Effect of Alcohol Washing and Autoclaving on Nucleotides of Soybean Meal

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Nucleotides from mature soybeans were extracted with trichloroacetic acid and separated into five major 260 mμ-absorbing fractions by anion exchange resin (Dowex-1) or polyethylene-imide-cellulose column chromatography. Two of the major fractions contained a large quantity of nucleotides. Another major fraction contained carbohydrates predominantly and a small amount of nucleotides. Gel filtration of the carbohydrate-rich fraction separated pigments from nucleotides. Trichloroacetic acid extracts from alcohol-washed or autoclaved meal contained more nucleotides than extracts from untreated meal. The nucleotide elution patterns of extracts from treated meals differed from those of extracts of untreated meal. Alcohol washing or autoclaving apparently disrupts pigment-nucleotide complexes, which permits a more complete extraction of nucleotides from meals so treated.

The mature soybean contains 8.5% nonprotein nitrogen (Circle, 1950) and about 1% of nucleic acid (DiCarlo *et al.*, 1955). A small quantity of this nonprotein nitrogen is believed to occur in the form of soluble nucleotides. The presence of pigments in plant nucleotide extracts makes separation and identification of individual nucleotides difficult (Christianson *et al.*, 1968). Therefore, little information is available on nucleotides in mature soybeans. Less is known about changes of nucleotides in soybean meals as a result of processing. Information on nucleotides in mature peas (Brown, 1962), hydrated soybeans (Key and Hanson, 1961), developing soybean seeds (Galitz and Howell, 1965), and soybean tissues (Medvedev and Khavkin, 1963) is available. This paper reports on the major nucleotides in mature soybeans and the changes in nucleotides when soybean meal is washed with 80% ethanol or autoclaved.

MATERIALS AND METHODS

Meal Preparation and Chemicals. Kanrich soybeans were used. About 7 kg. of the beans (stored at 4° C.) were dehulled, ground into meal with an Alpine mill, and kept at 4° C. for further use. Nucleosides, nucleotides, nicotinamide adenine dinucleotide (NAD), and nicotinamide adenine dinucleotide phosphate (NADP) used as standards in paper chromatography were purchased from Pabst Laboratories, Milwaukee, Wis., or from Sigma Chemical Co., St. Louis, Mo.

Extractions. One hundred grams of meal were stirred with 400 ml. of 10% trichloroacetic acid (TCA) for 20 minutes at 0° C. The mixture was centrifuged at 10,000 G at 0° C. for 15 minutes to collect the supernatant. The residue was washed once in a similar manner with 100 ml. of 5% TCA. The combined extract (500 ml.) was extracted with ice-chilled ether until the pH was between 5 and 6. The residual ether in the extract was evaporated under reduced pressure and the solution was lyophilized. The nucleotides were then dissolved in ice-cold water (100 ml.) and frozen until used. For the autoclaving treatment the meal was heated at 15 p.s.i. for 30 minutes, cooled immediately, and then extracted with TCA to obtain the nucleotides as described for unheated meal.

Nucleotides from the alcohol-washed meal were obtained in two parts as follows: The meal was washed with 80% ethanol at a solvent-to-meal ratio of 10 to 1 at room temperature for 2 hours. Nucleotides from the washed meal, after removal of the residual alcohol, were extracted with TCA as before. The alcoholic extract was evaporated under reduced pressure and then lyophilized. Nucleotides in the resulting sirupy solids were extracted with TCA by the procedure used for untreated meal. The two nucleotide fractions from alcohol-treated meal were combined for estimation of nucleotide content. Only the nucleotides from the washed meal were used for column chromatography to show the effect of washing.

