THE DETERMINATION OF PANTOTHENIC ACID IN WHEAT

by

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INTRODUCTION

Review of Literature

Williams and co-workers (31) found that water extracts of diverse tissues representing many different biological groups contain a material which is capable of stimulating in a striking way the growth of Gebruder Mayer yeast. These workers concluded that the ability of these extracts to stimulate yeast growth was due to the presence of a single acid substance which appeared to be of universal biological occurrence. They tentatively designated it as pantothenic acid, the name being derived from the Greek meaning "from everywhere".

Koehn and Elvehjem (8) isolated from a liver extract concentrate a factor which prevented pellagra-like symptoms in chicks. Jukes and Lepkowsky (7) called this factor the "filtrate factor" to distinguish it from the pellagra-preventive factor (riboflavin). Snell, Strong and Peterson (23) isolated from an alcohol-soluble liver extract, an acidic ether extractable substance, which is essential for the normal growth of 14 species of lactic acid bacteria. They recognized the similarity between the properties of this substance and that of pantothenic acid.

Numerous individuals have worked on the concentration and purification of pantothenic acid (29, 33, 34, 36). The formula of pantothenic acid was first published by Stiller et al. (25)

after they had crystallized a lactone from the concentrates of pantothenic acid hydrolyzates.

A partial synthesis of pantothenic acid was accomplished by Williams et al. (32) by treating the impure lactone with beta alanine ester and subsequently hydrolyzing the ester group. By heating the dry lactone with the dry sodium salt of beta alanine, approximately the theoretical yield of sodium pantothenate was obtained directly. The total synthesis of pure pantothenic acid was accomplished by Stiller et al. (24) who showed that the synthetic pantothenic acid had the expected biological activity when assayed on chicks and rats.

Lipmann et al. (9) have shown that a coenzyme containing more than 10 percent pantothenic acid is required for the acetylation of aromatic amines in liver tissue and that this is apparently the same coenzyme which is required to acetylate the choline of brain tissue. Lipmann's data indicated that a large part of the pantothenic acid in tissue is present in the form of coenzyme A (9). Since the coenzyme occurs so universally in cells, and since the two acetylations are different types of synthesis (one a peptide linkage and the other an ester bond), it seems likely that coenzyme A may be found to participate generally in carbohydrate, fat and steriod synthesis, as well as in acetate metabolism.

Although pantothenic acid is found in most living tissues, its exact role is not known. It prevents dermatitis in chicks, graying hair and hemorrhagic adrenal glands in young rats. In

dogs, deficiency results in hypoglycemia, convulsions, fatty liver and gastro-intestional disturbances. It is necessary for growth in chicks, rats and microorganisms. Most of its deficiency signs have been studied in tests employing chicks and rats. It is generally regarded as being important in human nutrition but little information is available on this subject. It has been observed that the level of pantothenic acid in the blood is about 25 percent below normal in humans deficient in other vitamins of the B complex. Possibly it is synthesized by microorganisms in the intestines of humans, since it is known to be synthesized similarly in the rumen of cattle and sheep (20).

Methods of Determination

The Biological Method. The chick growth method of analysis has been used most extensively by Jukes (6). This method involves a two week growth period, and the results are usually in good agreement with those obtained by microbiological assay using L. casei. When certain materials such as liver extract, rice bran extract and particularly yeast, are tested, the values obtained by the chick assay method are much higher than the values obtained by microbiological assay. These excessively high values appear to be due to the presence of growth promoting substances other than pantothenic acid in the materials tested (17).

The Microbiological Method. Several microbiological assay methods of pantothenic acid have been reported (2, 5, 16, 22, 26). Pelczar and Porter (16) showed that pantothenic acid is one of the growth factors required by Proteus morganii. This test organism was found to be capable of responding to a lower dosage of pantothenic acid than the organisms used in other methods. When as little as 0.0002 gram of synthetic calcium pantothenate was added per ml of the assay medium, visible growth occurred. Thus it is possible to determine the pantothenate content of extremely small amounts of natural substances, a fact which may be significant, since other nonspecific factors which might affect the test such as inhibitory substances and interference by color or opacity, can be avoided by dilution.

