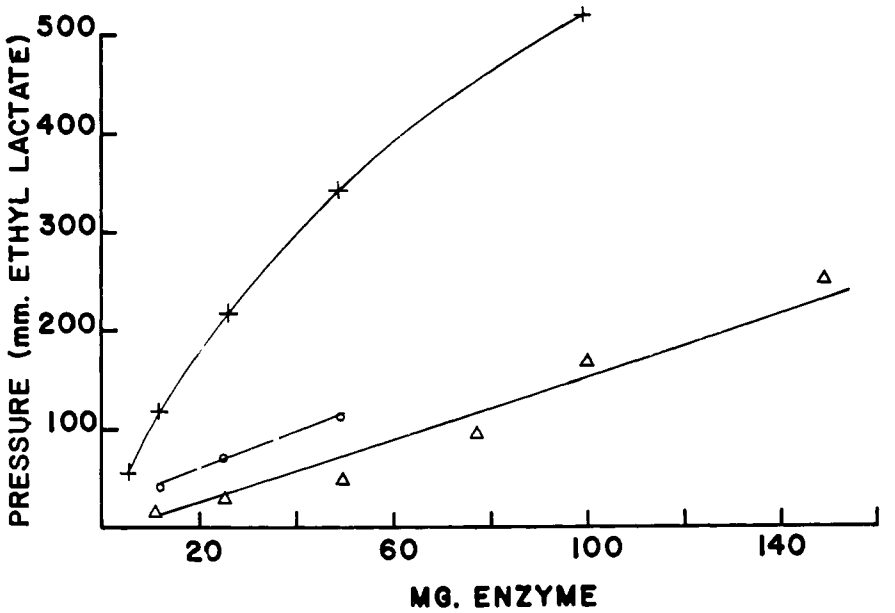

Figure 5. Fungal and bacterial lactase activity as influenced by enzyme concentration

(× fungal lactase A; ○ fungal lactase B; △ bacterial lactase)

Rickenberg, University of Indiana, Bloomington; B. Rotman, Veterans Administration Hospital, Albany, N. Y.; National Dairy Products Corp., Glenview, Ill.; and Rohm & Haas Co., Philadelphia, Pa. They furnished the bacterial extract from *E. coli*, the crude bacterial extract, the lyophilized extract of *E. coli*, the preparation from yeast, and the two fungal preparations, respectively.

Literature Cited

(1) Anderson, J. M., Rickenberg, H. V., *J. Bacteriol.* **80,** 297 (1960).
(2) Cohn, M., Horibata, K., *Ibid.*, **78,** 601 (1959).
(3) Colowick, S. P., Kaplan, N. O., "Methods in Enzymology," Vol. **1,** p. 241, Academic Press, New York, 1955.
(4) de Becze, G. I., *Wallerstein Lab. Commun.* **23,** 99 (1960).
(5) Difco Manual, 9th ed., p. 251, Difco Laboratories, Inc., Detroit, Mich.
(6) Dixon, M., Webb, E. C., "Enzymes," p. 508, Academic Press, New York, 1958.
(7) Hu, A. S. L., Wolfe, R. G., Reithel, F. J., *Arch. Biochem. Biophys.* **81,** 500 (1959).
(8) Linko, P., J. AGR. FOOD CHEM. **9,** 310 (1961).
(9) National Dairy Products Corp., Glenview, Ill., private communication, December 12, 1960.
(10) Pomeranz, Y., Miller, B. S., Miller, D., Johnson, J. A., *Cereal Chem.* In press.
(11) Rickenberg, H. V., University of Indiana, Bloomington, private communication, November 28, 1960.
(12) Rohm & Haas Co., Philadelphia, Pa., private communication, January 16, 1961.
(13) Rotman, B., *J. Bacteriol.* **76,** 1 (1958).
(14) Sandstedt, R. M., Blish, M. J., *Cereal Chem.* **11,** 368 (1934).
(15) Sumner, J. B., Somers, G. F., "Chemistry and Methods of Enzymes," 3rd ed., p. 111, Academic Press, New York, 1953.
(16) Tauber, H., Kleiner, I. S., *J. Biol. Chem.* **99,** 249 (1932).
(17) Umbreit, W. W., Burris, R. H., Stauffer, J. F., "Manometric Techniques," Burgess Publishing Co., Minneapolis, Minn., 1957.
(18) Wallenfels, K., Malhotra, O. P., in "Advances in Carbohydrate Chemistry," Vol. **16,** p. 259, Academic Press, New York, 1961.