Adsorption on Charcoal. Before column chromatography on Dowex-1, the nucleotides were adsorbed on charcoal-Celite (Darco G-60–Celite, 1 to 2) at 4° or 23° C. The charcoal-Celite mixture was pretreated with boiling 1*N* HCl, washed thoroughly with water, and dried at 55° C. Nucleotides were adsorbed by passing the solution through a Büchner funnel containing 200 grams of charcoal-Celite. After washing the charcoal-Celite with 0.01*M* (ethylenedinitrilo)tetraacetate (EDTA), pH 7.0, and then with cold water to remove salts and pigments, the adsorbed nucleotides were eluted by five separate batches of 50% ethanol containing 0.1% ammonia. The ethanol was evaporated under reduced pressure and the nucleotides were dissolved in cold water, yielding solutions of pH 6.0 to 6.5. Usually operations were carried out at 4° C., except in one experiment which was done at 23° C. Nucleotides chromatographed on polyethyleneimide (PEI)-cellulose columns were not pretreated with charcoal.

Chromatography. For Dowex 1 chromatography, 200 ml. of nucleotide solution (from 200 grams of meal) containing approximately 11,000 units (one unit per ml. is defined as 260 $m\mu = 1$) of nucleotides were put on a 2 × 20 cm. column. The resin (Dowex 1-X8, 200 to 400 mesh, formate form) was prepared as described by Hurlbert (1957). About 1750 units of bases and

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nucleosides were removed with 500 ml. of water. Nucleotides were then eluted with an ammonium formate–formic acid system, as used by Hurlbert (1957), contained in a mixer volume of 2 liters. Aliquots (15 ml.) were collected and the appearance of nucleotides was followed by determination of absorbance at 260 mμ. The nucleotides eluted from the column were divided into seven fractions. Each fraction was again treated with 5 grams of charcoal-Celite to separate nucleotides from formate and the nucleotides collected from charcoal-Celite were dissolved in 10 ml. of water for analyses.

The PEI-cellulose column (1 × 110 cm.) was prepared according to Christianson *et al.* (1968). Nucleotides from 7 grams of soybean meal were put on the column without charcoal treatment. The column was washed with water until absorbance approached zero and then eluted with a combination of LiCl and borate as described by Christianson *et al.* (1968). Nucleotides were monitored at 260 mμ with a continuous recording flow cell spectrophotometer (Vanguard, 1056-OD). Aliquots (5 ml.) were collected with a Beckman fraction collector. After all of the major peaks were pooled and concentrated, only three were analyzed. Other peaks were too low in concentration to be analyzed. Chromatography of the alcoholic extracts for nucleotides was impossible on either a Dowex-1 or PEI-cellulose column because lipids plugged the columns.

Gel filtration of carbohydrate-rich nucleotide fractions obtained by Dowex-1 or PEI-cellulose chromatography was carried out on a Sephadex G-25 column (1 × 113 cm.). The gel was thoroughly washed with water to remove ultraviolet-absorbing materials. Nucleotides in 5 ml. of water were poured onto the column and eluted with water at a rate of 23 ml. per hour. Three-milliliter samples were collected in a refrigerated collector. The material which eluted from the column was divided into three fractions for further analyses.

The procedure as described by Carter (1950) was used to chromatograph nucleotides on paper. Fractions that did not resolve in the two systems (5% ammonium citrate, pH 3.6 and 9.6, with isoamyl alcohol as an overlying nonaqueous phase) were hydrolyzed 1 hour in 1*N* HCl at 100° C. for purines and 2 hours in formic acid at 175° C. for pyrimidines. The resulting nucleosides were identified by paper chromatography.

Analyses of Nucleotides. Nucleotides in each fraction were identified by conventional methods. Aliquots from each fraction were analyzed for nitrogen by the Kjeldahl method (LePage, 1957), phosphate by the method of Fiske and SubbaRow (1925) pentose by reaction with orcinol (Mejbaum, 1939), and total sugars expressed as glucose by the phenol-sulfuric method (Dubois *et al.*, 1956). Ultraviolet-absorbing spots on chromatograms were detected by ultraviolet lamps. The characteristic ultraviolet-absorption curve of each fraction was measured with a Beckman spectrophotometer Model DK-2A at pH 2, 6, and 11. The ratio of absorbance at 250, 280, and 290 mμ to that at 260 mμ at different pH's served as a basis for identification, together with the mobility in paper chromatography and the elution position in the ion-exchange chromatography.

