Measurement of Ascorbic Acid and Dehydroascorbic Acid in Mammalian Tissues using HPLC with Electrochemical Detection

Debra A. Fry

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Measurement of Ascorbic Acid and Dehydroascorbic Acid
In Mammalian Tissues using HPLC with
Electrochemical Detection

by
Debra A. Fry
Bachelor of Science, North Dakota State University, 1989

A Thesis
Submitted to the Graduate Faculty
of the
University of North Dakota
in partial fulfillment of the requirements
for the degree of
Master of Science

Grand Forks, North Dakota
August 1991
This thesis, submitted by Debra A. Fry in partial fulfillment of the requirements for the Degree of Master of Arts from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

This thesis meets the standards for appearance, conforms to the style and format requirements of the Graduate School of the University of North Dakota, and is hereby approved.

Dean of the Graduate School
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Title  Measurement of Ascorbic Acid and Dehydroascorbic Acid In Mammalian Tissues Using HPLC With Electrochemical Detection

Department  Physiology

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To Pete and Rose Schell
ABSTRACT

The oxidized and reduced forms of ascorbic acid (AA) are reactive and unstable compounds making their measurement difficult. Detection of small amounts of AA and dehydroascorbic acid (DHAA) is essential for determining the biochemical function of the vitamin in cells and subcellular organelles. While a variety of techniques exist which detect millimolar levels of AA, accurate assessment of pmol levels of AA and DHAA in a variety of tissues has not been perfected. In the present study, a method was developed for quantitation of AA and DHAA that combines HPLC separation with the advantages of increased sensitivity and selectivity available with coulometric electrochemical detection.

Tissues were homogenized in 0.3% meta-phosphoric acid containing 1 mM thiourea and 0.1 mM EDTA. Portions of the homogenates were either placed in 10% meta-phosphoric for AA analysis or in a 120 mM sodium phosphate buffer (pH 7.2) containing 0.1 mM EDTA and 1 mM thiourea, for total AA (AA +DHAA) measurement. Total AA was determined by reducing the endogenous DHAA in a sample with 10 mM β-mercaptoethanol and allowing the reduction to occur for 10 minutes at room temperature. The reaction was stopped with the addition of 10% meta-phosphoric acid.

The developed method was extended to assess the levels of AA and DHAA in nondiabetic and diabetic rat liver, kidney, brain and adrenal tissues. Results indicated that the AA levels in diabetic liver and kidney tissues were significantly less than control levels but there was no difference in AA levels in diabetic and nondiabetic brain and adrenal tissues. When compared to controls, DHAA concentration was greater in diabetic rat liver, kidney, and adrenal glands but not in brain tissue.
I. History of Ascorbic Acid

Scurvy was first reported in the year 1498 and although awareness of scurvy existed for centuries, its cause was unknown. Symptoms of scurvy included swelling and bleeding of the gums, swelling of the legs, muscular ache, bone pain, weakness, hyperkeratosis, and hemorrhage of small blood vessels. Scurvy was thought to result from infection, toxication, or faulty diet. Studies eliminated the first two possible causes and it was concluded that scurvy was a nutritional disease (1).

Results of early studies showed that fat soluble A factor and water soluble B factor were essential nutrients. In 1919, Drummond designated a third essential nutrient as water soluble C factor (2); this was thought to be the antiscorbutic factor sought after for many years. However, it wasn't until 1928 that a Hungarian scientist, Albert Szent-Gyorgyi, working at Cambridge, first achieved the isolation of vitamin C (3). He observed that there was a high concentration of a powerful reducing chemical in the adrenal cortex. From his analysis of the crystals isolated from the adrenal cortex, he determined the molecular weight of the reducing agent and that the only elements contained in the compound were carbon, oxygen, and hydrogen. The ratio of these elements was shown to be 6:6:8, respectively, and so he gave it the name hexuronic acid. He also isolated this powerful reducing agent from cabbage and orange juice, two substances known to protect sailors from scurvy. However, there was disagreement as to whether hexuronic acid was really the antiscorbutic factor. Most of the
controversy arose because of experiments in which the addition of these crystals to experimental guinea pig diets failed to prevent scurvy (4). The suggestion was (5) that in their method of preparing the crystals for the animals' diet, an oxidation step was involved where most of the ascorbic acid (AA) was likely lost. In 1932, two separate studies (6, 7) reported that crystals obtained from lemon juice, when fed to guinea pigs at 0.5 mg per day, protected them from scurvy. This confirmed the initial hypothesis that hexuronic acid was the antiscorbutic factor. Hexuronic acid was first synthesized in 1933 by Haworth and Hirst (8) and renamed AA. Since that time, understanding the vital functions of vitamin C and its availability in food sources has been a major nutritional research focus.

II. Metabolism of Vitamin C

The ability to synthesize AA from carbohydrate sources, namely D-glucose or D-galactose, through the glucuronic pathway, is retained in a wide variety of plant and animal species. The synthetic ability is absent in humans, other primates, guinea pigs, insects, invertebrates, fish, and certain bats and birds (9). In terms of evolution, the synthesis of AA apparently began in the kidney of amphibians and reptiles and was transferred to the liver of mammals but lost in guinea pigs, flying mammals, and primates. The inability to synthesize AA results from the loss of the genetic material coding for the terminal enzyme, L-gulono oxidase (9, 10, 11). Ascorbic acid is required in the diet of species deficient in this enzyme.

The intestinal absorption of AA is an active process in scurvy prone animals but is a passive process in other animals (12). It was determined that the process in scurvy prone animals is that of Na+-dependent coupled-carrier transport (12, 13) across the brush border. The active AA absorbance
mechanism becomes saturated when the concentration of the vitamin is increased. This concurs with evidence that the intestinal absorption of AA decreases with increasing intake of the vitamin (14). Fresh solutions of dehydroascorbic acid (DHAA) were also shown to retain nutritional or antiscorbutic activity (4, 15). It was determined that this antiscorbutic activity is associated with DHAA's intestinal absorbance that occurs through a process of facilitated diffusion across the brush border, and subsequent reduction to AA (16, 17).

Transport of AA between plasma and tissues was also found to be by an active process in most tissues. Dehydroascorbic acid was shown to be the preferred form taken up by human neutrophils (18) and erythrocytes (19), and is quickly reduced to AA after it is taken up by the cells. Dehydroascorbic acid is continuously formed when AA is oxidized in the body. Its concentration is maintained at very low levels in tissues by mechanisms that recycle it to AA. Compiled tables of AA content in human organs indicate that the pituitary and adrenal glands and the leucocytes contain the highest concentration of AA (20). The ocular tissues, brain, liver, and spleen also have relatively high concentrations when compared to plasma levels. Plasma levels are approximately 0.4-1.0 mg/100 g wet tissue (100 times less than the concentration in the pituitary gland). The low levels of AA found in the plasma indicate that transfer of the vitamin from plasma to tissue occurs against a concentration gradient and therefore must be an active process.