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ASCORBIC ACID MEASUREMENT

Polarographic Determination of Total Ascorbic Acid in Foods

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NUMEROUS METHODS for the estimation of ascorbic acid have been described. In general, these are based on decolorization by ascorbic acid of certain oxidation-reduction indicators. Of these, the one most widely used is the 2,6 - dichlorophenolindophenol (2,6-DCIP) titration. Unfortunately, this reagent is not specific for ascorbic acid as it is also reduced by sulfhydryl compounds, reductones, and various inorganic cations.

Roe and coworkers (*15, 16*) have described a colorimetric method based on condensation of oxidized ascorbic acid (dehydroascorbic acid) with 2,4-dinitrophenylhydrazine (2,4-DNPH) to produce a red color in sulfuric acid. This method also lacks the desired specificity and is somewhat tedious.

In recent years, the development of the polarographic method (*3, 6, 11–14*) has provided another approach to the problem of ascorbic acid analyses. The principle advantages associated with this method are its specificity, comparative rapidity, and applicability to highly colored solutions which otherwise are not easily analyzed by titrimetric or colorimetric procedures.

Unlike ascorbic acid, dehydroascorbic acid is not oxidized by the dropping mercury electrode, and hence cannot be determined polarographically. Since both the dehydro and reduced forms of ascorbic acid are physiologically active, a limitation of the polarographic method is its inability to determine total ascorbic acid.

A method is described for the polarographic determination of total ascorbic acid. It is based on reduction of dehydroascorbic acid to ascorbic acid with homocysteine, treatment with *N*-ethylmaleimide to remove the interference of homocysteine, and subsequent polarographic determination of the total amount of ascorbic acid present. This method has been applied to a variety of food products, and the results have been compared with total ascorbic

A method is presented for the polarographic determination of both ascorbic acid and dehydroascorbic acid. Ascorbic acid is determined from the anodic wave produced between 0 and +0.2 volt in a phosphate buffer pH 6.8. Dehydroascorbic acid is determined by difference from the increase in wave height following reduction to ascorbic acid. Homocysteine, which is employed in the reduction step, interferes in the estimation of ascorbic acid. However, addition of *N*-ethylmaleimide following homocysteine reduction suppresses the anodic wave of the homocysteine and permits the subsequent determination of the ascorbic acid.

acid values obtained by the 2,6-DCIP and 2,4-DNPH methods. These findings indicate that the polarographic procedure offers a simple and rapid method for total ascorbic acid analysis.

Materials and Methods

Reagents. All chemicals were analytical reagent grade, except when specifically indicated.

ASCORBIC ACID STANDARD—50.0 mg. ascorbic acid (U.S.P. reference standard) in 100 ml. of 2% aqueous solution of HPO_3. The standard is prepared daily.

N-ETHYLMALEIMIDE—(Nutritional Biochemical Corp.) 1% aqueous solution. After preparation this reagent is stable for 1 week.

PHOSPHATE BUFFER—50 grams of K_2HPO_4 dissolved in 500 ml. of distilled water and adjusted to pH 6.8 by dropwise addition of 85% H_3PO_4.

Apparatus. A continuous recording Leeds and Northrup Electro-Chemograph (Type E) was used to obtain the current-voltage curves. The capillary had *m* and *t* values of 4.82 mg. per second and 1.87 seconds. The electrolytic cell was a Lingane H-type with a saturated calomel reference electrode. A derivative attachment constructed according to the specifications described by Delahay (*4*) was used in conjunction with the Electro-Chemograph for recording derivative polarograms.