To estimate the nucleotide content of soybean meal the total absorbance at 260 mμ of the TCA extract was measured. The solution was then passed through a Dowex-1 formate column and the column was washed thoroughly with water. Absorbance of the wash water was subtracted from the total absorbance and nucleotide content was expressed in absorbance units as defined earlier. Nucleotides in each treated meal were also estimated by pentose analysis and expressed in terms of adenosine.

RESULTS

Estimation of Nucleotides. Alcohol-washed or autoclaved meal yielded more nucleotides than meal not so treated. In Table I, nucleotides were estimated at 11.0 mg. per gram of untreated meal, whereas the autoclaved and alcohol-washed meals gave values of 17.2 and 19.4, respectively. The increase of N and P in extracts from autoclaved or alcohol-washed meals substantiated these results. There was also an increase of TCA-soluble sugars in alcohol-washed meal (15.2 mg. per gram) as compared to untreated meal (11.8 mg. per gram). In the autoclaved meal TCA-soluble sugar content was only 10.5 mg. per gram. Autoclaving may have liberated sugars from nucleotides.

Dowex-1 Chromatography. The temperature at which TCA extracts from soybean meal were treated with charcoal-Celite altered the nucleotide elution patterns. Five major 260 mμ-absorbing peaks (F_1, F_2, F_3, F_4, and F_6) were obtained from extracts treated at 4° C. (Figure 1, A). Extracts treated at 23° C. yielded seven peaks (F_1–F_4, F_6, F_8, and F_9) in Figure 1, B. Absorbances of F_3 and F_6 decreased significantly after sorption and desorption at 23° C. as compared to preparation at

Table I. Analyses of Trichloroacetic Acid Extracts from Kanrich Soybean Meal

Treatment of Meal	N, Mg./G.	P, Mg./G.	Total Sugar, Mg./G.	Pentose, Mg./G.	Estimated-Soluble Nucleotides: Total O.D. 260 units/g. of meal	Estimated-Soluble Nucleotides: O.D. units retained on Dowex-1 column	Estimated-Soluble Nucleotides: Mg./g. (pentose analysis)
Untreated	1.19	2.04	11.8	6.25	129	89 (69%)	11.0
Toasted	1.54	2.50	10.5	9.00	157	118 (75%)	17.2
Alcohol-washed[a]	2.04	3.21	15.2	9.25	237	188 (81%)	19.4

[a] Nucleotides removed by alcohol extraction were isolated and added to trichloroacetic acid extract of meal.

