Acknowledgements

My most sincere thanks are due to Dr. C. Godin for the help he has given me in the preparation and writing of this thesis. I also thank Dr. A. Desmarais for his kind help particularly in the absence of Dr. Godin.

I would like to express my appreciation to Dr. T.K. Murray and Dr. A.B. Morrison of the Food and Drug Directorate for allowing me to continue my studies and to perform the research reported in this thesis. I would also like to acknowledge the technical assistance received at various times from R. Madere, R. Brassard, E. Nagy and C. Desloges, and the help of D.A. Lyon, statistician, of the Food and Drug Directorate.

TABLE OF CONTENTS

Page No

Page No

ABSTRACT

The chromatographic separation of one part L-ascorbic acid (AA) from 100 parts of D-isoascorbic acid (IAA) was made on silicic acid impregnated glass fiber paper. Since the IAA specimen did not show any trace of AA, the effects obtained in the following studies were due to IAA only.

A specific analytical method for vitamin C was developed. AA was oxidized to dehydroascorbic acid (DHAA) with 2,6-dichloroindophenol; interfering substances, but not DHAA were reduced with homocysteine in presence of boric acid; osazones were formed from the coupling of DHAA with 2,4-dinitrophenyhydrazine. Blanks were obtained by reducing DHAA before the addition of boric acid. AA and DHAA, added to serum and urine, were completely recovered. Reproducibility of assays on different days was satisfactory.

The differential determination of IAA and AA was based on the different rates of osazone formation.

When a diet containing AA plus IAA was fed to guinea pigs, the organs retained a significant quantity of IAA which replaced a corresponding quantity of AA. The incorporated IAA could, in turn, be replaced by AA when AA only was subsequently given in the diet.

It was shown that IAA could cure scorbutic guinea pigs. Since the organs of guinea pigs treated with IAA during two months contained very little AA but a significant quantity of IAA, and since IAA caused an accelerated loss of AA from normal guinea pigs, IAA per se has antiscorbutic activity.

People having lower vitamin C levels retained or utilized more IAA than people having higher vitamin C levels, but IAA did not affect the utilization or urinary excretion of AA by humans.

Studies on the effect of cigarette smoking on vitamin C have shown that smokers had less vitamin C in their body than non-smokers. However, the vitamin C retained in the body has been utilized at the same speed by smokers and non-smokers. The difference in the vitamin C levels between the two groups was not due to a difference in the amount of ingested vitamin C but to a smaller quantity of vitamin reaching the body of the smokers after ingestion of the same dose as non-smokers.

The mode of absorption of L-ascorbic acid by guinea pigs was found very complex. There was a rapid and massive destruction of L-ascorbic acid in the gastrointestinal tract. The tissue of the small intestine was also the major site for the uptake of L-ascorbic acid-1-C^{14} with a maximal uptake at 45 minutes after oral administration. The appearance of labelled vitamin C in the blood and kidneys was maximal only one and a half hours after ingestion. Uptake of L-ascorbic acid-1-C^{14} by the liver and adrenals was maximal 6 hours after ingestion and in all other organs about 3 hours.

In a preliminary experiment, nicotine incorporated in the food of 4 guinea pigs depressed the vitamin C levels of their organs. Since nicotine could have acted directly on the gastrointestinal tract, the effect of nicotine administration by other ways was also studied.

Subcutaneous injections of nicotine to guinea pigs during vitamin C depletion did not accelerate their weight loss or the develop-

ment of scurvy; nicotine did not alter the metabolism of L-ascorbic acid-1-C^{14}, given orally to these animals. Thus, nicotine did not seem to modify the loss of vitamin C stored in the body.

Neither nicotine nor cigarette smoke condensate injected subcutaneously affected the storage of non-labelled vitamin C by guinea pigs, but nicotine caused a decrease in the incorporation of C^{14} by the body and an increase in the amount of $C^{14}O_2$ produced 3 to 6 hours after oral administration of L-ascorbic acid-1-C^{14}. Nicotine thus appears to modify the utilization of vitamin C.

RESUME

Une méthode chromatographique utilisant une feuille de fibre de verre imprégnée d'acide silicique a été améliorée de façon à permettre la séparation d'une partie d'acide L-ascorbique (AA) en présence de cent parties d'acide D-isoascorbique (AIA). Le spécimen d'AIA utilisé dans notre travail n'a montré aucune trace d'AA. Les effets obtenus sont donc dûs à l'AIA seulement.

