36–38° C. This concentrate was passed through a column of Sephadex G-10 in 1*N* acetic acid (Figure 1). The fractions rich in thiamine were combined, evaporated under vacuum, and then volumetrically diluted to 1.0 ml for paper chromatographic separation.

The phenol extraction method described by Iacono and Johnson (1957) was utilized to substantiate qualitatively results from gel filtration clarifications and to indicate the presence of any hydroxyethylthiamine (HET).

Thiamine and its derivatives were resolved using selected chromatographic systems described by Camiener and Brown (1960) and Carlson and Brown (1961). A bioautographic technique utilizing *Lactobacillus viridescens* (Camiener and Brown, 1960; Pearson, 1967) was used for the detection of thiamine derivatives on each chromatogram. Areas of the chromatogram containing metabolizable forms of the vitamin were discerned by corresponding areas of growth on seeded agar plates after overlay of the latter with the dried chromatogram for 7 sec and incubation for 12 hr.

Macromolecular Complexes. For investigation of thiamine-macromolecular complexes, extracts were prepared with 0.01*M* tris buffer (pH 7.0). Forty-ml aliquots of the extract were dialyzed against 4 l. of the buffer at room temperature for 6 days with constant stirring. The dialyzate buffer was changed after 3 days. Toluene was added to tubing contents and dialyzate to prevent microbial growth.

Department of Food Science, North Carolina State University, Raleigh, N.C. 27607

[1] Present address: Department of Food Science, University of Florida, Gainesville, Fla. 32601

[2] To whom correspondence should be addressed.

Each dialyzate was subsequently reduced to 100 ml *in vacuo* at 36–38° C. Assay for thiamine was made in the two dialyzates and the tubing contents. Companion studies were made incorporating 0.4 g papain into the extract prior to dialysis.

Extracts were prepared in 0.01*M* phosphate buffer (pH 7.0) for Sephadex gel filtration studies of thiamine complexing. Aliquots were filtered through a Sephadex G-100 column (2.7 × 42.0 cm) in the phosphate buffer at the rate of 0.5 ml per min. Five tube groups were analyzed for thiamine.

To determine the effect of the preparation technique on the associations observed, radioactive thiamine (thiazole-2-^{14}C, Amersham/Searle Corp., Des Plaines, Ill.) was incorporated into the blending medium. The isotope (3.3 μC) was blended with 50 g of peanuts in 150 ml 0.01*M* phosphate buffer (pH 7.0). HSC extract and pellet were obtained from aliquots of the homogenate as previously described. The pellet was dissolved in 0.1*N* HCl. Aliquots of the supernatant and pellet solutions were thereupon assayed for thiamine and monitored for radioactivity, using a phosphor mixture composed of PPO-POPOP in Triton X-100. These samples were counted in a Packard Model 574 Tri-Carb liquid scintillation spectrometer. Ratios of radioactivity and thiamine in the pellet *vs.* the supernatant liquid were determined.

Characterization of 27,000 × G Extract Precipitate. The pellet obtained from high speed centrifugation of extracts was characterized with several qualitative tests: Molisch for carbohydrates (Triebold and Aurand, 1963), biuret for protein (Robinson, 1963), ninhydrin for free amino groups (Clark, 1964), and iodine for starch. The latter test was performed by adding five drops of 0.1% iodine in 95% ethanol to 10 ml of an aqueous suspension of the material. These same tests were also performed after hydrolyzing one portion of the precipitate in 2*N* HCl at 100° C for 1 hr and a second portion in 6*N* HCl at 121° C for 10 hr.

Model System Binding Studies. Reversible binding of thiamine to peanut protein was studied by a method similar to that of Klotz *et al.* (1946). Proteins were obtained from a 1*M* NaCl extract of peanuts (50 g) by precipitation with ammonium sulfate (100% saturation). After centrifugation at 17,300 × G, the protein was redissolved in 400 ml 1*M* NaCl. Ten ml aliquots of the dispersion were placed in dialysis tubing and equilibrated for 48 hr against 0.01*M* phosphate buffer (pH 7.0) to free each sample of salt as well as other small molecules, including thiamine. The samples were then dialyzed against 75 ml quantities of the buffer containing thiamine pyrophosphate (0.20–3.75 μg). Each dialyzate contained 6.5 × 10^{-3}*M* $MgSO_4$. The total contents of each bag as well as a 10 ml aliquot of each corresponding dialyzate were assayed after 60 hr equilibration.