Sample Preparation. FOR SOLID AND LIQUID PRODUCTS. Fifty grams of the sample to be analyzed are placed in a Waring Blendor and 50 ml. of 2% HPO_3 added. Nitrogen gas is bubbled through the mixture for 5 minutes to displace the air from the container and the sample material. The blendor should be covered while the mixture is being homogenized to prevent oxidative losses of the ascorbic acid. Prior to analysis, the blend is clarified by vacuum filtration through Whatman No. 5 filter paper.

FOR CANNED PRODUCTS (containing solid material packed in a liquid—for example, canned vegetables, fruits). To a weighed amount of the sample is added enough solid HPO_3 to give approximately a 2% solution on blending. The mixture is then deaerated and blended as previously described.

Analytical Methods. POLAROGRAPHIC METHOD FOR TOTAL ASCORBIC ACID. An aliquot (1 to 4 ml.) of the clarified extract is added to a test tube calibrated to 10 ml. The volume is adjusted to 5 ml. with phosphate buffer pH 6.8, and 1 ml. of freshly prepared 0.5% aqueous solution of homocysteine (free base form, Nutritional Biochemical Corp.) is added. After mixing, the solution is left standing at room temperature for 15 minutes to ensure complete reduction of the dehydroascorbic acid. Then, 1 ml. of a 1% solution of *N*-ethylmaleimide is added and the solution diluted to 10 ml. with additional buffer. A measured aliquot of this solution is carefully transferred to the Lingane H-type cell of the polarograph and nitrogen bubbled through the solution for 5 minutes to displace any dissolved air. The polarogram is recorded anodically between −0.2 v. and +0.2 v.

REDUCED ASCORBIC ACID. The procedure for the determination of reduced ascorbic acid is the same as previously described with the exception that the 15-minute reduction period is omitted and the *N*-ethylmaleimide added immediately after the homocysteine.

For routine analyses of the same product, the concentration of total or reduced ascorbic acid can be determined from a calibration curve. However, to avoid the necessity of preparing curves daily for each of the products analyzed in this study, the Method of Standard Addition (*10*) was used to determine the ascorbic acid content of the test solution.

METHOD OF STANDARD ADDITION (*9*). Following the recording of the polarogram, 2 ml. of a standard solution of ascorbic acid (0.5 mg. per ml.) are added to the polarographic cell, and the solution is de-aerated with nitrogen for 2 minutes. The current-voltage curve for the solution is again recorded between −0.2 v. and +0.2 v. Based on the initial wave height obtained on the test solution and the increase in wave height following addition of the standard, the concentration of total ascorbic acid per milliliter of test solution is calculated from the following equation:

$$X = \frac{-n\,CH}{mh - H(m + n)}$$

X = concentration of ascorbic acid per ml. of test solution

n = ml. of standard solution of ascorbic acid added

C = concentration of ascorbic acid per ml. of standard solution

m = ml. of test solution in polarographic cell

h = wave height (microamperes) of test solution

H = wave height of test solution plus standard ascorbic acid

2,6 - DICHLOROPHENOLINDOPHENOL METHOD. The procedure employed in these studies for the determination of total ascorbic acid was that described in "Methods of Vitamin Assay" (*1*). Essentially, this consists of treating the test solution with hydrogen sulfide for 15 minutes, removing excess reagent with nitrogen, and titrating an aliquot of the sample with a standard solution of 2,6-DCIP. Ascorbic acid (reduced form) was determined by direct titration of the HPO_3-extract with 2,6-DCIP.