Metabolism of AA and DHAA is governed by a variety of specific and nonspecific mechanisms. For example, AA is oxidized to a free radical intermediate, semidehydroascorbate or ascorbate free radical (AFR), which is detected by electron spin resonance (21, 22). Ascorbate free radical is relatively nonreactive when compared to other free radical species. It can either
react with another AFR to regenerate AA or it can react with other radicals to terminate their propagation (Fig. 1). An enzyme, AFR reductase, has been described in rat liver (23), bovine adrenals, and a variety of rat and guinea pig tissues (24, 25). This enzyme reduces the AFR back to AA. In plants, ascorbate oxidase (EC 1.10.3.3), a copper containing enzyme, catalyzes the reaction of AA and O₂ to give DHAA and water (26). In tissues (17, 27, 28, 29) and plants (30), dehydroascorbate reductase (EC 1.8.5.1) converts DHAA to AA, using glutathione as a reducing agent. Some believe that, in mammals, DHAA is converted to AA mainly by nonenzymatic means (31, 32). Dehydroascorbic acid is hydrolyzed irreversibly to 2,3-diketogulonic acid (DKG) (Fig. 2), which is then rapidly degraded to other products such as xylose, threonic acid, xylonic acid, and lyxonic acid.

III. Biochemical Roles of Ascorbic Acid

Ascorbic acid has many diverse roles in the body. Most of these are attributed to its reducing and chelating abilities (33). One of its well known functions involves the formation of the mature collagen molecule. Ascorbic acid serves as a reducing agent for the hydroxylation of proline contained in the procollagen molecule (34,35). It is necessary for activation of prolyl hydroxylase (36), an Fe⁺⁺ requiring enzyme that carries out hydroxylation of proline. Ascorbic acid presumably keeps Fe⁺⁺ in its reduced or active state.

Ascorbic acid is also essential for hydroxylation reactions in the biosynthetic pathway of muscle carnitine (34, 35, 37). Two of the five steps in the pathway involve reactions where AA is required for the enzymes to perform hydroxylation. Studies show that vitamin C deficient guinea pigs have low carnitine concentrations (38).
Ascorbic acid also plays a vital role as an antioxidant. Ascorbic acid is suggested to play a role in the protection of various tissues against free-radical damage induced by various agents including cigarette smoke, radical species generated from white blood cells, and photooxidative damage (39). It accomplishes this task either directly by reacting with free radicals generated in the aqueous phase of the cell, or indirectly by revitalizing the antioxidant properties of vitamin E (alpha-tocopherol) (39). Ascorbic acid directly scavenges superoxide radical (40, 41), hydroxy free radical (42) and singlet oxygen (43) and is an effective inhibitor of lipid peroxidation (39). Lipid peroxidation occurs in areas rich in polyunsaturated fatty acids (44). Cell membranes contain large amounts of unsaturated fatty acids and are subject to damage which could result in cellular death (45) from unchecked peroxidation of the membrane lipids. Vitamin E is a fat soluble antioxidant found in the cell membranes. It interrupts the chain reaction that occurs after initial free radical attack on a membrane lipid by reacting with the generated lipoxy radical, resulting in vitamin E becoming a reactive free radical, the α-tocopherol radical. AA regenerates the α-tocopherol radical by reducing it back to α-tocopherol (46, 47). In this way, vitamin C protects the antioxidant capacity of vitamin E.

Vitamin C is also required in the nervous system for synthesis of norepinephrine. Dopamine-β-hydroxylase is the enzyme that catalyzes the final step in the formation of norepinephrine directly from dopamine (48). The enzyme requires Cu++ as a cofactor and Cu++ acts as an intermediate accepting electrons from AA; then Cu++ transfers the electrons to oxygen to form H₂O. Ascorbic acid is also involved in the processing of many neuropeptides. Ascorbate dependent monooxygenase, called peptidylglycine α-amidating monooxygenase (PAM), discovered in the pituitary (49, 50), catalyzes the conversion of peptides ending in glycine to their corresponding α-amidated
peptide by a mechanism similar to dopamine-β-hydroxylase (51, 52). PAM requires Cu++, AA, and O₂ for optimal activity. The process of α-amidation of peptides is an important mechanism in the biosynthesis of neuroendocrine peptides including α-melanocyte stimulating hormone (α-MSH), thyrotropin releasing hormone, oxytocin, vasopressin, and cholecystokinin (53, 54, 55).

Other roles of vitamin C include detoxification and metabolism of numerous compounds, occurring through the cytochrome (P450) mixed function oxidase (MFO) system. MFO is found predominantly in liver microsomes and reticuloendothelial tissues and is a system which includes enzymes that hydroxylate and methylate toxic agents produced by the body, such as bilirubin and steroid hormones (56). Numerous studies (57, 58) have shown that there is a relationship between the MFO system and AA in which increased drug metabolism decreases levels of AA and also decreased levels of AA hinders the drug metabolizing ability of MFO; however, there is no direct evidence for the site of action of AA. Ascorbic acid is also involved in cholesterol metabolism occurring within the MFO system although its site of action is again unknown. Studies suggest that a vitamin C deficiency in guinea pigs leads to a reduction in cholesterol metabolism (59).

Other functions of AA include facilitation of iron absorption (60), association with synthesis of prostaglandins, and involvement in the functioning of the immune system (61). Ascorbic acid may play an important role in a redox system involving NADH-free radical reductase, which functions in membrane energetics and in the regulation of cell growth, and is found in cell membranes and organelle membranes (62).
IV. Ascorbic Acid Metabolism in Diabetes

Ascorbic acid metabolism is abnormal in diabetic humans and animals, but disagreement exists as to how it is altered. For example, several studies have shown that plasma levels of AA in diabetic patients are significantly decreased while DHAA levels are increased (63, 64, 65). Other studies found normal DHAA levels in the plasma of diabetic patients (66, 67). Despite their ability to synthesize AA, decreased levels of AA are reported in various tissues of rats that were made diabetic with alloxan (68). Few studies have determined tissue levels of DHAA in diabetic animals but at least one attempt was made to measure levels of both AA and DHAA in the plasma, liver, testes, and kidneys of diabetic and non-diabetic rats (69). In this case an increase in DHAA levels was found only in the plasma; however, levels of AA were significantly decreased in all tissues except testes.

The mechanism by which the decrease in AA in diabetic animals occurs is not well understood. Mann (70) noted the structural similarity between L-AA and D-glucose and suggested that both may be involved in the same membrane transport system. He formulated two hypotheses to explain the decrease of AA that occurs in hyperglycemia (71). First, he proposed that the transport of AA across the cell membrane is hindered by glucose. Assuming they share a common transport mechanism, hyperglycemia would saturate the carrier with glucose. His second hypothesis was that the transport of AA in certain tissues is accelerated by insulin. In the absence of insulin a decrease in transport of vitamin C would occur. Later, Mann (72) showed that D-glucose inhibited the transport of DHAA into human red-blood cells, which are non-insulin dependent tissues. Other researchers observe inhibition of AA transport with glucose in human lymphocytes (73) and human cardiac endothelial cells (74) and concluded that glucose and AA share a common transporter (75).
It is also suggested that there may be a decreased number of glucose transporters in the plasma membrane of diabetic leukocytes, and since AA and glucose share the same transporter, this can explain the decrease in AA observed in diabetic tissues. This conclusion was deduced from studies (68) in which the rate of DHAA uptake by blood granulocytes and mononuclear cells was slower and the plasma concentrations of AA lower, among persons with diabetes mellitus. $V_{\text{max}}$ transport rates for DHAA, 2-deoxyglucose and 3-o-methylglucose were decreased each to the same degree in diabetic cells but $K_m$ values were not different from those observed in non-diabetic cells.