Ammonium Sulfate Precipitation. The association of thiamine with various protein fractions obtained by ammonium sulfate precipitation was also investigated. Three precipitate fractions were obtained from a 1*M* NaCl extract (HSC) by adding ammonium sulfate at rates representing 40, 80, and 100% saturation, while holding the pH constant at 6.5 (Naismith and McDavid, 1958). The precipitates were dissolved in glacial acetic acid and, along with the final supernatant liquid, taken to known volumes for thiamine assay.

RESULTS AND DISCUSSION

Estimation of general forms of thiamine derivatives by variations in the described thiochrome method of analysis indicated that 64% of the total vitamin was unphosphorylated, while 36% was phosphorylated. Reduction of extracts with cysteine caused no increase in thiamine, thus indicating

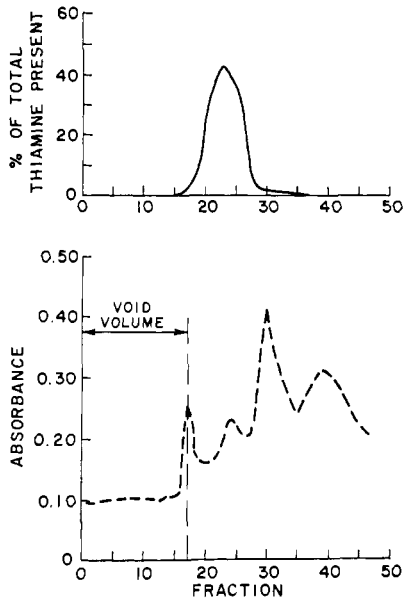

Figure 1. Sephadex G-10 gel filtration chromatogram of low molecular weight fraction obtained from filtration of HSC extract through Sephadex G-25. Lower curve: absorbance at 280 nm. Upper curve: % thiamine recovery per fraction group. Solvent: 1*N* acetic acid (pH 2.5). Column size: 2.7 × 42.0 cm

lack of thiamine disulfides or disulfide phosphates. Recoveries for authentic TCH, TPP, and thiamine propyl disulfide were 104, 98, and 93%, respectively, when added to the peanut slurries.

The correlation between elution pattern from Sephadex G-10 and the thiamine location in the low molecular weight fraction of peanut extract is shown in Figure 1. Unexpectedly, thiamine appeared near the void volume of the G-10 column (exclusion limit *ca.* 700) and somewhat ahead of the bulk of lower molecular weight material (primarily sugars). The molecular weights of TCH, TMP, and TPP are 265, 345, and 425, respectively. The explanation for the elution behavior of these compounds from Sephadex may lie in a dimerization phenomenon or the fact that the basic molecule is essentially flat with substituted groups extending parallel with the plane of each ring. Also, the approximate exclusion limits of the gels are estimated from data on carbohydrates and proteins, both of which assume more compact configurations than thiamine.

Results from paper chromatography of the combined thiamine-rich fractions is given in Table I. Data from three solvent systems are presented; these were verified with two additional systems. The presence of thiamine, thiamine monophosphate, and thiamine pyrophosphate is indicated. No hydroxyethyl thiamine was evidenced, and if present in the resting seed, must be in exceedingly small concentration. Overlay of the seeded agar plate with filter paper upon which was spotted 1 ng of thiamine stimulated formation of an easily visible growth zone by the *Lactobacillus* organism upon incubation. Johnson and Goodwin (1963) found hydroxyethyl thiamine in 3-day old corn seedlings, but not in the dormant seeds. By comparison with the extent of growth produced by known concentrations of thiamine, and analysis of total thiamine in the extract, it was estimated that the molar ratio of TCH:TMP:TPP was roughly 33:10:1.