Two lactic acid bacteria, L. casei and L. arabinosus, have been widely used as test organisms. The original method of Pennington, Snell and Williams (18) together with the modifications of Strong, Feeney and Earle (26) using L. casei has proved generally useful and satisfactory for routine determinations of pantothenic acid in a wide variety of materials. This method used Clarase or papain to release the vitamin. Certain difficulties were encountered with L. casei, however, even with the use of modified media and extraction procedures. The organism responded erratically in the presence of small amounts of starch, fats, and fatty acids so that it was necessary to digest samples of test materials enzymatically, and

to extract them with ether to remove excess fat prior to performing the assays (26). A more recent method advocated by Skeggs and Wright (22) as well as by Hoag et al. (5) made use of L. arabinosus 17-5, with a medium containing acid-hydrolyzed casein, fortified by cystine, tryptophan, and synthetic vitamins. This is the method most generally used today since L. arabinosus is not stimulated as much by fatty material as is L. casei.

L. arabinosus as well as L. casei measures only free pantothenic acid. Releasing it from its bound form in the protein molecule without destroying pantothenic acid itself is the real problem. This was made evident by repeated observations that none of the methods for releasing pantothenic acid by such enzymes as papain, Taka-diastase, Mylase-P or Clarase gave values which even approached the high values obtained by chick assay (15). Neither acid nor alkali hydrolysis can be used, since the pantothenic acid molecule, which is an acid amide, would be split into beta alanine and the substituted butyric acid. Neither of these products of hydrolysis is a nutrilite for L. arabinosus or L. casei (23). As shown by Lipmann et al. (9), coenzyme A is not broken down to release pantothenic acid by any means except autolysis, treatment with intestinal phosphatase, or pigeon or chick liver extract. By using a papain-Clarase digestion method, only about 0.15 percent pantothenic acid was liberated from this coenzyme, but with the use of intestinal phosphatase and pigeon liver enzyme, approximately 10

percent of the coenzyme was found to be pantothenic acid. This figure was confirmed by an independent assay of the beta alanine present, using the yeast method (21). Lipmann's recommendation for sample preparation prior to pantothenate analysis is, therefore, treatment of the sample with intestinal phosphatase plus chicken liver enzyme preparation. Samples, such as yeast or liver thus treated, give pantothenate values approximating those obtained by chick assay. Both enzymes are essential since it has been shown that 20 to 30 percent of the pantothenate is released by dephosphorylation and about 70 percent by the action of freshly prepared extract of chicken liver acetone powder (15).

The Chemical Method. Williams (30, p. 265) suggested a chemical method for the assay of pantothenic acid based upon the selective oxidation of pantoic lactone by lead tetraacetate. The oxidation was found to be erratic, however, and not very selective when applied on a micro scale to natural extracts. Wollish and Schmall (35) pointed out a rapid colorimetric method for the assay of panthenol (alpha, gamma-dihydroxy-beta, beta-dimethyl butyramide)[1] and pantothenates, which can be applied to the assay of panthenol and D-calcium pantothenate in pure form as well as in tablets and ampoules. The colorimetric method is based upon the formation of pantoyl

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[1] Panthenol is the biologically active hydroxy analog of pantothenic acid.

lactone by hydrolytic cleavage in an acid medium. The lactone reacts with hydroxylamine in the presence of alkali and the hydroxamic acid thus formed yields a purple color upon acidification in the presence of ferric chloride. This color, having a maximum absorption at 500 millimicrons, is utilized for photometric measurements. The results are in good agreement with those obtained using the microbiological method.