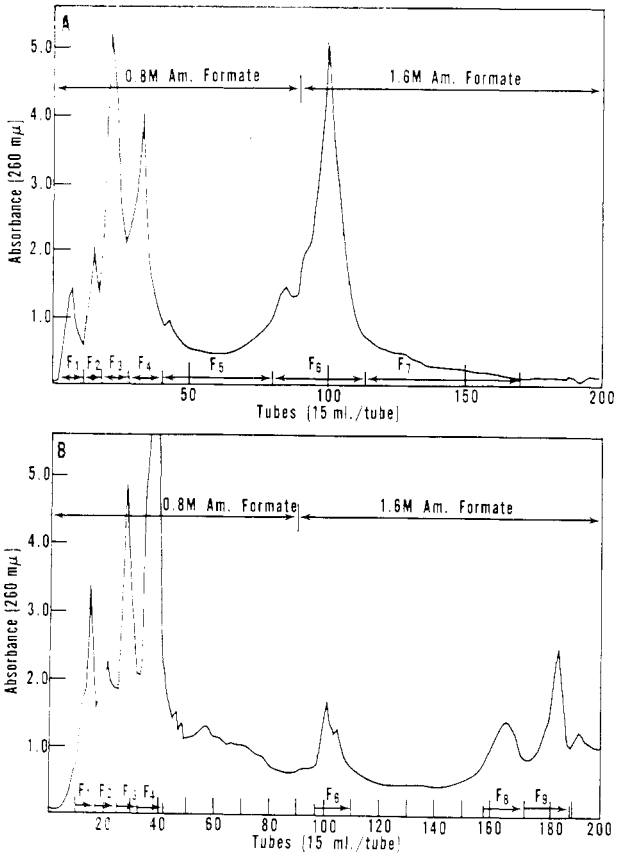

Figure 1. Dowex 1-X8 formulate column chromatography of TCA extract treated with charcoal-Celite

A. At 4° C.
B. At 23° C.

4° C. An increase in the absorbance of F_4 and appearance of new peaks (F_8 and F_9) resulted when the extract was treated at 23° C. Analyses of nucleotides in each fraction from the extracts treated at the two temperatures also revealed differences in sugar content, phosphate-to-pentose ratios, number of ultraviolet-absorbing spots on paper chromatography, and the ultraviolet-absorption curves (Table II).

Fractions F_1 and F_2 (Table II, Figure 1, *A*) were pigment-nucleotide complexes based on paper chromatographic behavior. Fraction F_3 was a complex containing a higher sugar content than the previous two fractions. These three fractions (F_1, F_2, and F_3) were not high in nucleotide content despite their high absorbances (Figure 1, *A*). Fractions F_4, F_5, and F_6 were the major nucleotide fractions; fractions F_4 and F_6 contained 56% of the pentose and 68% of the phosphate of the TCA extract. Peak F_4 had a phosphate-to-pentose ratio of 1 and peak F_6 had a ratio of 1.5. Fraction F_5 was a nucleotide-sugar complex. On paper chromatography fractions F_4, F_5, and F_6 showed multiple spots of nucleotides.

In Table II (Figure 1, *B*), changes occurred in fractions F_3, F_4, and F_6 as compared with the same fractions in Table II (Figure 1, *A*). Fraction F_3 contained less sugar [decreased from 11.7 μmoles in Table II (Figure 1, *A*) to 3.9 μmoles in Table II (Figure 1, *B*)]. Fraction F_4 lost about equal amounts (>60%) of its phosphate and pentose —for example, 19.9 μmoles of pentose in Table II (Figure 1, *A*) to 6.7 μmoles in Table II (Figure 1, *B*). In fractions F_6, there were losses in sugar content (3.9 μmoles), in the number of ultraviolet-absorbing spots on paper chromatography, and in phosphate and pentose contents. A decrease in phosphate-to-pentose ratio from 1.5 [Table II (Figure 1, *A*)] to 0.33 [Table II (Figure 1, *B*)] occurred. All these changes in fraction F_6 indicated a disappearance of nucleotides as a result of treating the TCA extract with charcoal-Celite at 23° instead of 4° C. No explanation can be given for this temperature effect. Disappearance of fraction F_6 coincided with appearance of fractions F_8 and F_9. Both fractions had higher phosphate and pentose contents than any of the other fractions in Figure 1, *B*. The ratio of phosphate to pentose in fraction F_8 was 1.4; in fraction F_9, 1.7. Sugars appeared in equal amounts in fractions F_8 (3.4 μmoles) and F_9 (3.3 μmoles). Fraction F_9 revealed multiple ultraviolet-absorbing spots on paper chromatography.

Yellow color was observed in some fractions. In Figure 1, *A*, fractions F_3, F_5, and F_6, and in 1, *B*, F_4, F_8, and F_9 contained almost all of the yellow pigment. In addition, paper chromatography of fractions F_1 to F_9 revealed some fractions with an orange fluorescence and all fractions contained ultraviolet-absorbing material having a high R_f value. These results indicate that charcoal-Celite treatment did not remove all of the pigment at either temperature.