Dehydroascorbic acid is structurally similar to alloxan, a compound used experimentally to induce diabetes. It is known to be toxic and daily intravenous injections of DHAA into rats at a dose of 20 mg/kg was found to result in lasting hyperglycemia after 3 weeks (76, 77). A third study confirmed this effect and attributed it to damage sustained by the beta cells of the pancreas (78).

The disturbance of AA metabolism in diabetes is of interest because it may be an important factor in the pathogenesis of many diabetic complications (74, 79, 80, 81), such as cataract, keratopathy, retinopathy, neuropathy, microangiopathy, and nephropathy. In one study (82), it was found that diabetic subjects had low plasma ascorbate levels but those with retinopathy had significantly lower plasma ascorbate status compared to diabetic subjects who did not have vascular complications.

Mann and Newton (72) proposed that a decrease in AA levels in tissues of diabetic patients would lead to the development of a "local scurvy" due to impaired transport. The resulting lesion would not be the same as classical scurvy since the deficit is in a local AA supply rather than an extracellular supply. Ascorbic acid is also important in regulating the intracellular redox state and scavenging reactive free radicals. Therefore, AA deficiency may lead to
increased susceptibility to oxidative damage in diabetic subjects. Ascorbic acid deficiencies may also be responsible for some of the collagen abnormalities in diabetes, such as decreased capillary integrity and poor wound healing. In addition, both DHAA and 2,3-diketogulonic acid, oxidation products of AA, are known to initiate cross-linking of lens proteins (83, 84, 85).

The sorbitol or polyol pathway is implicated as a source of disturbances that lead to various complications of diabetes (86, 87), including cataracts, neuropathy, retinopathy, and nephropathy. The sorbitol pathway involves the reduction of D-glucose to sorbitol catalyzed by aldose reductase and the oxidation of sorbitol to fructose by sorbitol dehydrogenase. Normally the flux of glucose through the sorbitol pathway is very low but it can be increased and has been reported to account for 1/3 of the glucose metabolism in rabbit lens at high glucose concentrations (86). A consequence of hyperglycemia in diabetes is increased metabolism of glucose by the polyol pathway. Sorbitol is not permeable to cell membranes and tends to accumulate in the cell. There is no known physiological role for sorbitol. Levels are negligible in most tissues but under hyperglycemic conditions, the accumulation of sorbitol in tissues is increased. Accumulation of sorbitol is found in diabetic tissues in proportion to the concentration of extracellular glucose in sensitive tissues (87). Aldose reductase inhibitors prevent the buildup of sorbitol, which is thought to decrease the propensity for diabetic complications. The interaction between AA and the sorbitol pathway is not well understood, but AA supplemented patients are found to have reduced erythrocyte sorbitol accumulation by 27-56% depending upon the dosage of AA given (79). Other studies have shown that low plasma AA levels in diabetic rats could be normalized by dietary AA supplement and also by the aldose reductase inhibitor, tolrestat.(80).
V. Measurement of AA and DHAA

Since AA was first discovered to be the antiscorbutic factor vital to our diets, researchers were interested in determining the basic biochemistry of the vitamin, its content in foods, and tissue concentrations associated with disease. Attainment of this knowledge required the development of assays to measure levels of the vitamin in various substances. Earlier developed assays were non-specific and not satisfactory for detailed work. Most assays involving measurement of AA and DHAA take advantage their potential to function in a redox system.

Methods developed include fluorometric, spectrophotometric, chromatographic, and colorimetric assays. One of the earliest (88) and most frequently used methods involves the ability of AA to reduce and subsequently decolorize the indicator, 2,6-dichlorophenol indophenol. A limitation of this method is its nonspecificity in that other reducing agents are able to decolorize the indicator. Another colorimetric assay developed involves the ability of DHAA to react with 2,4-dinitrophenylhydrazine to form an osazone and then conversion of osazone to furfural which can be colorimetrically detected (89). However, this method is also nonspecific because others compounds in the samples are capable of reacting to form osazones.

Spectrophotometric methods are also used and involve direct detection where ultraviolet light absorption of AA (265 nm) and of DHAA (230-280 nm) is followed. These assays have a limited value because of interference by many other biological substances absorbing in this region.

Chromatographic methods hold the most promise for specific methods in the detection of AA, DHAA, and 2,3-DKG. A method involving paper chromatography was developed to measure AA and various breakdown products (90). However, high-pressure liquid chromatography (HPLC) has
proven to be a powerful and efficient technique for the separation, identification, and quantitation of small quantities of AA and its metabolites. Combining HPLC with other methods allows an assay, that was nonspecific before the addition of HPLC, to become specific because the separation of the compound of interest from other constituents of the sample can be achieved. Numerous procedures using HPLC were developed to measure AA in foods and tissues (91, 92, 93). Variations in the procedures include the column type, mobile phase, detection system, and stabilization of samples.

Ascorbic acid is subject to oxidation in biological solutions and is therefore unstable. The first oxidation product, DHAA, is extremely unstable and reactive. It is rapidly hydrolyzed irreversibly to DKG with a half life of six minutes at a temperature of 37° C and pH 7.0 (94). Metaphosphoric acid (MPA) and trichloroacetic acid (TCA) are the reagents of choice for stabilizing AA and DHAA acting by precipitating proteins and maintaining a reduced pH in the sample (95). Long term stability of AA in acidic medium has been reported from 30 days at -20°C (96) to 5 weeks at -70°C (97).

Detection methods used with HPLC include ultraviolet (98, 99, 100), radiochemical (101), fluorometric (102), and electrochemical analysis (103, 104, 105). These methods all require stabilization of the sample and vary in sensitivity. One method to analyze AA and DHAA involves derivatization with o-phenylenediamine to separate DHAA from AA (106). The AA contained in the sample is measured electrochemically and the DHAA is measured by UV analysis. The use of HPLC and electrochemical (EC) detection in the analysis of AA has been applied to food and animal tissues with excellent success, whereas the application of HPLC-EC to the measurement of DHAA in a variety of tissues has not been as successful, mainly due to the instability of the molecule. However, electrochemical detection of AA and DHAA in the samples
offers many advantages. It requires small injection volumes, has increased selectivity for AA, and is capable of low detection limits.

There are two types of EC detection, amperometric and coulometric. Theoretically, all EC detectors are amperometric, meaning the potential applied to the detecting electrode is held constant while the resulting currents are monitored as analytes flow past the detector. Most amperometric sensors cause approximately 1-5% of the passing analyte to undergo charge transfer. However, coulometric electrochemical detection utilizes porous, flow-through electrodes. An oxidation reaction takes place at the surface of the electrodes and the charge that is transferred is measured. In this way, the electrode reacts with 100% of the analyte, resulting in increased sensitivity.

