AN ABSTRACT OF THE THESIS OF

Raymond LeRoy Pederson for the M. S. in Organic Chem.
(Name) (Degree) (Major)

Date Thesis presented June, 1944

Title Microbiological Assay of Nicotinic Acid in Natural Materials

Abstract Approved [redacted]
(Major Professor)

During the routine determination of the vitamin contents of dehydrated foods, it was observed that nicotinic acid values were often lower than expected. The methods of extraction of the food samples were therefore studied, together with the manner of preparation of the assay medium in an effort to secure optimum assay conditions.

The procedure followed in the improvement of the assay method was to use extracts of natural materials in the basal medium provided for the growth of Lactobacillus arabinosus. These extracts were rendered nicotinic acid-free by adsorption on Lloyd's reagent. Of the materials investigated, yeast and peptone were included in the medium. These extracts furnished several of the known vitamins and also possessed extra stimulatory ingredients required by the organism. The improved medium was simple and afforded an increased sensitivity of the organism to nicotinic acid; also the standard curve obtained by use of this medium was linear over a wide range. The time required to establish a satisfactory standard curve has been shortened from 72 hours to 36 hours.

MICROBIOLOGICAL ASSAY OF NICOTINIC ACID IN NATURAL MATERIALS

by

RAYMOND LEROY PEDERSON

A THESIS

submitted to the

OREGON STATE COLLEGE

in partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE

June 1944

APPROVED:

[redacted]

Associate Professor of Chemistry

In Charge of Major

[redacted]

Head of Department of Chemistry

[redacted]

Chairman of School Graduate Committee

[redacted]

Chairman of State College Graduate Council

ACKNOWLEDGMENTS

The author wishes to express his gratitude to Dr. V.H. Cheldelin, Associate Professor of Chemistry, for having made this research possible, and for the direction and advice received during the study.

The author also wishes to have expressed to Dr. H. P. Sarett, Research Associate in Chemistry, gratefulness for the regular consultations received in the laboratory during the progress of this work.

The author is indebted to the Committee on Food Composition of the National Research Council for supplying the dehydrated foods on which part of the work for this thesis was done.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

MICROBIOLOGICAL ASSAY OF NICOTINIC ACID IN NATURAL MATERIALS

INTRODUCTION

Nicotinic acid, popularly called niacin, has received widespread attention since its discovery in 1937 as the anti-pellagra vitamin (6). The vitamin occurs chemically combined as di- and tri-phosphopyridine nucleotides and also occurs free as nicotinamide and nicotinic acid. Its quantitative measurement in biological materials is extremely important in the study of nutrition.

Two types of methods exist for the measurement of nicotinic acid. They are (a) chemical methods (4,13), which depend upon the formation of a colored product from nicotinic acid with cyanogen bromide and an aromatic amine (8), the resulting color being measurable in a colorimeter, and (b) microbiological methods utilizing lactobacilli (18) or dysentery bacilli (5). The method used in this laboratory utilizes Lactobacillus arabinosus 17-5. This method is based on the fact that the organism requires nicotinic acid for growth and that a direct product of its metabolism is lactic acid, which can be measured by titration with standard alkali. All variations of these methods require preliminary extraction of the material to release the vitamin from the structure in which it is incorporated. The methods of extraction in general use are hot digestion with sulfuric acid, sodium hydroxide, water, and digestion at 37° C.

with enzymes.

PART I

COMPARISON OF EXTRACTION METHODS

A comparison of extraction methods by Cheldelin and Williams (3) has indicated that for fresh foods of animal origin and for most fresh vegetables maximum extraction of nicotinic acid could be effected by enzyme, acid, or alkaline digestion. Hot water extraction has been found generally insufficient (3,13). Enzyme extraction has therefore been relied upon in this laboratory as an effective means of releasing nicotinic acid from fresh materials of the types described above.