Recently Szalkowski, Mader and Frediani (27) developed a chemical method based on the hydrolysis of calcium pantothenate and its cleavage to beta alanine and alpha, gamma-dihydroxy-beta, beta-dimethyl butyric acid. Beta alanine, when oxidized with potassium permanganate in the presence of potassium bromide under properly regulated conditions, yields an insoluble hydrazone with 2,4 dinitrophenylhydrazine. Since the ratio between the amount of hydrazone formed and the hydrolyzed pantothenate oxidized is constant, the calcium pantothenate may be estimated by dissolving the dinitrophenylhydrazone in pyridine, diluting with sodium hydroxide and measuring the resulting blue color spectrophotometrically at 570 millimicrons. Certain interfering compounds, with the exception of ascorbic acid, are eliminated by chromatographic absorption in an aluminum oxide column.

The vitamin content of wheat is known to vary with the variety and environmental conditions such as soil type and weather (11, 12, 19). Several workers (2, 3, 6, 10, 13, 26, 28) have determined the calcium pantothenate content of wheat

and wheat products employing either the biological or the microbiological methods of assay. It has been the object of this work to determine by chemical as well as microbiological methods of assay the calcium pantothenate content of several species and varieties of wheat grown under comparable environmental condition.

MATERIAL AND METHODS

Material

Sixteen samples of wheat comprising eight species from the 1950 crop grown at Brawley, California under comparable environmental conditions were assayed for calcium pantothenate. The samples were prepared for assay by grinding in a Wiley mill to pass a 1 mm wire screen. Moisture and crude protein were determined using methods reported in Cereal Laboratory Methods (1).

Calcium pantothenate used for preparing the calibration curve for both the chemical and microbiological methods was obtained from Merck and Co., Inc. The test organism L. arabinosus 17-5, was obtained from the American Type Culture Collection, Georgetown University, School of Medicine, Washington, D. C.

Adaptation of a Chemical Method to the Analysis of Calcium Pantothenate in Wheat

Calcium pantothenate was determined in a trial sample of wheat using the chemical procedure outlined by Szalkowski et al. (27). The amount of calcium pantothenate thus measured was about 500 µg/gm, although values reported in the literature ranged between 5 and 19 µg/gm (2, 3, 6, 10, 13, 26, 28). This high result obtained in the trial run indicated the possible interference of compounds such as carbohydrates, thiamine hydrochloride, niacin and niacinamide (27). The presence of carbohydrate material in the chromatographic column was demonstrated by a positive Molisch test and by the development of a dark brown color on boiling the eluate with the sulphuric acid used to hydrolyze the calcium pantothenate.

The calcium salts of pantothenic acid have been described by Stiller et al. (24) as microcrystalline, hygroscopic powders which were readily soluble in water and the lower alcohols. Ford (4) observed that calcium pantothenate was soluble to the extent of more than 50 gm/100 ml of methanol. Methanol instead of water was used, therefore, to extract the pantothenates from wheat. In this new treatment, the possibility of interfering compounds, such as sterols and certain fat-soluble vitamins, which are soluble in methanol and yield insoluble dinitrophenylhydrazones after oxidation with potassium permanganate, was considered. Cereals, however, are poor sources for vitamins A and

K and furthermore these vitamins are not adsorbed on the alumina column from methanol. Carotenoids, although soluble in methanol, are strongly adsorbed on aluminum oxide and are not easily eluted. To test the effect of soluble lipid material on the results, a 10 gram sample of wheat was extracted with petroleum ether, and the extract dissolved in 15 ml of methanol. The data in Table 1 show that the petroleum ether soluble compounds did not affect the analysis of calcium pantothenate.

Table 1. The effect of lipid material on the determination of calcium pantothenate.