While it has been suggested that plants in general contain very little phosphorylated thiamine (Pearson, 1967; Tauber, 1937), it appears that legumes, in fact, do. Weakley *et al.*

Table I. Paper Chromatographic Properties of Thiamine Derivatives Obtained from Peanuts

(A) Gel filtration chromatography
(B) Undigested extract[c]
(C) Diastase-digested extract[c]

Source	R_f Values[a] A	B	C
Authentics			
Thiamine (unphosphorylated)	0.56	0.30	0.29
Thiamine monophosphate	0.25	0.22	
Thiamine pyrophosphate	0.09	0.14	
Hydroxyethylthiamine		0.35[b]	0.48[b]
Peanut extracts			
Spot 1	0.57	0.30	0.28
2	0.25	0.23	
3	0.09	0.15	

[a] Developing systems: Extract A, 1 propanol/water/1*M* acetate buffer, pH 5.0 (7/2/1); Extract B, 1-butanol/acetic acid/water (4/1/5, upper phase); Extract C, 1-butanol/ethanol/water (4/1/1). [b] Reported by Carlson and Brown (1961). [c] Iacono and Johnson (1957).

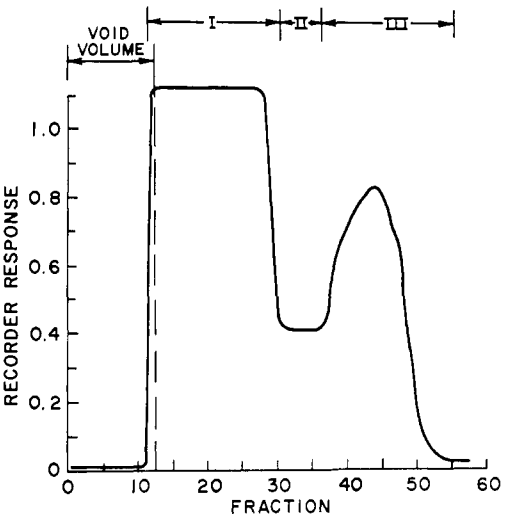

Figure 2. Ultraviolet (280 nm) absorbance profile of NHSC extract (10 ml) filtered through Sephadex G-100 column (2.7 × 42.0 cm). Solvent: 0.01*M* phosphate buffer (pH 7.0). Flow rate: 0.5 ml/min

(1961) found a large proportion of the vitamin in soybeans to be in the phosphate form. It has been shown herein that the phosphate esters are present in significant amounts in peanuts. Furthermore, the monophosphate ester predominates over the pyrophosphate, possibly due to vagary of the biosynthetic system or simply to the greater chemical stability of the monophosphate during curing and storage.

Upon dialysis of NHSC extracts a small amount of thiamine was ascertained as being tightly-bound to macromolecular material (Table II). Although this represented less than 1% of the total thiamine, the concentration within the tubing was over 20 times greater than that of the dialyzate on a unit volume basis. A portion may have been bound to enzyme (Morey and Juni, 1968) as a considerable amount (*ca.* 50%) of the residual vitamin was in the phosphate form. Additional thiamine (21–78%) was released by incorporation of papain in samples before dialysis.

Having thus demonstrated a small quantity of rather tightly-bound thiamine, reversible association of the vitamin with peanut protein was investigated. As indicated in Table III, there is little binding of TPP with peanut protein in dilute aqueous environment. The small quantity indicated as bound (0.24 to 0.58 μg per g) approximates that portion of thiamine which could not be removed from the protein preparation via extended dialysis against dilute phosphate buffer. Similar results were obtained with TCH and when dialyzing either TCH or TPP against 1*M* NaCl. Likewise, no association was evidenced between thiamine and starch in dilute buffer. While preparing this manuscript, the work of Leichter and Joslyn (1969) has appeared in the literature. These researchers, while investigating the protective effect of casein toward sulfite splitting of thiamine, found that the vitamin did not appreciably combine with this protein. Indeed, as the casein content was increased in the range of 5–20%, the quantity of bound thiamine decreased. When the casein content was held constant while increasing the thiamine content, an increase in association occurred. Within the range tested, the results with peanut protein do not confirm this latter observation (Table III); however, maximum concentration of vitamin used in the present study was less than one-half that employed by Leichter and Joslyn.