Values obtained for nicotinic acid in dehydrated foods* after enzyme digestion have often appeared surprisingly low. In view of the stability of this compound to most chemical changes, it appeared unlikely that dehydration would be accompanied by destruction of the vitamin. It was therefore decided to compare the amounts of nicotinic acid obtained for these materials by enzyme digestion with the amounts obtained after acid and alkaline extraction.

---

* Assays of dehydrated foods were performed as part of a collaborative study conducted by the Committee on Food Composition of the National Research Council.

EXPERIMENTAL

Method of Extraction

The following methods of extraction were used:

(a) Acid--A one gram sample in 30 ml. of 1N sulfuric acid was autoclaved at 15 pounds pressure for 20 minutes, cooled, neutralized to pH 4.5, diluted to final volume, filtered, and neutralized to pH 6.8.

(b) Alkali-- As in (a) except that 1N sodium hydroxide was used instead of sulfuric acid.

(c) Enzyme-- One gram of sample was suspended in 30 ml. of 1% acetate buffer having a pH value of about 4.5. To this was added 1 ml. each of a suspension of takadiastase and papain. (Each suspension contained 20 mg. of the enzyme per ml.) The mixture was then incubated with occasional agitation at 37° C. for 24 hours under benzene, steamed 30 minutes, diluted to volume, filtered and neutralized as above.

Method of Assay

The microbiological method of Snell and Wright (18) was employed in this study. Acid production was measured after 72 hours' growth by titration with 0.1N sodium hydroxide solution.

Results

The nicotinic acid contents of a number of dehydrated foods as obtained by acid, alkaline, and enzyme extraction are recorded in Table I.

TABLE I

Nicotinic Acid Content
Obtained by the Various Extraction Methods

Sample No.	Material	Nicotinic Acid Content Micrograms Per Gram Acid	Alkaline	Enzyme
1	Beet	7.7		6.3
2		14		9.0
3		13		13
1	Cabbage	28		28
2		21		24
3		24		14
4		32		24
5		29		15
6			18	18
1	Carrot	17	14	10
2		33		22
3		14		14
4		16		13
5		24		16
6		32		16
7		22		10
1	Cranberry	7.3		7.1
1	Onion	12		11
2		12		10
1	Pea Soup	27		25
1	Pea Soup (Yellow)	43		23
1	Pea Soup (Green)	30		24
1	Potato (White)	26		18
2		42		25
3		50		20
4		36		8.0
5		19		12
6		16		11
7		35	34	9.0
8		[illegible]	28	12
9		52	48	16
10		38	35	10
11		30	23	12
12		30	34	13

TABLE I (cont.)

1	Sweet Potato	16	18	14
2		16	17	
3		13	13	
4		10	11	
5		12	17	
6		14		15
7		13		7
1	Tomato	53		54
1	Turnip	57	43	25
1	Liver, B concentrate	1430		1280
1	Liver, B injection sample	820		770
1	Yeast	500		490

DISCUSSION

It is evident from Table I that yields of nicotinic acid from dehydrated foods are in general much higher after acid and alkaline extraction than after enzyme digestion. This is in direct contrast to the findings for fresh foods (3) and suggests that part of the nicotinic acid present in these materials may become tightly "bound" during dehydration, possibly to tissue proteins. It is not known, however, what fraction of the vitamin found as a result of alkaline or acid treatment is available nutritionally. Animal assay methods which are reliable for low potency materials of this sort have not been developed. Chemical assays of dehydrated foods would be of little value for comparison, since in these methods all samples are digested with acid or alkali prior to assay. A second explanation of the difference in nicotinic acid values obtained from the various extraction methods may be that an incomplete assay medium was used. (This is discussed in Part II). Extracts prepared by acid or base treatment serve to complete the medium, whereas extracts prepared by enzyme treatment do not.