Nous avons développé une méthode d'analyse spécifique pour la vitamine C. La méthode consiste à: (1) oxider l'AA en acide dehydroascorbique (ADHA) avec le 2,6-dichloroindophenol; (2) réduire les substances gênantes, mais non l'ADHA, avec l'homocystéine en présence d'acide borique; (3) produire des osazones par réaction de l'ADHA avec le 2,4-dinitrophenylhydrazine. En réduisant l'ADHA avant d'ajouter l'acide borique on obtient les "témoins". La méthode a permis le recouvrement complet d'AA et d'ADHA ajoutés au serum et à l'urine. L'analyse d'un même échantillon, plusieurs jours différents, a fourni des résultats semblables.

La différentiation quantitative de l'AA et de l'AIA a été réalisée grâce à leur vitesse différente de formation d'osazones.

Des cobayes recevant un mélange d'AA et d'AIA retiennent dans leur organisme une quantité importante d'AIA qui prend la place d'une quantité correspondante d'AA. L'AIA ainsi retenu peut être remplacé par l'AA donné seul dans la nourriture. Nous avons démontré que l'AIA peut prévenir le scorbut chez le cobaye. Puisque les organes de cobaye traité durant deux mois avec l'AIA contiennent très peu d'AA, mais une quantité importante d'AIA et que l'AIA cause une perte accélérée

d'AA chez le cobaye normal, l'AIA exerce par lui-même cette activité antiscorbutique. L'organisme du cobaye perd l'AIA accumulé plus rapidement que l'AA; ceci expliquerait en partie l'activité moindre de l'AIA.

Les personnes ayant un taux de vitamine C plus bas retiennent ou utilisent plus d'AIA que des personnes ayant un taux de vitamine C plus élevé, mais la présence d'AIA n'influence pas l'utilisation et l'élimination dans l'urine de l'AA.

Des études sur l'effet de la cigarette sur la vitamine C démontrent que les fumeurs ont moins de vitamine C dans l'organisme que les non-fumeurs. Cependant, la vitamine C retenue par l'organisme est utilisée à la même vitesse par les fumeurs et les non-fumeurs. La différence dans le taux de vitamine C entre les deux groupes n'est pas due à une différence dans la quantité de vitamine C ingérée mais à une moindre quantité de vitamine fournie à l'organisme des fumeurs après ingestion de la même dose que les non-fumeurs. Le test de surcharge de vitamine C a démontré d'une manière efficace l'état de vitamine C chez l'individu.

Le mode d'absorption de la vitamine C par le cobaye s'est avéré être très complexe. Il y a destruction rapide et massive de la vitamine C dans le tube digestif. Le tissu de l'intestin grêle est celui qui retient le plus d'acide L-ascorbique-1-C^{14} et ceci environ 45 minutes après ingestion orale. La vitamine C radioactive atteint une concentration maximale dans le sang et le rein seulement une heure et demie après ingestion. La vitamine C radioactive retenue par le foie et les surrénales atteint son maximum 6 heures après ingestion et dans les autres organes 3 heures après seulement.

Une expérience préliminaire indique que la nicotine incluse dans l'alimentation du cobaye diminue le taux de vitamine C dans ses organes. Comme la nicotine aurait pu agir directement sur le tube digestif, l'effet de la nicotine administrée par d'autres voies a été étudié.

L'injection sous-cutanée de nicotine à des cobayes privés de vitamine C n'accélère pas la perte de poids ni l'apparition du scorbut; la nicotine ne modifie pas le métabolisme de l'acide ascorbique-1-C^{14} ingéré par ces animaux. La nicotine ne semble donc pas modifier le taux de perte de la vitamine C accumulée dans l'organisme.

La nicotine et le condensé de cigarette par injection sous-cutanée n'affecte pas l'emmagasinage de la vitamine C non radioactive par le cobaye, mais la nicotine cause une diminution dans la quantité de C^{14} incorporé dans l'organisme et une augmentation de la production de $C^{14}O_2$ au cours de la période de 3 à 6 heures après injection de l'acide ascorbique-1-C^{14}. La nicotine semble donc influencer l'utilisation de la vitamine C.