2,4 - DINITROPHENYLHYDRAZINE METHOD. The procedure employed in these analyses was an adaptation of the method of Roe *et al.* (*15, 16*) and is described in detail in "Methods of Vitamin Assay" (*2*). Basically, dehydroascorbic acid is determined by conversion to diketogulonic acid, followed by coupling with 2,4-DNPH under carefully controlled conditions of time and temperature (37° C. for 3 hours) and colorimetric estimation of the resulting osazone. Total ascorbic acid is determined by bromine oxidation of ascorbic acid and subsequent reaction of the dehydroascorbic acid with 2,4-DNPH. The difference between the amount of dehydroascorbic acid determined before and after bromine oxidation is equal to the amount of ascorbic acid (reduced form) present.

Results and Discussion

Hydrogen sulfide reduction of dehydroascorbic acid to ascorbic acid is frequently used in the determination of total ascorbic acid by 2,6-DCIP titration. The flammability, toxicity, and difficulty in ensuring complete removal of excess reagent are disadvantages associated with its use. Aside from this, the reagent does not specifically reduce dehydroascorbic acid so that other substances formed in the reduction may be determined in the titration.

To circumvent the difficulties in-

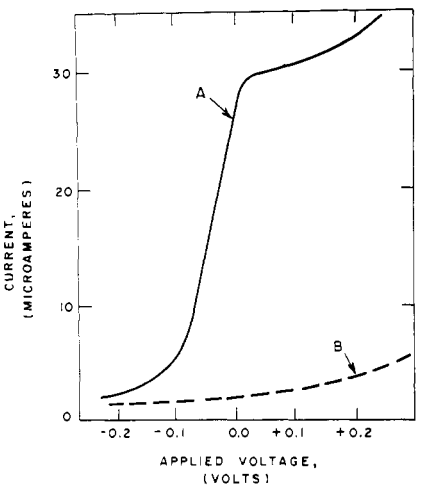


Figure 1. Polarographic wave of homocysteine (*A*) and its suppression by *N*-ethylmaleimide (*B*)

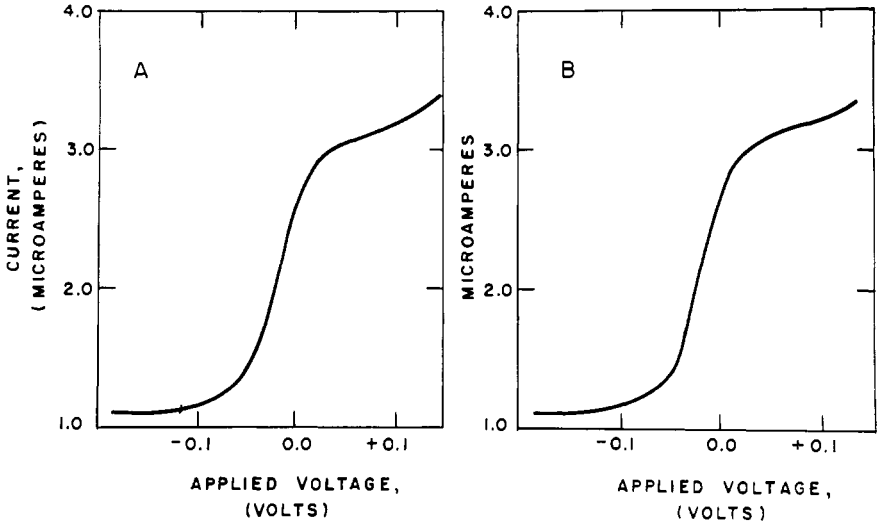


Figure 2. Polarographic wave of ascorbic acid alone (*A*) and in the presence of *N*-ethylmaleimide (*B*)