Spectral curves of nucleotides at pH 2, 6, and 11, and ratios of absorbances at 250, 280, and 290 mμ to that at 260 mμ were measured to aid identification. In general, there was no spectral difference between similar fractions shown in Figure 1, *A* and *B*, except in fractions F_3 and F_4. For example, at pH 6 fraction F_3 in Figure 1, *A*, showed a single λ max at 250, but a multiple peak at 253, 258, and 263, with inflection at 325 mμ in Figure 1, *B*. Fraction F_4 in Figure 1, *A* (pH, 6.0) possessed a λ_{max} at 257 but λ_{max} 273 mμ in 1, *B*. The presence of pigment and the occurrence of nucleotide mixtures (based on appearance of multiple spots during paper chromatography) complicated interpretation of the ultraviolet-absorbing curves.

PEI-Cellulose Chromatography. Christianson *et al.* (1968) recently reported that PEI-cellulose is superior to polystyrene anion exchange resins for chromatography of plant extracts containing ultraviolet-absorbing contaminants such as phenolic compounds. Occurrence of phenolic constituents in soybeans (Daubert, 1950) prompted the use of PEI-cellulose chromatography for analysis of soybean nucleotides. TCA extracts, not treated with charcoal-Celite, from 7 grams of untreated meal, as well as those from autoclaved and alcohol-washed meals, were chromatographed on PEI-cellulose column and yielded the elution patterns shown in Figures 2 to 4.

Free bases, nucleosides, and possibly contaminants

Table II. Identification of Soybean Nucleotides in Figure 1, *A* and *B*

Fractions	Pentose, μmoles	P, μmoles	Sugar as Glucose, μmoles	Amount of[a] Nucleotide, μmoles	Base[b] Type	R_f Values in Solvents[c] 1	2
				FIGURE 1,*A*			
1	5.0	3.5	6.1	14	G, A	0.67[d]	0.55
						0.75	0.61
						0.93	0.91
2	7.4	9.0	7.2	12[e]	C	0.85	0.85
3	7.8	7.3	11.7	7	Unknown	0.93	0.89
4	19.9	19.5	7.6	122	NAD,	0.66[d]	0.50[d]
					A, G, C?, U	0.71	0.67
						0.86	0.83
						0.92	0.91
5	11.7	7.6	10.6	155	A, G	0.71	0.69
						0.83	0.88
						0.89	—
6	21.7	31.5	9.8	168	NAD, NADP,	0.78	0.32
					A, G, C?, U	0.85	0.67
						0.91	0.75
							0.82
							0.89
7	1.1	4.0	1.5	2	Unknown	0.87	0.88
				FIGURE 1,*B*			
1	4.0	4.5	7.6	44	G, A	0.88	0.91
2	7.4	9.0	7.2	10	C	0.89	0.72
							0.86[d]
3	4.7	10.0	3.9	39	Unknown	0.88	0.86
4	6.7	6.0	7.8	55	A, NAD	0.82[d]	0.80[d]
						0.89	0.86
6	6.0	2.0	5.9	43	NAD, NADP,	0.61[d]	0.46[d]
					A	0.89	0.85
8	10.1	14.5	3.4	4	Unknown	0.89	0.66
							0.86
9	14.1	24.5	3.3	46	NAD, NADP,	0.59[d]	0.31[d]
					A, G, C?, U	0.89	0.64
							0.72
							0.77[d]
							0.85[d]