Certain data indicated that thiamine will associate with peanut protein at or near the point of destabilization.

Appearance of Thiamine in the Large Molecular Weight Fraction from Gel Filtration of 0.01*M* Phosphate Buffer Extracts of Peanuts. The elution of profile of these extracts from Sephadex G-100 are shown in Figure 2. Analyses of the three fractions (Table IV) indicated 6.4% of the total thiamine in fraction I, corresponding in molecular weight to 100,000 or greater. When identically rechromatographed (Figure 3), this fraction yet contained 4.7% of the total while fraction

Table II. Thiamine Content of Fractions Obtained from Dialysis of Peanut Extract in 0.01*N Tris* Buffer (pH 7.0)

Source	Diastase digestion	Trial A Thiamine (μg)	Trial A Concentration (μg/l)	Trial A Ratio[a]	Trial B Thiamine (μg)	Trial B Concentration (μg/l)	Trial B Ratio[a]
Dialysis tubing (3 days)	+	4.38	82.6	1.8	3.05	55.00	1.5
Dialysis tubing (3 days)	−	3.16			2.22		
Dialyzate (3 days)	+	181.0	45.3		150.00	37.50	
Dialyzate (3 days)	−	181.00			143.00		
Dialysis tubing (6 days)	+	1.03	19.4	23.2	0.80	14.60	26.1
Dialysis tubing (6 days)	−	0.51			0.47		
Dialyzate (6 days)	+	3.35	0.84		2.25	0.56	
Dialyzate (6 days)	−	2.65			1.75		

[a] Concentration ratio of thiamine in dialysis tubing: thiamine in dialyzate on unit volume basis.

Table III. Equilibrium Binding of Thiamine Pyrophosphate (TPP) by Peanut Protein Suspensions

Dialyzing mediums: 0.01M phosphate buffer (pH 7.0) containing 6.5 × $10^{-3}M$ $MgSO_4$. Protein concentration: 0.124 g per sample. Time (T): 60 hr

Total TPP (μg) added to dialyzate, T = 0 hr	Relative concentration TPP in dialyzate, T = 0 (μg/g protein)	TPP (μg/10 ml) in dialyzate T = 60 hr	TPP (μg/10 ml) inside tubing T = 60 hr	Bound TPP (μg/g protein)
0.20	1.61	2.7	6.4	0.30
0.30	2.41	3.6	9.7	0.49
0.40	3.22	4.5	10.0	0.45
0.50	4.03	6.3	11.5	0.42
0.75	6.03	8.6	12.7	0.32
1.50	12.07	16.4	21.0	0.37
2.25	18.10	24.0	29.4	0.44
3.00	24.14	36.0	42.7	0.54
3.75	30.17	43.0	46.6	0.29

Table IV. Thiamine Content of Fractions from Chromatography of NHSC Extract on Sephadex G-100

Fraction	Thiamine Concentration (μg)	Thiamine % of Original
I	1.50	6.4
II	0.65	2.8
III	20.00	84.7
Rechromatography		
I_1	1.10	4.7
I_2	0.50	2.2
III_1	0.40	1.7
III_2	20.40	86.4
Control	23.60	100.0

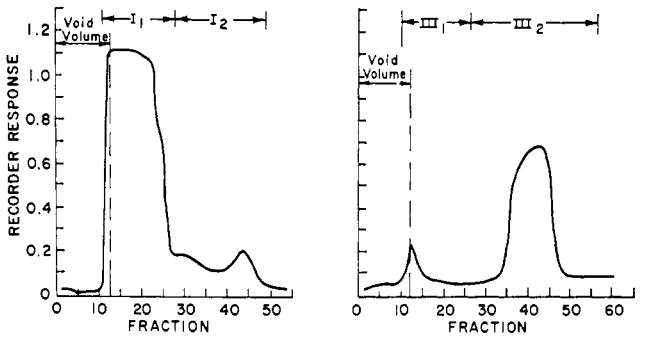

Figure 3. Rechromatography of fraction groups I and III under the same conditions as Figure 2

III showed 1.7% of the total thiamine in a high molecular weight fraction.