Detection of AA by electrochemical detection offers the advantage of increased selectivity for AA. This is accomplished by placement of the electrodes in series, where the first electrode reacts with and eliminates contaminating compounds so they will not interfere at the second electrode, where oxidation of AA occurs and is detected. Dehydroascorbic acid is not electrochemically active and so it must be measured indirectly. The procedure involves the measurement of total AA (DHAA + AA). Ascorbic acid is measured first, DHAA is then reduced to AA in the sample, and then subsequent measurement of total AA can occur. The challenge in measuring DHAA in biological samples is its extreme instability. Success in measuring DHAA with electrochemical detection has almost exclusively occurred in biological fluids including plasma (97, 98, 107, 108, 109), protein free human milk (107), cerebrospinal fluid (109), and leukocyte extracts (107) rather than in whole tissues.
STATEMENT OF THE PROBLEM

The purpose of the present study was two-fold. The first purpose was to develop a sensitive and reliable method for accurately measuring ascorbic acid (AA) and dehydroascorbic acid (DHAA) in mammalian tissues. The challenge in accomplishing this goal is the instability of DHAA. The verification of a reliable assay requires the assay's ability to linearly recover exogenous DHAA and AA added to tissue homogenates. The second objective was to apply the method to the measurement of AA and DHAA in normal and diabetic tissues.
MATERIALS AND METHODS

I. Experimental Animals

Male Spraque-Dawley rats, obtained from Bio-Labs Corporation (St. Paul, MN), were used for this study. The number of animals used in the determination of AA and DHAA in normal and diabetic tissues was 23; the number used in the initial development of the method was undetermined since tissues were donated by another research group using the same strain of rats.

The animals were kept at the Animal Resource Facility (UND Medical School, Grand Forks, ND). Environment conditions were maintained at a temperature of 72°F and a 12 hour/12 hour light/dark cycle. Animals were kept in stainless steel, mesh floor cages with an area of 160.9 in². Two animals were housed in each cage. The only time the animals were removed from this environment was for tissue analysis. Rats were maintained ad libitum on Purina Rodent Lab Chow 5001 and water.

II. instrumentation for Analysis

A) High-Pressure Liquid Chromatography (HPLC) System

The equipment used for analysis consisted of a Beckman System Gold HPLC containing two Beckman Model 110B high pressure pumps, a System Gold 502 autosampler with a 20 µl injection loop, and a Waters Radial Compression Separation System consisting of a radial compression module model RCM-100 with a Waters Radial-Pak cartridge, Resolve C₁₈ reverse-phase column. Data was collected with the assistance of a System Gold
Analog Interface Module 406 and an IBM model 55 SX personal computer. The computer was used to integrate and interpret the resulting chromatogram, with the aid of System Gold software. Results were printed on an Epson FX-850 printer and data were stored on floppy diskettes. The samples were injected onto the column and AA was eluted with a mobile phase consisting of 0.2 M KH₂PO₄, pH 3.0, filtered through 0.22 μm filters (Millipore Corp., Bedford, MA) prior to its use. The flow rate was maintained at 1 ml min⁻¹. The system was cleaned periodically with 10% methanol and then equilibrated with the mobile phase prior to use.

B) Electrochemical Detector System

1) Instrumentation

The content of AA in the samples was quantified by means of an ESA Model 5100A Coulochem Multi-Electrode Electrochemical Detector (ESA, Inc., Bedford, MA) containing an ESA Model 5010 Analytical Cell. In-line high pressure carbon filters (ESA, Inc.) were placed between the analytical column and the electrodes.

2) Initialization of Electrochemical Detector for AA Analysis

The appropriate detector settings for optimal AA sensitivity and selectivity were determined by making a current-voltage (C-V) curve, which is a graph of response (current) versus potential (voltage). By reviewing the C-V curve, the optimal voltage was selected for both electrodes so a maximum response was obtained while minimizing background noise from interfering compounds.

The procedure for making a C-V curve begins with the preparation of samples containing the analyte of interest, AA. Since DHAA is not electrochemically active, the developed method involved detection of endogenous AA and total AA. Total AA included endogenous AA and endogenous DHAA that was reduced to AA. A 1 mM solution of AA was
prepared in double glass distilled water and kept on ice until analysis. The model 5010 Analytical Cell contains two coulometrically efficient porous graphite working electrodes placed in series. In preparing the C-V curve, detector 1 is set at a very low potential (-0.5 V) because the optimal potential for detector 2 needed to be determined. The potential at the detector 2 was varied with each injection. To begin the determinations, detector 2 was set at -0.4 V, the AA solution was injected and the response was recorded. For the next injection, the potential at detector 2 was increased by 0.05 V to -0.35 V, the sample injected, and the response recorded. This procedure was repeated until the response had reached its maximum, i.e., there was no change in response (peak height) for increases in potential.

III. Experimental Compounds

A) Mobile Phase

The mobile phase consisted of 0.20 M KH$_2$PO$_4$ that was brought to a pH of 3.0 with KOH. The final solution was filtered through a 0.22 μm filter (Millipore) before its use. Monobasic potassium phosphate and 85% ortho-phosphoric acid were purchased from Fischer Scientific and were HPLC grade. Potassium hydroxide (45%) was prepared from a stock of KOH pellets purchased from Fisher Scientific and was A.C.S. grade.

B) Buffer

The buffer used in the analysis of DHAA consisted of 120 mM dibasic sodium phosphate brought to a pH of 7.2 with 120 mM monobasic sodium phosphate. The buffer also contained 0.1 mM EDTA and 1mM thiourea for stabilization of AA and DHAA. Dibasic sodium phosphate was purchased from Fischer Scientific and its quality was enzyme grade. Monobasic sodium phosphate was purchased from Matheson, Coleman and Bell and was A.C.S.
grade. The EDTA was purchased from Sigma and was Sigma grade. The buffer was also filtered through a 0.22 μm filter (Millipore Corp.) before use.

C) Meta-Phosphoric Acid

Two solutions of meta-phosphoric acid were used in the assay. For initial stabilization and homogenization of the tissue sample, a 0.3% solution was used containing 1 mM thiourea (Sigma), and 0.1 mM EDTA (Sigma). The second solution was a 10% meta-phosphoric solution also containing 0.1 mM EDTA and 1 mM thiourea and was used for the reaction involved in the analysis of AA and the termination of the reducing reaction for measurement of total AA. The solutions were made by dissolving meta-phosphoric acid in double glass distilled water. The meta-phosphoric acid was purchased from Fisher Scientific and was A.C.S grade.

D) β-Mercaptoethanol

The reducing agent, β-mercaptoethanol, or 2-mercaptoethanol, used in the analysis was purchased from Sigma Chemical Co. (St. Louis, Mo.).

IV. Procedure for Analysis

A) Obtaining the Tissue samples

Tissues were obtained from rats four weeks after injection with streptozotocin or vehicle. After anesthetization of the rats with ether, various tissues were surgically removed. Blood was either removed through a heart puncture or the common iliac artery, which was the technique of euthanizing the animals. The tissues were weighed and immediately placed in 3 mls of 0.3% meta-phosphoric acid, 0.1 mM EDTA, and 1 mM thiourea per gram of tissue to stabilize the analytes of interest.
B) Analysis of Compounds

1) Ascorbic Acid

Samples were placed in meta-phosphoric acid and homogenized using tissue grinders purchased from Curtin Matheson Scientific, Inc. (Eden Prairie, MN). The homogenates were centrifuged at 19,000g for 20 minutes. One hundred µl of the supernatant were removed and used for AA analysis. Nine hundred µl of 10% meta-phosphoric acid were added to the sample to stabilize and allow for analysis by HPLC.