[a] Estimated as adenosine based on molar absorbance λ_{max} at pH 7.0 of 15.7×10^3.
[b] A, G, C, U, NAD, and NADP. Adenine, guanine, cytosine, uracil, nicotinamide adenine dinucleotide, and nicotinamide adenine dinucleotide phosphate, respectively.
[c] Solvent 1, 5% citric acid was titrated to pH 3.6 with NH_4OH, with isoamyl alcohol as overlying nonaqueous layer. Solvent 2, same as Solvent 1 except to pH 9.6.
[d] UV fluorescent.
[e] Estimated as cytosine based on molar absorbance of 7.7×10^3 at pH 7.

were removed with water elution. Five to six major 260 mμ-absorbing peaks were eluted with buffer. Variations due to treatments of the meal occurred as follows:

Untreated Meal. Five major peaks were observed in Figure 2. Peaks 1, 2, and 5 corresponded to fractions F_3, F_4, and F_6 in Figure 1, *A*. Peaks 3 and 4, present in lesser quantity, were not identified. Identification of peaks 1, 2, and 5 was based mainly on paper chromatographic behavior and spectrophotometric measurements.

Autoclaved Meal. A significant change occurred in the nucleotides extracted from autoclaved meal. The twin peaks 1 and 2 of Figure 2 disappeared and two new peaks (1′ and 2′) and an unknown peak eluted earlier in Figure 3, *A*. However, on storage of the extract in a refrigerator for 6 weeks, the elution pattern (Figure 3, *B*) from the autoclaved meal reverted to that from the untreated meal (Figure 2). Apparently the

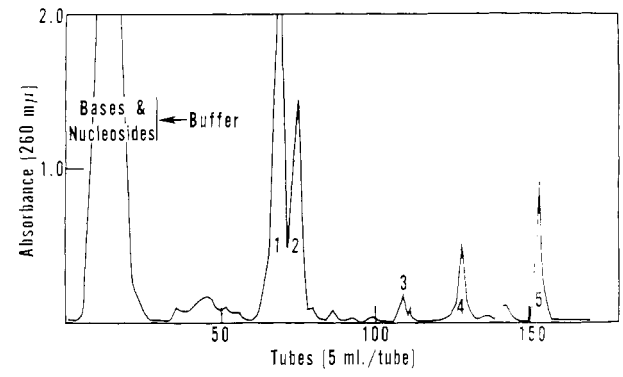

Figure 2. Polyethyleneimide (PEI)-cellulose column chromatography of trichloroacetic acid (TCA) extract from untreated meal

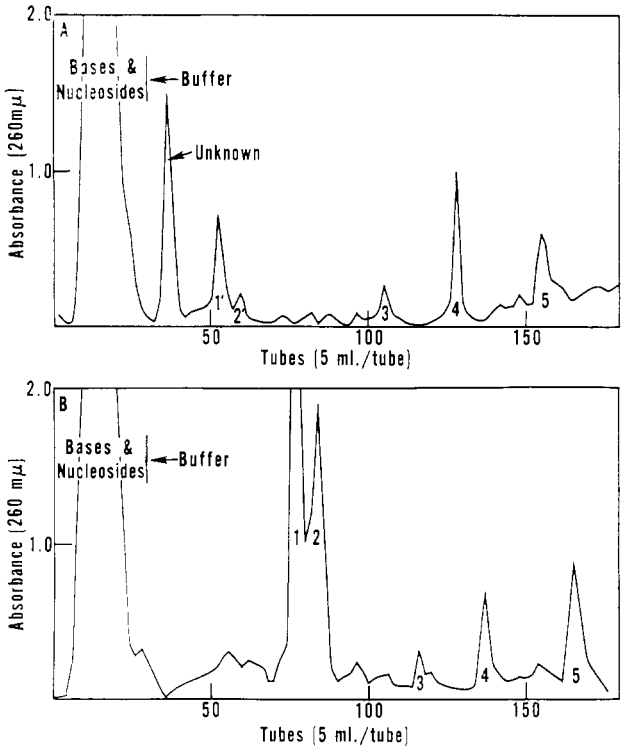

Figure 3. PEI-cellulose column chromatography of TCA extract

A. Autoclaved meal
B. After the extract stored at 4° C. for 6 weeks

effect of autoclaving on the nucleotide elution pattern was reversible.