The existence of at least one unknown precursor of nicotinic acid has been demonstrated in cereals (1,3). It possesses no growth-promoting activity for L. Arabinosus, but is very readily converted to nicotinic acid by alkali,

less readily by acid, and only with difficulty by hot water. This precursor has been assumed to be responsible for the increased values obtained by acid and alkaline extraction over enzyme digestion in the assay of cereals. Studies in this laboratory suggest its presence in fresh potatoes as well. This may account in part for the high values obtained for dehydrated potatoes after acid or alkaline extraction.

Recently Bovarnich (2) has produced nicotinic acid in small amounts (up to 350 micromoles per mole) by prolonged heating of asparagine with glutamic acid or other amino acids in the presence of oxygen or hydrogen peroxide. This work was repeated in this laboratory using asparagine and glutamic acid with comparable results. It was therefore considered possible that nicotinic acid might be produced in varying amounts from amino acids contained in the samples during the preparation of extracts. To check this possibility, dehydrated potatoes were prepared for assay by enzyme, acid and alkaline digestion in the presence of 100 mg. each of asparagine and glutamic acid per gram of sample. When the nicotinic acid contents were compared to those obtained in the absence of these amino acids, only slight increases (from 0-10%) were found as a result of the added glutamic acid and asparagine.

Although acid and alkaline extraction usually release much greater amounts of nicotinic acid than does enzyme di-

gestion, the two former procedures do not always yield comparable results. Acid extraction of white potatoes often gives higher yields of the vitamin than does alkaline extraction. In sweet potatoes the situation is reversed. It would seem then that the completeness of extraction (or conversion of precursors) may be dependent upon the nature of the material being examined. In this connection the observations of Dann and Handler (4) are of interest, wherein they found a considerably higher nicotinic acid value in fresh meats extracted with acid than in those extracted with alkali.

PART II

AN IMPROVED MICROBIOLOGICAL ASSAY FOR NICOTINIC ACID

The microbiological method of determination of nicotinic acid as established by Snell and Wright (18) has been used extensively in vitamin research and routine assays of foods. Results obtained from routine work performed in this laboratory, such as those obtained in Part I of this paper, indicated that the growth medium employed was inadequate.

The chief difficulties encountered when the Snell-Wright medium was employed were: (1) a standard curve which was linear over a very limited range. This condition suggested that factors other than nicotinic acid were limiting growth in regions where nonlinearity existed. (2) On the above medium the organism was incapable of producing maximum amounts of acid. As shown in Figure 1, the maximum quantity of acid produced in each culture using this medium was approximately 9 ml., whereas the addition of extra glucose and acetate (19) together with 10 mg. of yeast or liver resulted in the production of approximately 20 ml. of acid per culture (7). (3) When increasing amounts of materials were assayed, the calculated nicotinic acid values frequently exhibited an upward or downward trend so that the extreme values obtained over a fourfold concentration range

often showed discrepancies as great as 30%.

To remedy these difficulties two methods of attack were considered. The first method, which has been used by Landy and Dicken in studying Lactobacillus casei (11) and by others (9,10,12,16) for L. arabinosus, has been to investigate as completely as possible the growth requirements of the organism and to provide the required nutrients (other than the one being assayed) in optimum concentrations. This procedure has resulted in the development of synthetic media in which the only component of unknown composition was hydrolyzed vitamin free casein. Such media have contained as many as 20 ingredients, and where the casein hydrolysate has been replaced by pure amino acids, the number of ingredients has risen to 36 (12). These media are relatively difficult to compound, and great care must be exercised to prevent contamination and/or deterioration of the components. In addition, the omission of natural extracts which contain stimulatory ingredients of unknown nature (thus preventing duplication by synthetic materials) limited the growth of the organism. (7)

The second method, which has been followed in the present work, has been to prepare media containing extracts of yeast, liver, peptone or other natural materials which have been treated to remove the vitamin being assayed. The unknown stimulatory substances mentioned above are meanwhile retained in the basal medium in varying amounts and

tend to overcome the extra stimulatory effects produced by the materials to be tested. The removal of nicotinic acid, however, from materials has been difficult, due to the stability of the vitamin towards most reagents. Complete adsorption of the vitamin from peptone or yeast on charcoal cannot be readily effected. Selective adsorption is thus difficult, and charcoal treatment has been of little value in producing nicotinic acid extracts which still possess appreciable growth-promoting activity.