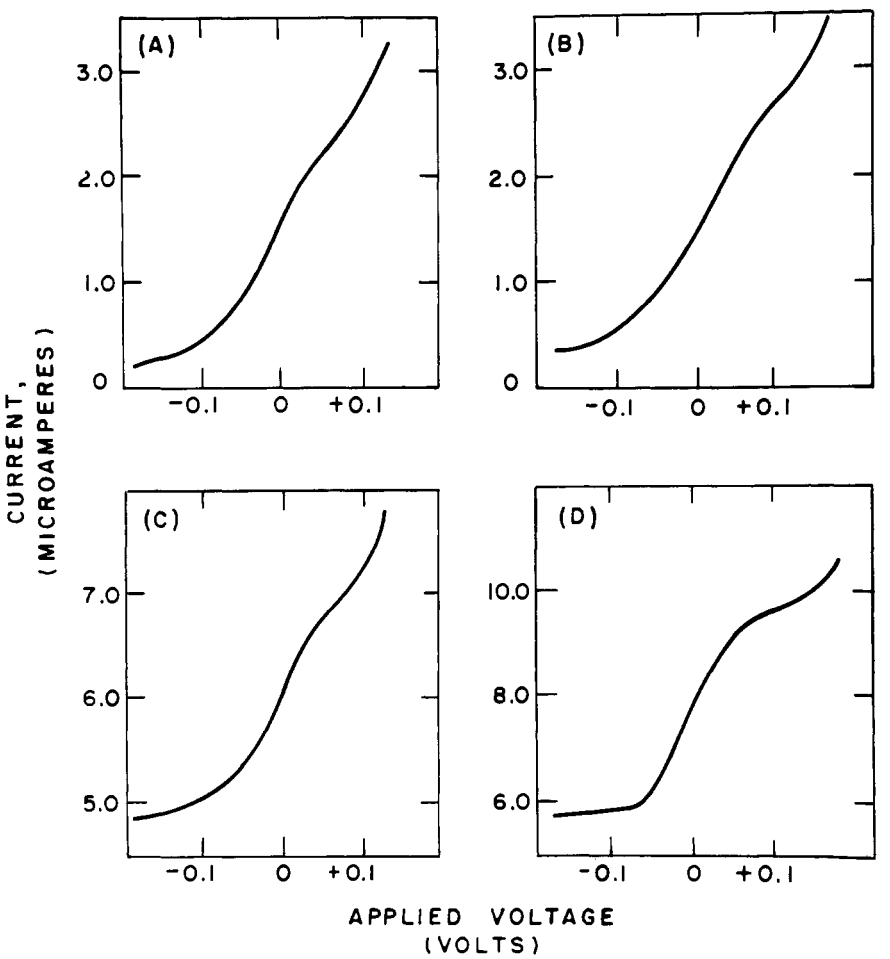


Figure 3. Polarograms of several food products showing the wave for tota ascorbic acid

(*A*) Sauerkraut, (*B*) Canned spinach. (*C*) Onions. (*D*) Watercress.

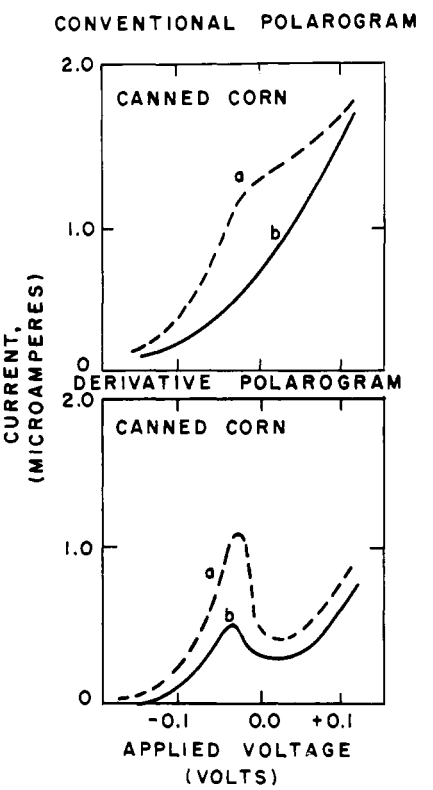


Figure 4. Comparison of conventional and derivative polarograms

(*a*) Product and added ascorbic acid
(*b*) Product

volved in the use of hydrogen sulfide reduction, Hughes (*7*) employed homocysteine as the reducing agent in the estimation of total ascorbic acid with 2,6-DCIP. The recommended conditions for reaction consist of the additions of a 40-fold excess of reagent to buffered solutions (pH 6.8) of dehydroascorbic acid with reduction being complete in 15 minutes at room temperature. Unfortunately, this method still lacks the desired specificity associated with 2,6-DCIP titrations (*7*).