Alcohol-Washed Meal. Chromatography of the TCA extract from alcohol-washed meal on a PEI-cellulose column gave the elution pattern shown in Figure 4. (Nucleotides in the alcohol extract were not included because the presence of lipids caused plugging of the column.) Comparison of Figures 2 and 4 shows considerable decreases in amounts of all components in the latter elution pattern, presumably the result of partial extraction of nucleotides during alcohol washing of the meal.

The PEI-cellulose chromatography revealed that the nucleotide elution patterns of extracts from treated meals differed from extracts of untreated meals. The significance of the differences in nucleotide elution patterns is under investigation.

Gel Filtration. To characterize fraction F_3 of Figure 1, *A* further, this fraction was subjected to gel filtration on a Sephadex G-25 column. Three major peaks were obtained (Figure 5). The first peak had a characteristic yellow color which absorbed in the 320- to 340-mμ region. No ultraviolet-absorbing spots were observed on paper chromatography of the yellow material and the R_f of the peak corresponded to a pteridine derivative (Kwietny and Bergman, 1959). The second peak absorbed at 273 mμ (pH 7.0 in water) and appeared to be pigment-nucleotide complex for the following reason: When it was lyophilized and extracted with 5 ml. of 95% alcohol, the alcohol-insoluble materials absorbed at 260 mμ (pH 7.0 in water), whereas the alcohol-soluble material was yellow and absorbed at 340 mμ (pH 7.0 in 95% alcohol). When the alcohol-soluble material was dissolved in 0.1*N* NaOH, a shift to 360 mμ occurred. These results indicate that a complex is present which can be broken into pigment and nucleotide. The third peak consisted of nucleotide on the basis of ultraviolet absorbance on paper chromatography, pentose, and phosphate contents, and spectrophotometric measurements (λ_{max} = 259 mμ).

A similar Sephadex G-25 separation was obtained with peak 1 of Figure 2 in which the TCA extract was not adsorbed on charcoal-Celite. This result substantiates our previous conclusion that peak 1 from the PEI-cellulose column corresponds to F_3 from the Dowex 1 column.