Lloyd's reagent has been employed successfully by Perlzweig, Levy, and Sarett (15) and by Dann and Handler (4) for quantitative adsorption of nicotinic acid. This adsorption is effected in a strongly acid medium in which the formation of hydrogen ions by nicotinic acid is repressed and the basic properties of the vitamin predominate. This method of removal of nicotinic acid has been applied successfully in the present method to peptone and yeast extracts, and a basal medium has been developed which is believed to be reliable for assay of nicotinic acid.

EXPERIMENTAL

Organism

The organism used was Lactobacillus arabinosus 17-5. Inocula were prepared by transfer from stock cultures to an inoculum tube containing the basal medium described below, to which had been added one microgram of nicotinic acid per tube. After incubation at 37° for 24 hours the cells were centrifuged and resuspended in 0.9% sodium chloride solution. One drop of the resulting suspension was then added aseptically to each assay tube.

Basal Medium

The proposed nicotinic acid free medium is listed in Table II.

TABLE II

Basal Medium for Nicotinic Acid Assay

Vitamin free acid hydrolyzed casein	10	g.
Peptone (treated with Lloyd's reagent)	5	g.
Yeast Extract (")	2	g.
Glucose	40	g.
Sodium Acetate, anhydrous	40	g.
Cystine	400	mg.
Calcium pantothenate	200	micrograms
Biotin	0.4	micrograms
Inorganic Salts--Solutions A and B	10	ml. each
Distilled water to make one liter		

The ingredients of the above medium were prepared as follows:

Peptone--Ten grams of Bacto-Peptone were dissolved by

autoclaving 15 minutes in 100 ml. of water. The solution was acidified to pH 1.0 with concentrated HCl, and ten grams of Lloyd's reagent were added. This mixture was agitated five minutes and centrifuged. The supernatant liquid was decanted and the pH was adjusted to 1.0. The adsorption was then repeated twice with Lloyd's reagent as above. The resulting liquid was filtered through Celite to remove traces of dispersed adsorbent and neutralized to pH 4.5. Two grams of K_2HPO_4 were added and the extract steamed to precipitate dissolved Lloyd's reagent. The extract was then neutralized to pH 6.8, filtered and diluted to 200 ml. This solution was stored in a refrigerator.

Yeast--Ten grams of Difco Yeast Extract were treated with Lloyd's reagent in the manner described for peptone.

Casein--Bitamin free casein (Harris) was hydrolyzed as described by Pennington, Snell and Williams (14).

Glucose--Mallinckrodt C.P. anhydrous.

Sodium Acetate--An amount of $NaC_2H_3O_2 \cdot 3H_2O$ equal to 40 grams of the anhydrous salt was used per liter of medium.

Cystine--500 mg. of l-cystine were dissolved in a minimum amount of hot concentrated hydrochloric acid and diluted to 500 ml. with water. The solution was preserved under toluene.

Calcium Pantothenate--A weighed amount was dissolved in water to obtain a solution containing 100 micrograms per ml. and stored in the refrigerator.

Biotin--Crystalline biotin was dissolved in water and diluted to a concentration of one microgram per ml. The solution was stored in the refrigerator.

Inorganic salts--The salt solutions A and B were prepared as described by Snell and Strong (17).

Nicotinic Acid--A standard solution (100 micrograms per ml.) was prepared and stored in the refrigerator. A secondary standard containing 0.10 microgram per ml. was prepared daily from the above solution for use in assays.