Mixture	Calcium pantothenate	
	Calculated	Found
	µg	µg
2 ml methanol solution + 25 ml methanol, chromatographed	no color developed	
2 ml methanol solution + 10 gm wheat/100 ml methanol; 25 ml chromatographed	18.2	18.2
4 ml methanol solution + 10 gm wheat/100 ml methanol; 25 ml chromatographed	18.2	18.0
2 ml methanol solution + 25 ml calcium pantothenate solution; 25 ml chromatographed	16.0	16.0
4 ml methanol solution + 25 ml calcium pantothenate solution; 25 ml chromatographed	16.0	16.0

The chemical method used in this work was the same as that outlined by Szalkowski et al. (27) except for using methanol to extract the pantothenates. The concentrations of solutions were also changed to conform to the concentration of pantothenates in wheat. Three portions of wheat, 8, 12, and 16 grams, were treated separately. Each portion was suspended in 100 ml methanol, shaken vigorously and centrifuged for 10 minutes or until a clear solution was obtained. A 25 ml aliquot of the clear solution was chromatographed, eluted, oxidized and measured as described by Szalkowski et al. (27). The standard curve obtained for pure calcium pantothenate, using the modified chemical method, is shown in Fig. 1.

The recovery of calcium pantothenate using different levels of both wheat and calcium pantothenate and the chemical method of analysis is summarized in Table 2. The recovery was 98.9±2.9 percent. The standard deviation for 9 determinations of pure calcium pantothenate solution containing 80 µg/ml was 3.13 µg/1 ml. The standard deviation for 7 determinations of calcium pantothenate contained in 8 grams of wheat (144.32 µg) was 6.5 µg/analysis.

Table 2. Recovery of calcium pantothenate.

Mixture	Calcium pantothenate		
	Calculated	Found	Recovery
	µg	µg	%
10 gm wheat + 80 µg Ca pantothenate	262	255	97.3
15 gm wheat +100 µg Ca pantothenate	373	380	101.8
20 gm wheat +200 µg Ca pantothenate	564	550	97.5
Average			98.9±2.9

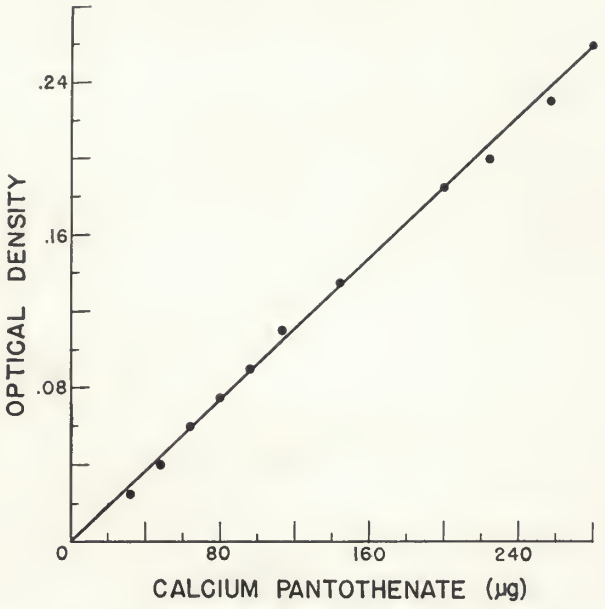

Fig. 1. Calibration curve using the modified chemical method.

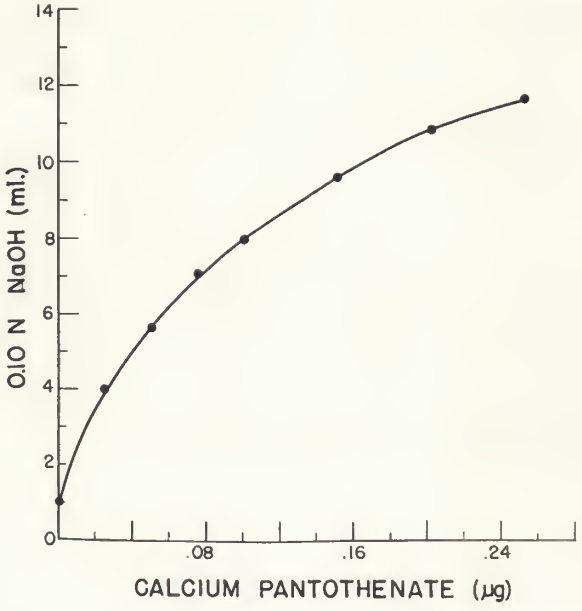

Fig. 2. Calibration curve using the microbiological method.