2) Dehydroascorbic Acid

Total AA analysis included endogenous AA and endogenous DHAA that was reduced to AA. By subtracting the endogenous AA, determined in the previously mentioned assay, from the total AA, the endogenous DHAA could be quantified. One hundred µl of the supernatant were removed from the homogenized sample that was centrifuged as for the AA determination. The reducing agent, β-mercaptoethanol, was added to the sample to a final concentration of 10 mM in 390 µl of sodium phosphate buffer (pH 7.2). This solution was allowed to react for 10 minutes at room temperature and the reaction was stopped with the addition of an equal volume of 10% meta-phosphoric acid.

3) Sample Analysis using HPLC-EC System

The samples that were analyzed were either analyzed immediately or frozen for later analysis. The data generated from an individual sample were obtained in the form of a chromatogram in which the AA peak was identified and peak area integrated. Unknowns were compared to standard peaks of known AA concentration, to yield a concentration of AA in the sample.

The standards were made at the time of analysis. They were prepared by making solutions of different concentrations of AA in 5% meta-phosphoric acid
and injecting them into the HPLC-EC system. A computer program called System Gold version 510 was used to integrate peak areas.

V. **Determination of Appropriate Assay Conditions**

A) **Stability of the Frozen Sample**

Liver homogenates were used to determine the stability of prepared samples ready for analysis but frozen for various lengths of time before analysis. The samples were acidic since all contained meta-phosphoric acid.

B) **Assay Conditions**

The determination of appropriate assay conditions for the reduction of DHAA was accomplished by varying one of the conditions while holding the others constant. The optimal condition was chosen on the basis of percent conversion of DHAA to AA of an exogenously added amount of DHAA in the samples. Liver samples were used for the determinations of optimal conditions. The exogenous DHAA that was added was from a solution of AA which was oxidized to DHAA by bromination. The bromine was removed by blowing a stream of N₂ over the solution. The conditions that were varied were reaction temperature, reducing agent concentration, reaction time and buffer pH.

C) **Recoveries**

In the determination of optimal reaction conditions, exogenous AA and DHAA were recovered in a linear fashion from tissue homogenates. Recoveries of exogenous DHAA and AA added to tissue samples and buffer were used for verification that the assay was working. Ten μl of 10 mM AA was added to 100 μl of tissue homogenates, analyzed for AA in diabetic studies and addition of 890 μl of 10% MPA followed. Ten μl of 10 mM DHAA was added to 100 μl of tissue homogenates, prepared from diabetic and nondiabetic animals followed by addition of 380 μl of sodium phosphate buffer and 10 μl of β-
mercaptoethanol. The reaction was stopped with 500 μl of 10% MPA followed. The exogenous AA and DHAA were also recovered from buffer in these studies to evaluate the actual amount of AA and DHAA added to tissues.

VI. Diabetic Studies

A) Streptozotocin Administration

Thirteen rats, 180 to 200 gram body weight, were made diabetic with a single intraperitoneal injection of 75 mg streptozotocin (Sigma Chemical Co.) per kg body weight. The streptozotocin solution was dissolved in 10 mM citrate buffer with a pH of 4.5. Ten control rats received an intraperitoneal injection of 10 mM citrate buffer, pH 4.5.

B) Blood Glucose Measurements

In diabetic studies, blood glucose was measured to verify a diabetic state. The GOD-Perid (Sigma) procedure, based on the oxidation of chromagen by a coupled reaction involving glucose oxidase and peroxidase, was used to establish blood glucose levels. Two-tenths of a ml of whole blood were placed in 0.8 mls cold water. This ml of sample was added to 1.0 ml of 8% Ba(OH)\(_2\)H\(_2\)O. After 1 minute 1.0 ml of 2% ZnSO\(_4\)H\(_2\)O was added and the mixture was vortexed. The precipitated protein was removed by centrifugation at 1850 rpm for 15 minutes in a table top centrifuge at room temperature. One-half ml of the resulting supernate was removed as samples from control animals and as samples from diabetic animals, 0.1 ml of supernate was diluted with 0.4 ml of water and analyzed for glucose concentration.

Two ml of the GOD-Perid reagent was added to the above test tubes and the reaction was allowed to go to completion in 25 minutes at room temperature and absorbance was determined at 505 nm. The assay contained a calibration blank and 4 standards containing 0.005, 0.01, 0.03, and 0.05 mg glucose.
Using the values obtained from the standards and the appropriate dilution factors, the concentration of glucose was calculated.

VII. Statistical Analysis

Data analysis included analysis of variance and linear regression. The accepted level of significance for $F_{obs}$ was $\alpha \leq 0.05$. Linear regression was used to produce standard curves for the recovery of exogenous AA and DHAA from buffer and tissues. Multivariate ANOVAS were performed to compare the conditions tested in the assays involving the determinations of optimal pH, reaction time, reaction temperature, and reducing agent concentration. Univariate ANOVAS were performed to compare the AA and DHAA concentrations in diabetic and nondiabetic tissues.
RESULTS

I. C-V Curve

The results for the C-V (current to voltage) curve for AA are presented in figure 3. Observations that can be made from this curve and the determination of optimal potentials for both detectors are as follows. $E^\circ$ is the formal potential for AA. At potentials negative to $E^\circ$ little or no oxidation of AA occurs. At potentials positive to $E^\circ$ the current quickly reaches its limiting value and the half-wave potential, $E_{1/2}$ is equivalent to the formal potential. The half wave potential is defined as that potential at which current equals one-half of the limiting value. Detector 1 is set at the foot of the C-V curve for the analyte of interest. This is done to decrease background currents and prevent unwanted peaks that result from eluants that oxidize at lower potentials than the analyte of interest. The potential of detector 2 is set at the plateau of the C-V curve, where maximum response is observed for the analyte of interest. The detector potentials for optimal AA measurement were determined to be -0.3 V for detector 1 and 0.0 V for detector 2.

II. Stability of Frozen Samples

Samples of liver homogenates were prepared for analysis of total AA (AA + DHAA) and were frozen before HPLC-EC analysis. Samples were removed from the freezer at various lengths of time after initial freezing and total AA was measured. These values were then compared to initial total before freezing AA concentration.
The results are graphed in figure 4 and indicate that there is no significant loss of total AA in the sample in a time period up to 55 days.

III. Conditions for Reducing DHAA to AA

The results for the determination of appropriate buffer pH, reaction time, reaction temperature, and reducing agent concentration for the reduction of DHAA to AA in tissue homogenates are presented in figures 5, 6, 7, and 8. The optimal conditions were selected by determining the maximum conversion of exogenous DHAA to AA added to the reaction mixture. The process of making DHAA through bromination of AA results in a loss of approximately 20% of the original AA. In order to obtain actual values of AA, DHAA, and DKG in commercial samples of AA and in DHAA solutions obtained from AA by bromination, $^{14}$C radiolabelled AA was used. The radioactive AA is separated by HPLC, where the degradation products of AA, DHAA and DKG, along with AA are separated. The fractions are collected and the concentration of these products is determined by counting the radiolabelled components by liquid scintillation spectometry. The results presented in table 1 indicate that the commercial radiolabelled AA is about 75% AA and 16% DHAA, therefore the total AA (DHAA + AA) in the original sample is 91%. About 69% of the original total AA results in DHAA after bromination with the remainder degrading to DKG. Therefore, a 22% loss of DHAA is expected with bromination, so a 78% or greater recovery of exogenous DHAA would be 100% of what is present. Data are presented in this manner for the determination of optimal conditions.