Procedure

A medium having the composition specified in Table II is prepared using the ingredients described above. Care must be exercised to avoid contamination when aliquots are removed from stock solutions. The medium is neutralized to pH 6.8 and diluted to the desired volume. Samples for assay are chosen to contain 0.05 to 0.3 micrograms of nicotinic acid. Tubes containing 0, 0.05, 0.10, 0.15, 0.20, 0.30, and 0.50 micrograms of nicotinic acid are used to establish the standard curve. The contents of all the tubes are then diluted with water, and 5 ml. of the basal medium are pipetted into each tube. The tubes are plugged with cotton and autoclaved at 15 pounds pressure for 15 minutes. When cool the test is inoculated as described previously.

Samples to be assayed are digested with either sul-

furic acid or with takadiastase and papain as described on page 4.

Results

The response to added nicotinic acid as determined by the present medium and the Snell-Wright medium is shown in Figure 1. On the present medium, nearly direct proportionality exists between the quantity of acid produced and the amounts of the vitamin added over the range 0.05 to 0.30 micrograms. The response produced by the vitamin over this range is 1.30 ml. per 0.05 micrograms of nicotinic acid. The standard curve obtained with the original medium, however, slopes rapidly away from the linear above 0.2 micrograms of nicotinic acid, and the corresponding response produced by the vitamin is 0.90 ml. per 0.05 micrograms.

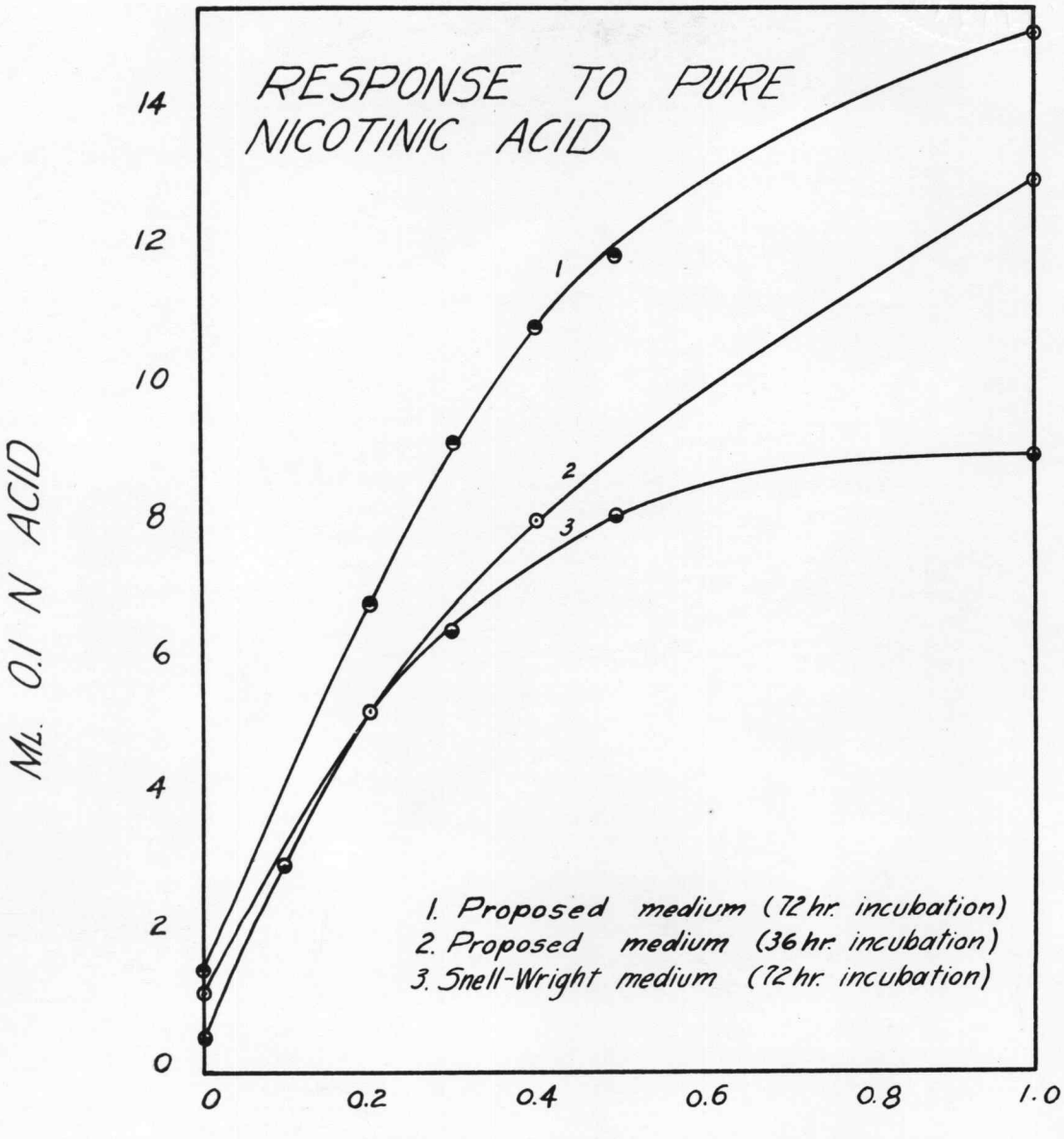
RESPONSE TO PURE NICOTINIC ACID
Ml. 0.1 N ACID
14
12
10
8
6
4
2
0
0
0.2
0.4
0.6
0.8
1.0
1
2
3
1. Proposed medium (72 hr. incubation)
2. Proposed medium (36 hr. incubation)
3. Snell-Wright medium (72 hr. incubation)
Micrograms Nicotinic Acid