Microbiological Method

The microbiological method outlined by Skeggs and Wright (22) was used without modification. Pantothenates were released by digesting wheat samples with alkaline phosphatase and chicken liver extract (15). A typical calibration curve is shown in Fig. 2.

RESULTS AND DISCUSSION

The calcium pantothenate content of the different species of wheat as determined by both the chemical and microbiological methods is recorded in Table 3. Chemical values are the average of three separate analyses of three different levels in duplicate. The microbiological values are the average of two analyses each made with four different levels in duplicate. The crude protein content, and the calcium pantothenate content in the various samples are recorded on a 14 percent moisture basis.

A comparison of the mean differences between the two methods used (Table 3) shows the nature of the difference, with the chemical method consistently yielding higher values. The difference ranged from about one-half of one percent to ten percent of the overall mean, with the average difference being about three percent of the mean. Such differences are statistically significant when compared with the sampling differences.

Table 3. The calcium pantothenate content of whole wheat.

Species and varieties of wheat	Crude protein	Calcium pantothenate: Chemical method	Calcium pantothenate: Micro-biological method	Calcium pantothenate: Difference
	%	µg/gm	µg/gm	µg/gm
T. monococcum	12.9	12.2	11.4	.8
T. diococcum, vernal	11.7	20.5	20.6	- .1
T. orientale	8.8	10.1	9.8	.3
T. pyramidale	8.1	12.0	10.7	1.3
T. persicum	9.2	11.2	10.1	1.1
T. durum				
Stewart	8.7	12.2	11.5	.7
Pentad	10.3	14.3	13.7	.6
T. sphaerococcum	11.8	10.1	10.1	.0
T. vulgare				
Baart (White)	10.5	9.8	8.9	.9
Federation (White)	9.9	11.5	11.2	.3
Dawson (White)	13.5	16.7	16.1	.6
Thatcher (Hard Red Spring)	11.3	11.2	11.0	.2
Marquis (Hard Red Spring)	12.6	17.1	16.4	.7
Trumbull (Soft Red Winter)	14.8	19.9	19.3	.6
Comanche (Hard Red Winter)	12.6	15.3	14.6	.7
Red Chief (Hard Red Winter)	11.8	14.8	14.5	.3

The analyses of variance for the chemical method, the microbiological method and a comparison of the estimates of variances for the corresponding sources in the two methods are tabulated in Table 4. None of the tested sources of variation other than species and varieties within species showed any real effects on the chemical method of determination. For the microbiological method of determination, except for the species and variety in species, only the (variety in species) x (levels in duplication) interaction was significant. This difference indicated that the relative differences among the varieties

was not consistent from one level of action to another, which may be regarded as a disadvantage of this method.

The comparison of the estimates of variance for the corresponding sources in the two methods showed homogeneity, which permitted combination of the entire set of results into a single analysis. In the pooled analysis, the mean square between methods was considerably larger than any of the estimates of variance as computed from the interactions or sampling variation. This indicates that the likelihood of obtaining such differences due to chance variation is exceedingly small.

The largest contributors to the variation in results were the species and variety within species differences. Since these samples were grown under the same environmental conditions, the large differences between the calcium pantothenate content of the species and varieties within species (Table 3) are probably due to genetic variation.

Table 4. Analyses of variance.