In figure 5, the appropriate buffer pH was determined for recovery of DHAA from a sodium phosphate buffer. Based on previous reports (110), three pH's 6.2, 7.2, and 8.2 were used for screening. It was determined that the recovery of DHAA at pH 8.2 was significantly less than the recovery at pH 6.2 and pH 7.2.
Although greater recovery was not statistically significant at a pH of 7.2 it was chosen as the buffer pH for the assay.

In figure 6, the results are presented for the determination of the optimal reaction time for the reducing reaction. The conversion of DHAA to AA at times 2, 5, 10, and 20 minutes were all significantly different than conversion at time zero and two minutes. The reaction time chosen for the assay was 10 minutes.

Figure 7 shows the results from experiments in which optimal reaction temperature are shown. There was no significant difference in conversion of exogenous DHAA to AA added to liver homogenates when the reaction was carried out at room temperature or in ice. For convenience, room temperature was chosen as the reaction temperature for the assay.

In figure 8, the results are presented for the determination of the optimal concentration of reducing agent, \( \beta \)-mercaptoethanol. A concentration of 1 mM was not as effective as 5, 10, or 20 mM. The \( \beta \)-mercaptoethanol concentration for maximum conversion of DHAA to AA was chosen as 10 mM. From the data presented in figures 5, 6, 7, and 8, the conditions for reducing DHAA to AA in liver homogenates are: a buffer pH of 7.2, a reaction time of 10 minutes, a reaction temperature at room temperature, and a 10 mM concentration of \( \beta \)-mercaptoethanol.

IV. **Standard Curves of Recoveries**

Figures 9, 10, and 11 are the results obtained from liver, brain, and kidney homogenates, respectively, in which varying amounts of AA were added and recovered. These figures indicate that AA was recovered in a linear fashion from all homogenates.

Figures 12, 13, 14, and 15, are standard curves obtained from buffer, liver, brain, and kidney homogenates, respectively, in which DHAA was added to the
homogenates and recovered in a linear fashion using the reducing reaction explained in the methods section.

The standard curves obtained for the recoveries of both AA and DHAA in tissue homogenates indicates that stabilization and measurement of the analytes is satisfactory and the method described is capable of accurately measuring endogenous levels of AA and DHAA.

V. Diabetic Study

Once the optimal conditions for reducing DHAA to AA were established and recoveries of AA and DHAA were verified, the method was applied to measurement of endogenous levels of AA and DHAA in diabetic rats. Table 2 contains blood glucose measurements from diabetic and control rats used in the study. Diabetic animals had significantly greater blood glucose concentrations as compared to control animals. This measurement is used to confirm that animals were in a uncontrolled diabetic state.

Figure 16 shows results of endogenous AA measurements in liver, kidney and brain tissues from diabetic and control animals. Diabetic liver and kidney tissues had significantly decreased levels of AA compared to control tissues. There was no significant difference of AA levels in diabetic and control brain tissues.

Figure 17 shows the results of endogenous DHAA measurements in liver, kidney, and brain tissues from diabetic and control animals. Levels of DHAA in diabetic liver and kidney were significantly increased compared to control liver and kidney tissues. There was no DHAA detected in the nondiabetic livers. There was no significant difference in the levels of DHAA measured in diabetic and control brain tissues.
In figure 18 (A & B) the results for the levels of AA and DHAA measured in diabetic and control adrenal glands are shown. There was no significant difference in AA levels between diabetic and control adrenal glands. DHAA in diabetic adrenal glands was significantly increased compared to levels found in control adrenals, in which DHAA was not detectable.

Recoveries of exogenous AA and DHAA added to diabetic and control brain, kidney, and liver tissues were done to verify that the assay was working. Results are presented in figure 19. Recoveries of AA and DHAA from both diabetic and nondiabetic tissues were greater than 90%.
DISCUSSION

Ascorbic acid has many important functions and is essential for the maintenance of health. Since AA and DHAA levels in various tissues of healthy subjects are maintained at very low levels, accurate detection of these compounds is required. DHAA and AA are unstable and reactive compounds making their measurement difficult, especially in tissues which contain substances capable of reacting with AA and DHAA and therefore increasing their instability. A highly sensitive, selective, and reliable method for measuring DHAA in tissues has not so far been reported in the literature.

Methods for measuring AA and DHAA have improved with the advent of HPLC combined with electrochemical (EC) detection. HPLC allows for increased selectivity and EC detection utilizes the unique redox properties of AA. Coulometric electrochemical detection increases the sensitivity of the assay by completely oxidizing the analyte at the electrode's surface. The detectors are set at potentials that are optimal for the oxidation of AA and thus, for its determination. Assays for determining DHAA using HPLC-EC include biological fluids, such as cerebrospinal fluid (109), plasma (97, 98, 107, 108, 109) human milk (107), and leukocyte extracts (107). These methods are reliable for these fluid measurements but have not been extensively applied to tissue samples. One method (98) involves derivatization of DHAA with 2,4-diphenylhydrazine so that simultaneous measurement of AA by EC detection and DHAA by ultraviolet detection is possible. However, low detection limits of DHAA are not possible with this method, and therefore it is not satisfactory for
determining differences in animal tissues that normally have very low levels of DHAA. At least one group developed a method (108) which measures both AA and DHAA in tissues, in addition to biological fluids, and foods. In this assay, homocysteine is the reducing agent. The reaction converts endogenous DHAA to AA, over a period of 30 minutes at room temperature and at a pH of 7.1, for electrochemical measurement. The detection limits for this assay are suggested to be 50 pg/20 μl injection volume, while the presently developed method has detection limits of 2 pg/20 μl injection volume. The instability of DHAA and AA is not appropriately addressed in their procedure. The reduction of DHAA for 30 minutes at room temperature and pH 7.1 appears to be inappropriate considering the half-life of DHAA is 6 minutes at a pH of 7.0 and a temperature of 37°C (94).

Ascorbic acid is involved in a variety of functions in the body. It functions in collagen formation (34, 35, 36), acquirement of energy for muscle tissues (34, 35, 37), synthesis of neurotransmitters and neurohormones (48-55), protection against reactive and potentially damaging free radicals (39-47), and the immune response (61). It is understandable then, that a deficiency of this vitamin could produce some damaging effects. The symptoms of scurvy occur when the deficiency is taken to its full extent. It is possible that there is an intermediate level of deficiency that could produce clinical symptoms not exactly similar to those seen in scurvy (72). The present study has shown that in diabetic animals, there is a decrease in concentrations of AA and an increase in DHAA in some tissues.