Incorporation of Significant Quantities of Thiamine into the HSC Pellet During Centrifugation. Considerable difficulty was encountered at times with flow characteristics of G-100 columns using NHSC extract. It was suspected that a fine precipitate was forming, thus causing flow rate to drop drastically. Resort was thus made to 27,000 × G centrifugation to obtain the HSC extract. When HSC extracts were compared with NHSC extracts for gel filtration chromatograms, a similar elution profile was obtained but the quantity of thiamine in the high molecular weight fraction was reduced. An examination of the HSC pellet for thiamine indicated that while it accounted for a very small volume of the extract (*ca.* 2.5%), it contained from 3.4–5.8% of the vitamin. The level in such precipitates could have easily accounted for the difference in thiamine associated with the higher molecular weight fractions in gel-filtered HSC and NHSC extracts. The addition of radioactively labeled thiamine to the blending system at the time of extract preparation resulted in approximately equal distribution of total thiamine (96.4%:3.4%) and radioactivity (95.3%:4.7%) between supernatant liquid and HSC pellet after high-speed centrifugation.

Table V. Characterization of Thiamine-Rich 27,000 × G Precipitate by Qualitative Tests Before and After Hydrolysis

Test	Before Hydrolysis	After Hydrolysis in 2N HCl (1 hr)	After Hydrolysis in 6N HCl (10 hr)
Molisch	Negative	Negative	Negative
Iodine	Negative	Negative	Negative
Biuret	Strongly positive	Slightly positive	Negative
Ninhydrin	Slightly positive	Strongly positive	Strongly positive

Table VI. Thiamine Content of Fractions Obtained from Ammonium Sulfate Precipitation of 1M NaCl Extract

Fraction	Thiamine (μg)	% of Total Thiamine
40% Saturation	14.25	12.9
80% Saturation	10.25	9.3
100% Saturation	12.90	11.7
Supernatant liquid	73.50	66.1

Qualitative characterization of the HSC pellet (Table V) strongly indicated the material as being proteinaceous. When submitted to polyacrylamide gel electrophoresis in Na_2HPO_4-NaOH buffer (0.01M, pH 11.3), the pellet material yielded a pattern identical with total peanut protein obtained from 1M NaCl extraction of the defatted nuts.

Whether the HSC pellet protein precipitates from the buffer because of properties not elucidated by electrophoresis, *i.e.*, specific protein-protein interactions, or whether it is simply due to saturation of the extraction medium, is not known at this time.

Presence of Inordinately Large Concentrations of Thiamine in Protein Fractions Obtained by Ammonium Sulfate Precipitation from a 1M NaCl Peanut Extract. The data in Table VI indicate that approximately 34% of the total thiamine of the peanut was distributed into the protein fractions upon precipitation by $(NH_4)_2SO_4$. Most likely, the unfavorable competition for water molecules created by ammonium sulfate caused the protein to rapidly associate with thiamine at or near the point of precipitation. Inagaki and Fukuba (1959) found that dehydrated bean curd adsorbed thiamine strongly when dipped into solutions of the vitamin. Complete recovery was possible only after digestion of the curd with a proteinase.

As water is removed from the peanut cotyledon during curing, a favorable environment may be established for reversible association of thiamine with cytoplasmic protein. The water content at this time drops from 40% or higher to around 8%. Furthermore, Altschul *et al.* (1961) have shown nonparticulated protein occurring to the extent of approximately 25% in the resting peanut cotyledon. It has also been demonstrated that the bulk of intracellular thiamine is nonparticulated and hypothesized that the water content of dormant peanut cotyledons is likely bound (Dougherty and Cobb,

1970b). Additionally, a subcellular fraction containing high concentrations of nonparticulated protein also exhibited a moderately high amount of thiamine (Dougherty and Cobb, 1970b).

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