Figure 1

APPLICATION OF METHOD

TABLE III

Nicotinic Acid Assay Values
and Recoveries of Added Nicotinic Acid

Material	Amount of Material per Assay Tube (mg.)	Nicotinic Acid Found (Micrograms)	Nicotinic Acid (Micrograms per Gram)	Recovery of Added Nicotinic Acid (%)
Carrot	4.0	0.045	11.3	108
	8.0	.100	12.5	101
	12.0	.145	12.1	
	16.0	.184	11.5	
			Ave. 11.3	
Doughnut	6.0	0.177	19	100
	12.0	.220	18	
	18.0	.305	17	
	24.0	.440	18	
			Ave. 18	
Ham	4.0	0.174	43	
	6.0	.257	43	
	8.0	.365	46	
			Ave. 44	
White Potato	4.0	0.068	17	105
	8.0	.127	16	103
	12.0	.174	15	
	16.0	.235	15	
			Ave. 16	

The nicotinic acid contents of some common foods are recorded in Table III, together with recoveries of nicotinic acid added to these substances.

It may be seen from Table III that good agreement is

obtained among values calculated from increasing assay levels of materials, and that recoveries of added nicotinic acid are very nearly the theoretical values. A slight downward trend of values is noted, but it is less than has often been observed using the original medium in this and in other laboratories (9,18).

DISCUSSION

The principal advantages of the present medium are; (1) the greater response of the organism to nicotinic acid, (2) the extension of the region of linear response, (3) the shortening of the time required to establish a satisfactory standard curve (See Curve 2, Figure 1), and (4) the relatively small number of ingredients.

Yeast and peptone extracts appear to furnish adequate amounts of the B vitamins and purines which are required by L. arabinosus for growth. The addition of riboflavin, biotin, pantothenic acid, adenine, guanine, and uracil to the basal medium did not improve the response of the organism to nicotinic acid. However, biotin and pantothenic acid are being included in the medium until the effect of their presence can be determined during the assay of a greater variety of materials.

The composition of the medium used has been determined from a large number of experiments in which the ingredients were present in varying concentrations. The large amounts of glucose and sodium acetate used conform to the recommendations of Stokes and Martin (19) for L. casei.