AA concentrations were decreased in diabetic liver and kidney but no decrease was found in brain or adrenal tissues (Fig. 17 & 19). The mechanisms for the decreased concentrations observed in diabetes are unclear. Mann (71) proposed that there is competition for the transporter by glucose and DHAA.
This then could be a possible explanation for decreased levels of AA since hyperglycemia is associated with diabetes. The high glucose levels could more effectively compete for available transport mechanisms. One problem with this explanation is that a decreased concentration of AA was not observed in all the tissues. Low concentrations of AA in diabetes could be associated with its high metabolic turnover, resulting in increased consumption of AA by oxidation to DHAA (64). This could explain low concentrations of AA found in diabetic human tissues, in which intake of AA may not be adequate due to the rapid turnover. In the present study, rats, which are capable of synthesizing ascorbic acid, were evaluated. In this case, a decreased production of AA, one that is not capable of meeting its rapid turnover, maybe a possible explanation for decreased tissue levels of AA.

DHAA concentrations were increased in liver, adrenals, and kidney tissues but not in brain tissues of diabetic rats (Fig. 18 &19). There are a few possibilities to explain this occurrence. For example, an increased degradation of AA from its rapid turnover in diabetic subjects could lead to increases in its oxidized product, DHAA. There also could be an insufficiency in the mechanisms that regenerate ascorbic acid from its oxidized products. For example, reduction of glutathione levels in various tissues (111, 112) may reduce the potential for nonenzymatic mechanisms to appropriately regenerate ascorbate. Therefore, utilization of AA by its involvement in various biochemical reactions could lead to a buildup of DHAA because at least one mechanisms for regenerating AA is inadequate. Some tissues prefer DHAA as the form for uptake. When DHAA is taken up by these tissues, it appears in the cell as AA (113). Although the mechanism is unclear, it is possible that these tissues have reducing mechanisms (i.e. proteins) in close proximity to the transporter that are not optimally functioning. This would result in a decrease in tissue AA
concentrations because of the tissue's inability to transport this form into the tissue; and DHAA concentrations would be increased because of the tissue's preference for uptake of DHAA and the subsequent decreased ability for DHAA to be reduced.

It is known that in diabetes glycosylation of proteins occur that can reduce or inhibit their activities (114,115). Altered AA metabolism in diabetics could result from decreased function, due to glycosylation, of AA or DHAA transport proteins or enzymes involved in keeping AA in its reduced form.

Altered AA metabolism in certain tissues could account for some of the complications associated with diabetes. Decreased levels of AA may lead to an accumulation of sorbitol since AA plays a role in inhibiting the sorbitol pathway. AA inhibits this pathway either by competing for glucose entrance into this pathway and thereby keeping the reaction products low, or by inhibiting the enzymes responsible for this conversion of glucose to sorbitol. Sorbitol may be a contributor in many of the diabetic complications (86, 87). Increased levels of DHAA could also account for some of the diabetic complications. It is known to be involved in glycosylation reactions and increased concentrations would therefore be expected to increase these types of reactions (83, 84, 85). DHAA does not retain the antioxidant capabilities that AA has which could be associated with increased cell damage (116). DHAA is also known to be toxic to cell membranes (78). Studies have shown that diabetic patients with complications, such as retinopathy, had lower plasma AA concentrations that diabetic patients without complications (82).

It is interesting to speculate as to why brain tissue did not have altered levels of AA and DHAA in diabetic compared to nondiabetic tissues. It may be that AA is critical to the nervous system and is therefore, conserved. It is also possible that whatever mechanisms account for the inappropriate metabolism of AA in
other tissues are not involved in the brain. The fact that vitamin C is such an important component in many biochemical reactions and that its metabolism in diabetes is altered renders it an important part of the diabetic syndrome and suggests further endeavors should be taken to understand its involvement in this disease.
A reliable method for measuring AA and DHAA was established. Its reliability has been verified by recoveries of exogenous AA and DHAA in mammalian tissues. Optimal conditions for reducing DHAA in tissue samples were determined to be: a buffer pH of 7.2, a reaction time of 10 minutes, a reaction temperature at room temperature, and a 10 mM concentration of β-mercaptoethanol.

AA and DHAA were measured in diabetic and nondiabetic rat tissues using this method and the following results were obtained:

1. AA in the kidney of diabetic rats was 1/2 that of nondiabetic kidney. DHAA in the diabetic kidney was 7 times more than nondiabetic concentrations.

2. AA in diabetic liver was 1/2 that of nondiabetic liver. DHAA was 136 nmol/gram in diabetic liver and was not detectable in the nondiabetic liver.

3. AA and DHAA concentrations in diabetic rat brain tissues were not significantly different from the levels found in nondiabetic brain tissues.

4. AA concentrations in diabetic adrenal glands was not different from nondiabetic concentrations. DHAA in the diabetic adrenai glands was 401 nmol/gram and was not detectable in the nondiabetic animal.
APPENDIX A

FIGURES AND TABLES
### Table 1

**Composition of Radiolabeled AA and Prepared DHAA Solutions**

<table>
<thead>
<tr>
<th>Solution</th>
<th>n</th>
<th>%AA</th>
<th>%DHAA</th>
<th>%DKG</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA Standard</td>
<td>15</td>
<td>74.9 ± 1.2</td>
<td>16.2 ± 1.0</td>
<td>8.9 ± 0.8</td>
</tr>
<tr>
<td>DHAA Standard</td>
<td>37</td>
<td>1.8 ± 0.5</td>
<td>69.2 ± 2.7</td>
<td>29.1 ± 2.9</td>
</tr>
</tbody>
</table>

DHAA was prepared by brominating a solution of AA and blowing off the excess bromine with a stream of N₂.

Values are expressed as means ± S. E.
<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>[glucose] mM</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic Rats</td>
<td>10</td>
<td>21.211*</td>
<td>1.085</td>
</tr>
<tr>
<td>Control Rats</td>
<td>10</td>
<td>6.504</td>
<td>0.311</td>
</tr>
</tbody>
</table>

*p = 0.001
FIGURE 1. Ascorbate Free Radical Formation and Reduction
Diketogulonic Acid $\rightarrow$ DHAA $\rightarrow$ Asc

$R^* + \text{Asc} \rightarrow R + \text{AFR}$

DHAA-Reducase

AFR Reductase

-e, -H , +e
FIGURE 2. Metabolism of Ascorbic Acid
Ascorbic Acid

\[
\begin{align*}
\text{HO—C—} & \overset{-2\text{H}}{\rightleftharpoons} \text{c—c—H} \\
\text{I I I I I} & \\
\text{I I I I I} & \\
\text{OH} & \end{align*}
\]

Dehydro-L-ascorbic Acid

\[
\begin{align*}
\text{OH—C—} & \overset{\text{+2H}}{\rightleftharpoons} \text{c—c—H} \\
\text{I I I I I} & \\
\text{I I I I I} & \\
\text{OH} & \end{align*}
\]

2,3 Diketogulonic Acid

\[
\begin{align*}
\text{HO—C—C—C—C—CH•CH}_{2}\text{OH} & \\
\text{O O O O OH} & \end{align*}
\]
FIGURE 3. C-V Curve for Ascorbic Acid  Curve was made using a 1 mM ascorbic acid solution. Detector one (D₁) was set at -0.4 V while the potential at detector two (D₂) was varied at increments of 0.05 V and the response was followed.
FIGURE 4. Stability of Frozen Samples of a Liver Homogenate

Samples were frozen after the reduction reaction (to measure total AA) was completed by the addition of metaphosphoric acid. Measurement of samples were taken at various intervals after being frozen.
FIGURE 5. Determination of Optimal Buffer pH The pH for a 120 mM sodium phosphate buffer used for the reducing reaction of DHAA to AA in liver homogenates. Percent conversion of 1 mM DHAA to AA added to liver homogenates in buffers of pH 6.2, 7.2, and 8.2 was determined.