Polarographic analysis is more specific for the determination of ascorbic acid than 2,6-DCIP titration , since it provides through the diffusion current and the half-wave potential both a quantitative and qualitative index for this substance. Nevertheless, the inability to estimate dehydroascorbic acid polarographically has somewhat limited the use of this technique to the determination of reduced ascorbic acid. Hydrogen sulfide reduction for the polarographic determination of total ascorbic

Table I. Total Ascorbic Acid Recoveries by Homocysteine Reduction and Polarographic Analysis

Amount Added		Total Ascorbic Acid		
Ascorbic acid, mg.	Dehydroascorbic acid, mg.	Present, mg.	Found,[a] mg.	Recovery, %
0.100	0.010	0.110	0.114 ± 0.003	104
0.010	0.100	0.110	0.113 ± 0.002	103
0.500	0.500	1.000	0.976 ± 0.009	98
1.000	0.250	1.250	1.253 ± 0.008	100
0.200	1.000	1.200	1.195 ± 0.009	99

[a] Values given are the average of five determinations ± std. dev.

Table II. Total Ascorbic Acid Analyses of Various Foods

Comparison of Polarographic Method with 2,6-DCIP and 2,4-DNPH

	2,6-DCIP		2,4-DNPH		Polarographic	
Fresh Products	Reduced AA (mg. %)	Total AA (mg. %)	Reduced AA (mg. %)	Total AA (mg. %)	Reduced AA (mg. %)	Total AA (mg. %)
Brussel sprouts	105.3	130.4	99.4	127.5	99.4	125.0
Cabbage	48.1	52.1	45.6	50.0	44.5	48.7
Celery	37.5	43.5	30.6	45.3	32.5	38.8
Lemon juice	41.2	42.0	41.0	43.8	42.4	42.5
Onion	11.8	11.8	7.9	7.9	8.4	8.4
Orange juice	51.2	51.2	51.5	50.2	48.8	48.8
Parsley	70.1	88.0	71.0	88.5	68.0	84.9
Spinach	41.1	49.4	41.3	48.4	40.5	48.0
Tomato	21.2	23.2	18.5	22.4	18.0	19.0
Watercress	55.0	60.0	45.6	60.8	50.3	61.4
Canned Products						
Asparagus	32.8	32.8	15.4	17.4	14.2	14.6
Grapefruit juice	29.2	30.8	28.1	28.5	23.1	24.2
Lemon juice	41.7	42.6	40.4	41.2	39.6	40.0
Lima beans	8.8	8.8	6.4	8.7	6.8	8.0
Mixed vegetable juice	25.3	25.3	19.5	24.0	20.2	20.4
Orange juice	49.4	53.0	47.1	53.0	46.3	52.2
Peas	11.0	12.5	10.0	12.0	10.4	11.2
Sauerkraut	18.0	18.8	17.7	18.1	14.6	17.6
Tomato juice	16.4	16.4	14.2	16.0	14.6	14.6
Tomato puree	47.3	53.8	40.2	46.3	44.5	50.5

acid has been studied by Krauze and Bosyk (*9*). These investigators, however, have shown that the formation of polysulfides following treatment with hydrogen sulfide has an adverse influence on the polarographic wave produced by the ascorbic acid.

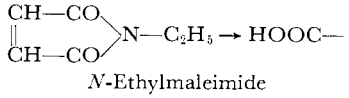
In view of the advantages afforded by the use of homocysteine over hydrogen sulfide, it was thought that the combination of polarographic analysis and the use of homocysteine would result in a rapid and specific method for determining total ascorbic acid.