During the progress of this research an assay method for nicotinic acid was developed by Krehl, Strong, and Elvehjem (9). The basal medium used by these workers was the synthetic type described on page 11, so that the only

natural ingredient was vitamin-free hydrolyzed casein. Using this medium results were reported which were comparable to those described here.

Numerous unsuccessful attempts have been made in this laboratory to duplicate the results obtained by the Wisconsin workers. In the author's experience their medium was only slightly superior to the Snell-Wright medium and possessed the disadvantage of being relatively complicated to prepare. No explanation is offered for the low response obtained with the medium of Krehl et al, in spite of the fact that it was used repeatedly with several changes in the combination of the ingredients in an effort to discover deficiencies among them. It is possible that the casein hydrolysates used in the two laboratories may have differed in quality.

SUMMARY

1. Comparisons have been made of the nicotinic acid content of dehydrated foods after extraction with acid, alkali and enzymes.

2. Acid or alkaline extraction yields higher nicotinic acid values for a number of dehydrated foods than does enzyme digestion. This may be due to (a) conversion of an unknown precursor of nicotinic acid to the active vitamin, or (b) a more complete liberation of nicotinic acid whether free or combined. The question of nutritional availability of nicotinic acid obtained by acid or alkali treatment has not been settled.

3. The relative yields of nicotinic acid after acid and alkaline extraction seem to depend upon the type of material assayed.

4. An improved medium for the assay of nicotinic acid is described in which the yeast and peptone present have been rendered nicotinic acid free by adsorption on Lloyd's reagent. The medium is simple and affords increased sensitivity of the organism to nicotinic acid. The range of linearity in the standard curve is also increased.

5. The time required for establishment of a satisfactory standard curve has been shortened from 72 hours to 36 hours.

BIBLIOGRAPHY

1. Andrews, J.S., Boyd, H.M., and Gortner, W.A., Ind. Eng. Chem., Anal. Ed., 14, 663 (1942).

2. Bovarnick, M.R., J. Biol. Chem., 151, 467 (1943).

3. Cheldelin, V.H., and Williams, R.R., Ind. Eng. Chem., Anal. Ed., 14, 671 (1942).

4. Dann, W.J., and Handler, P., J. Biol. Chem., 140, 201 (1941).

5. Dorfman, A., Koser, S.A., Horwitt, M.K., Berkman, S., and Saunders, F., Proc. Soc. Exptl. Biol. Med., 43, 434 (1940).

6. Elvehjem, C.A., Madden, R.J., Strong, F.M., and Woolley, D.W., J. Am. Chem. Soc., 59, 1767 (1937).

7. Hoag, E.H., Personal Communication.

8. Kónig, W., J. prakt. Chem., 69, 105 (1904).

9. Krehl, W.A., Strong, F.M., and Elvehjem, C.A., Ind. Eng. Chem., Anal. Ed., 15, 471 (1943).

10. Kuiken, K.A., Norman, W.H., Lyman, C.M., and Hale, F., Science, 98, 266 (1943).

11. Landy, M., and Dicken, D.M., J. Lab. Clin. Med., 27, 1086 (1942).

12. McMahan, J.R., and Snell, E.E., J. Biol. Chem., 152, 83 (1944).

13. Melnick, D., Robinson, W.D., and Field, H., Jr., J. Biol. Chem., 136, 131 (1940).

14. Pennington, D., Snell, E.E., and Williams, R.J., J. Biol. Chem., 135, 213 (1940).

15. Perlzweig, W.A., Levy, E.D., and Sarett, H.P., J. Biol. Chem., 136, 729 (1940).

16. Shankman, S., J. Biol. Chem., 150, 305 (1943).

17. Snell, E.E., and Strong, F.M., Ind. Eng. Chem., Anal. Ed., 11, 346 (1939).

18. Snell, E.E., and Wright, L.D., J. Biol. Chem., 139, 675 (1941).

19. Stokes, J.L. and Martin, B.B., J. Biol. Chem., 147, 483 (1943).