Source of variation	Degrees of freedom	Mean square
The Chemical Method		
Species	7	41.19**
Varieties in species	8	36.44**
Levels in action	2	.12
(Species) x (Levels)	14	.05
(Variety in species) x (Levels)	16	.05
The Microbiological Method		
Species	7	249.98**
Varieties in species	8	189.01**
Duplication of method	1	.35
(Species) x (Duplication)	7	.43
(Variety in species) x (Duplication)	8	.05
Levels within duplications	6	.73
(Species) x (Levels in duplication)	42	.34
(Variety in species) x (Levels in duplications)	48	.53**
Determinations in levels	8	.14
(Species) x (Determinations in levels)	56	.08
(Variety in species) x (Determinations in levels)	64	.08
Both Methods		
Species	7	290.63**
Varieties in species	8	224.97**
Methods	1	13.56**
(Species) x (Methods)	7	.69
(Variety in species) x (Methods)	8	.49
Sampling variation	220	.28

A comparison between the results obtained in this investigation and those given in literature is shown in Table 5. The quantities of calcium pantothenate obtained by both methods of analysis agree well with the values reported by Teply et al. (28) who determined calcium pantothenate in a number of varieties of T. vulgare grown in various parts of the United States. These workers, in contrast with the present work, found that neither varietal nor environmental differences had any significant effect on the amount of calcium pantothenate present in four varieties of dark hard winter wheat grown at four localities. Values for miscellaneous varieties representing all classes of T. vulgare grown in Kansas, Minnesota, Oklahoma, Texas and Washington indicated that genetic differences were of little consequence in affecting the calcium pantothenate content of soft red winter wheat, while environmental differences had considerable effect. Statistical analyses of these data were not available.

Table 5. Calcium pantothenate content in wheat as determined by different workers.

Source of determination		Calcium pantothenate	No. of samples	Method of assay
		µg/gm		
This work		9.8 - 20.5	16	Chemical
		8.9 - 20.6	16	Microbiological
Atkin et al.	(2)	8.3 - 8.9	3	Microbiological
Cheldelin & Williams	(3)	13.1	1	Microbiological
Jukes	(6)	12.2	2	Chick assay
Loy	(10)	9.8	1	Microbiological
Moran & Drummond	(13)	5.5	1	Microbiological
Strong et al.	(26)	9.0	1	Microbiological
Teply et al.	(28)	9.9 - 19.0	55	Microbiological

CONCLUSIONS

The chemical method for calcium pantothenate determination (27) was modified to be applicable to the extraction and concentration of pantothenates in wheat. Comparing the chemical results with those from the presently accepted microbiological method showed fairly consistent differences from one species and variety to another between the two methods. The chemical method, generally, gave higher (averaging 3 percent) values than did the microbiological method for almost all species and varieties studied.

Differences among species and varieties in species were significant in both methods of analysis. The analyses of variance indicated no effect due to differences in level of activity on the results in either method, although some interaction for the several varieties was in evidence in the microbiological method. The analyses also showed that the technique of laboratory analysis was sufficiently refined that no appreciable variation was introduced in the results.

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AN ABSTRACT OF A THESIS

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1952

A chemical method for the determination of calcium pantothenate was adapted to the assay of wheat. The adaptation of the method consisted of the extraction and concentration of the pantothenates from wheat with methanol instead of water which was used in the original method. Investigations showed that possible interfering compounds such as sterols and certain fat-soluble vitamins, which are soluble in methanol and which yield insoluble hydrazones after oxidation with potassium permanganate have no effect on the analysis of calcium pantothenate under the conditions employed. Recovery of calcium pantothenate using the modified chemical method averaged 98.9 percent.

Sixteen varieties of wheat representing eight species grown under similar environmental conditions were assayed for calcium pantothenate using both the modified chemical method and the microbiological method with L. arabinosus 17-5 as the test organism. A comparison of the mean difference in the assay values obtained by the two methods showed that the chemical method yielded consistent high results (averaging 3 percent). This difference was statistically significant. Differences among species and varieties in species were significant in both methods used. Since the samples were grown under the same environmental conditions, the large differences between the calcium pantothenate content of the species and varieties are probably due to genetic variation. The range of calcium pantothenate in the samples assayed (8.9-20.6 µg/gm) agreed well with the values reported in the literature.