At pH 6.8, a condition optimum for the reduction of dehydroascorbic acid by homocysteine, the half-wave potential for ascorbic acid ($E^{1/2}$ −0.06 v. *vs.* S.C.E.) is independent of concentration and suitable for quantitative analysis. It is not possible, however, to determine polarographically dehydroascorbic acid as ascorbic acid directly following homocysteine reduction. This results from the interference of homocysteine which, as shown in Figure 1, also produces an anodic wave ($E^{1/2}$ −0.06 v. *vs.* S.C.E.) at voltages similar to that of ascorbic acid.

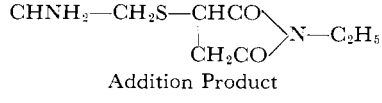
When *N*-ethylmaleimide is added to solutions of homocysteine, the anodic wave resulting from oxidation of the sulfhydryl group of the homocysteine at the dropping mercury electrode is not obtained because the *N*-ethylmaleimide complexes with sulfhydryl compounds (*5*). With homocysteine, the reaction is represented as follows:

$$HOOC—CHNH_2—CH_2—CH_2SH +$$

Homocysteine



N-Ethylmaleimide



Addition Product

The suppression of the anodic wave of homocysteine following the addition of *N*-ethylmaleimide is demonstrated in Figure 1. Figure 2 shows that the reagent is without effect on the anodic wave of ascorbic acid.

Selective removal by *N*-ethylmaleimide of the interference of homocysteine permits the polarographic determination of total ascorbic acid following homocysteine reduction (Table I). Typical polarograms obtained from some of the food products investigated in the study are illustrated in Figure 3.

Table II shows total and reduced ascorbic acid values obtained on a variety of food products analyzed by the present method. In all cases, the concentration of ascorbic acid in the extracts of the samples was determined by the Method of Standard Addition. This technique was used because it avoided the influence that each of these products had on the wave height and half-wave potential of ascorbic acid as well as compensating for any variation in temperature or capillary characteristics. Further, comparison of the half-wave potential of ascorbic acid before and after the addition of the standard solution to the unknown also eliminated the possible estimation of substances with half-wave potentials similar to ascorbic acid.

Also shown in Table II are total and reduced ascorbic acid values determined on these food products by the 2,6-DCIP and 2,4-DNPH methods. These data show conclusively that the polarographic method agrees substantially with these other methods and, therefore, is applicable for the determination of total ascorbic acid. In some products where more significant differences were obtained, for example, asparagus, celery, and onion, the lower polarographic value is probably the more reliable due to the greater specificity of the method.

By the technique described, 1 mg. of ascorbic acid per 100 grams of solution was easily detectable in model buffered solutions. It was not possible to discern through a well-defined wave this concentration of ascorbic acid in a food product. For example, food extracts shown to contain 3 mg. total ascorbic acid per 100 grams (mg.%) of product by 2,4-DNPH analyses gave no evidence polarographically of this substance being present. Only when these extracts were adjusted by the addition of ascorbic acid to contain more than 3 mg. per 100 grams of product did the characteristic ascorbic acid wave become apparent. This would seem to limit the method to concentrations higher than this amount. However, more recent investigations in the authors' laboratories have shown that the sensitivity of the method can be increased below the 3-mg.% level through the application of derivative polarography. In the derivative technique, the polarogram is recorded as a plot of $\frac{\Delta i}{\Delta E}$ *vs.* E in which i and E represent diffusion current and applied potential, respectively. The half-wave potential is thus represented as a maximum with the peak height proportional to concentration (*8*).

Derivative polarography will separate substances with oxidation or reduction potentials within 0.05 volt of the final current rise so that waves not appearing in conventional polarograms are more readily detected. This is illustrated in Figure 4 by a typical

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

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

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

Acknowledgment

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Literature Cited

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

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PROTEIN ISOLATION

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

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

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

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

Experimental

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