* p < 0.05 for pH 8.2 compared to pH 6.2 and pH 7.2
FIGURE 6. Determination of the Optimal Reaction Time The optimal reaction time for the reducing reaction was evaluated by adding 1 mM DHAA to liver homogenates and determining the conversion of DHAA to AA when the reducing reaction was carried out at 0, 2, 5, 10, and 20 minutes.

* p < 0.05 for time 0 minutes compared to 2, 5, 10, and 20 minutes and for time 2 minutes compared to 5, 10, and 20 minutes
FIGURE 7. Determination of Optimal Reaction Temperature The optimal reaction temperature for the reducing reaction involved in measuring total AA was evaluated by adding DHAA to liver homogenates. Conversion of the exogenous DHAA was determined in homogenates in which the reducing reaction was carried out at room temperature or on ice.
FIGURE 8. Determination of Optimal Reducing Agent

Concentration The optimal concentration of the reducing agent concentration, β-mercaptoethanol, was evaluated in liver homogenates. One mM DHAA was added to liver homogenates which then was reduced to measure total AA. Percent conversion of DHAA in homogenates using 1, 5, 10, and 20 mM β-mercaptoethanol was determined.

* p < 0.05 for recoveries at 1 mM compared to 5, 10, and 20 mM
FIGURE 9. Standard Curve of Recovered Exogenous AA Added to Liver Homogenates  One mM AA was added to liver homogenates in various amounts and recovered in a linear fashion using the reducing reaction explained in the methods section. 
$R^2 = 0.999$
FIGURE 10. Standard Curve of Recovery of Exogenous AA Added to Brain Homogenates  One mM AA was added to brain homogenates in various amounts and recovered in a linear fashion. $R^2 = 1.000$
FIGURE 11.  Standard Curve of Recovery of AA in Kidney Homogenates  One mM AA was added in varying amounts to kidney homogenates and recovered in a linear fashion.  
$R^2 = 0.996$
FIGURE 12. Standard Curve of Recoveries of DHAA Added to Buffer

DHAA was added to buffer in various amounts and recovered in a linear fashion.

$R^2 = 0.990$
The graph shows the relationship between \( \mu \text{M DHAA Added} \) and \( \text{pmol DHAA Recovered} \). A linear trend is observed, with error bars indicating variability at different levels of DHAA addition.
FIGURE 13. Standard Curve of Recoveries of DHAA in Liver

Homogenates  DHAA was added to liver homogenates in various amounts and recovered in a linear fashion by reduction to AA and subsequent measurement.

$R^2 = 0.992$
FIGURE 14. Standard Curve of Recovery of DHAA Added to Brain Homogenates  

DHAA was added to brain homogenates in varying amounts and reduced to AA for measurement and recovered in a linear fashion.  

$R^2 = 0.996$
FIGURE 15. Standard Curve of Recovery of DHAA From Kidney Homogenates  DHAA was added to kidney homogenates in varying amounts, reduced to AA, and recovered in linear fashion. $R^2 = 0.997$
Endogenous AA levels in diabetic and nondiabetic rat brain, kidney, and liver tissues were determined using HPLC-EC.

* p < 0.05 for diabetic compared to nondiabetic measurements
nmol Ascorbic Acid/gram

- **BRAIN**: DIABETIC > CONTROL
- **LIVER**: DIABETIC > CONTROL
- **KIDNEY**: DIABETIC > CONTROL
FIGURE 17. Endogenous Measurements of DHAA in Diabetic and Nondiabetic Rat Tissues

Endogenous DHAA levels in diabetic and nondiabetic rat liver, brain, and kidney tissues were determined by measurement of total AA and calculation of DHAA based on these measurements and AA measurements.

* p< 0.05 for diabetic compared to nondiabetic measurements

ND = not detectable
FIGURE 18 A & B. Endogenous Measurements of AA and DHAA in Diabetic and Nondiabetic Rat Adrenal Glands Figure 18 A presents the measurement of endogenous AA in diabetic and nondiabetic rat adrenal glands. Figure 18 B presents the measurements of endogenous DHAA in diabetic and nondiabetic rat adrenal glands calculated from measured total AA (AA + DHAA) and endogenous AA.

* p < 0.05 for diabetic compared to nondiabetic measurements

ND = not detectable
(A) nmol Ascorbic Acid/gram

(B) nmol Dehydroascorbic Acid/gram

CONTROL DIABETIC

(A)

ND

*
FIGURE 19. Recovery of Exogenous AA and DHAA  One mM AA and 1 mM DHAA were added to diabetic and nondiabetic tissues used in the analysis of endogenous levels of AA and DHAA. Recoveries in brain, kidney and liver tissues were evaluated and grouped into diabetic and nondiabetic measurements.
## APPENDIX B

### KEY TO ABBREVIATIONS USED IN TEXT

#### Compounds

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Ascorbic acid</td>
</tr>
<tr>
<td>AFR</td>
<td>Ascorbate free radical</td>
</tr>
<tr>
<td>Ba(OH)$_2$8H$_2$O</td>
<td>Barium hydroxide</td>
</tr>
<tr>
<td>DHAA</td>
<td>Dehydroascorbic acid</td>
</tr>
<tr>
<td>DKG</td>
<td>2,3-diketogulonic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>MFO</td>
<td>Mixed function oxidase</td>
</tr>
<tr>
<td>N$_2$</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide, reduced form</td>
</tr>
<tr>
<td>PAM</td>
<td>Peptidylglycine α-amidating monooxygenase</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>ZnSO$_4$7H$_2$O</td>
<td>Zinc Sulfate</td>
</tr>
</tbody>
</table>

#### Units of Measurement

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>L</td>
<td>liter</td>
</tr>
<tr>
<td>M</td>
<td>Molar (moles / liter)</td>
</tr>
<tr>
<td>m</td>
<td>milli</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>mol</td>
<td>moles</td>
</tr>
<tr>
<td>n</td>
<td>nano ((10^{-9}))</td>
</tr>
<tr>
<td>p</td>
<td>pico ((10^{-12}))</td>
</tr>
<tr>
<td>μ</td>
<td>micro ((10^{-6}))</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td><strong>Miscellaneous</strong></td>
<td></td>
</tr>
<tr>
<td>(E^o)</td>
<td>Formal potential</td>
</tr>
<tr>
<td>(E_{1/2})</td>
<td>Half-wave potential</td>
</tr>
<tr>
<td>EC</td>
<td>Electrochemical</td>
</tr>
<tr>
<td>g</td>
<td>Acceleration of gravity</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>pH</td>
<td>-log hydrogen ion concentration</td>
</tr>
</tbody>
</table>
BIBLIOGRAPHY