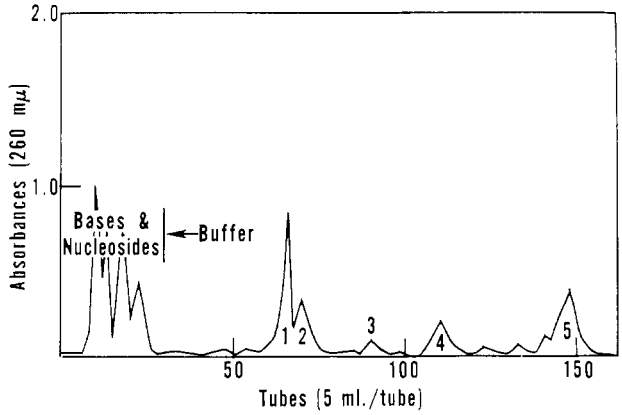

Figure 4. PEI-cellulose chromatography of TCA extract from alcohol-washed meal

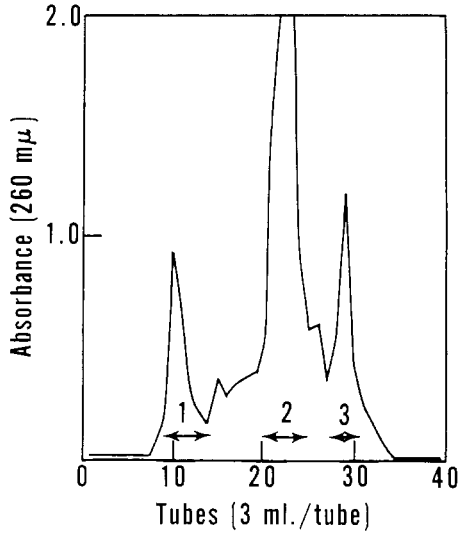

Figure 5. Gel filtration of fraction F_3 from Figure 1, *A*

DISCUSSION

Trichloroacetic acid extracts of mature soybeans contain nucleotides and ultraviolet-absorbing contaminants. Analysis of the extracts for nucleotides usually requires removal of these nonnucleotide materials. Charcoal-Celite treatment of the extract removes some, but not all, of the pigments. On a Dowex 1 column the TCA extract separates into five major ultraviolet-absorbing peaks when the extract is treated at 4° C. Of the five major peaks nucleotides accumulate in two fractions (F_4 and F_6 in Figure 1, *A*). These two fractions contain 56% of the pentose and 68% of the phosphate of the sample, and show multiple ultraviolet-absorbing spots on paper chromatography. Another major peak (F_3 in Figure 1, *A*) contains carbohydrates predominantly and a small amount of nucleotides. When the extract is treated at 23° C., seven major peaks are found. At either temperature some chromatographic fractions still contain yellow pigment even after a second charcoal-Celite treatment. Studies with the PEI-cellulose column and gel filtration chromatography suggest the presence of a pigment-nucleotide complex.

Use of PEI-cellulose permits direct analysis of the TCA extract without charcoal-Celite treatment. Free ultraviolet-absorbing materials from mature soybeans are readily eluted with water. Thus nucleotides are separated without losses from preliminary treatments. Chromatography of the TCA extract on PEI-cellulose resulted in five major ultraviolet-absorbing peaks containing nucleotides as noted with Dowex 1. Likewise two of the peaks contain most of the nucleotides while another consists of a carbohydrate-rich fraction. Alcohol-washing the soybean meal removes the carbohydrate-rich fraction and some of the nucleotides. Autoclaving soybean meal causes changes in the nucleotides and in the nucleotide elution pattern. PEI-cellulose column chromatography shows these changes effectively.

Gel filtration separates the carbohydrate-rich fraction (F_3 in Figure 1, *A*, or peak 1 in Figure 2) into pigments and nucleotides. The separated pigments are greater in size than the nucleotides, judging from their elution position ahead of nucleotide-containing peaks. Polymerization of free pigments may have occurred. Pigments are, perhaps, in some way associated with nucleotides. Supporting evidence includes the following: Pigments are separated from nucleotides by lyophilization and alcohol extraction of the pigment-nucleotide complex; the TCA extract from autoclaved meal shows no pigment-nucleotide complex peak but on storage of the TCA extract the elution pattern reverted to the pattern for an extract from unautoclaved meal. The latter result indicates that pigments and nucleotides can form complexes in vitro. The existence of such complexes in vivo and their importance in the utilization of soybean protein for food need more study.

Key and Hanson (1961) secured a carbohydrate-containing fraction in a TCA extract from hydrated soybean seeds. Key and Wold (1961) identified the carbohydrate as ascorbic acid. Our results indicate that nucleotide extracts from mature soybeans also contain a carbohydrate-rich fraction. This fraction can be destroyed by autoclaving or removed by washing the meal with alcohol. Gel filtration separates this fraction into pigment and nucleotide.

In the processing of soybeans for foods or feeds, autoclaving or alcohol washing has been used to improve its flavor and feeding efficiency (Beckel and Smith, 1944; Klose *et al.*, 1948). These two processes are different in nature yet achieve similar results. No findings have ever clearly indicated the cause for such improvement. Certain nucleotides are known to be flavor enhancers (Kuninaka *et al.*, 1964). Coloration of nucleotide fractions is observed. The changes in soybean nucleotides as a result of treatment of the meal may be significant in relation to flavor, color, and feeding efficiency. Further studies of the effect of nucleotides on physical, chemical, and physiological functions of the meal are needed.

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