GENERAL INTRODUCTION

The serious consequences of scurvy for mankind have been known for centuries and records dealing with the prevention of scurvy go back as far as the middle of the sixteenth century when Jacques Cartier during his exploration of Canada found that the natives prevented and cured scurvy by drinking extracts from the bark and needles of pine trees. Although this vitamin is widely distributed in the animal and plant kingdoms, the enzymes for its synthesis from D-glucose or D-galactose in man, monkey, guinea pig, indian fruit bat, and several species of passeriform birds are lacking due to a genetic defect. In 1933, Szent-Gyorgy and Haworth proposed the name ascorbic acid to designate the antiscorbutic principle. On the basis of degradation data, the optically active L-ascorbic acid was shown to be L-threo-hexono-1,4-lactono-2-ene.

Although it became obvious, after reviewing the literature on vitamin C, that many problems regarding this vitamin needed elucidation, priority was given to some of the more acute or chronic problems as follows:

(1) The yearly publication of several methods for the analytical determination of vitamin C indicated a continuous dissatisfaction with existing methods, and an obvious need for a practical and specific analytical method for vitamin C (L-ascorbic acid and dehydroascorbic acid). Because of the increasing use of D-isoascorbic acid as an antioxidant in foods, it was also important to develop an analytical method capable of differentiating between L-ascorbic acid and D-isoascorbic acid; such a method would permit the study of the metabolism of the latter in relation to vitamin C.

(2) In view of conflicting reports in the literature, there was a need to determine if D-isoascorbic acid did or did not have anti-scorbutic activity for the guinea pig. It was not known if this stereoisomer was taken up by guinea pig organs, what was its turnover and its effect on the turnover of L-ascorbic acid in guinea pigs, and how it could affect the vitamin C nutrition of humans.

(3) An insufficient vitamin C intake will result in impaired collagen synthesis which will eventually be manifested by scurvy. A daily intake of 10 mg vitamin C is apparently sufficient to prevent frank scurvy in humans, but it is not known if this intake will prevent all impairments of collagen synthesis. Furthermore, there has been several reports suggesting a requirement for vitamin C in other biological functions, and the association of certain diseases with lower vitamin C levels. A vitamin C intake or a vitamin C utilization sufficient to prevent scurvy might not be adequate for maintaining good health. Because there had been reports suggesting lower vitamin C levels in cigarette smokers and cigarette smokers are more susceptible to certain diseases than non-smokers, it appeared worthwhile to use a highly specific analytical method and to determine by controlled experiments the vitamin C status of cigarette smokers in comparison to non-smokers and to investigate how this difference was produced.

A review of the literature in relation to the above problems and the progress achieved in solving these problems is herewith presented.

PART I

THE ANALYTICAL DETERMINATION OF L-ASCORBIC ACID AND D-ISOASCORBIC ACID*

* Part of the results reported in this section has been published as indicated in the following references:

1 Pelletier, O. 1968. Determination of vitamin C in serum, urine and other biological materials. J. Lab. Clin. Med. 72, 674-679.

2 Pelletier, O. 1969. Differential determination of D-isoascorbic acid and L-ascorbic acid in guinea pig organs. Can. J. Biochem. 47, 449-453.

3 Pelletier, O., and C. Godin, 1969. Vitamin C activity of D-isoascorbic acid for the guinea pig. Can. J. Physiol. Pharmacol. 47, 985-991.

INTRODUCTION

The nature, properties and usage of L-ascorbic acid and D-isoascorbic acid

L-ascorbic acid is a white crystalline substance of molecular formula $C_6H_8O_6$. On the basis of degradation data and as confirmed by the synthesis of L-ascorbic acid from L-xylosone, its structure was established as L-threo-hexono-1,4-lactono-2-ene (Wagner and Folkers 1964).

L-ascorbic acid by oxidation with moderate oxidants will yield dehydroascorbic acid which by reduction can be reconverted to ascorbic acid; dehydroascorbic acid can be further oxidized to diketogulonic acid but diketogulonic cannot be reduced back to dehydroascorbic acid and for this reason has no antiscorbutic activity (Borsook et al. 1937). The antiscorbutic activity of dehydroascorbic acid is equal to that of L-ascorbic acid (Borsook et al. 1937; Linkswiller 1958).

L-ascorbic acid can be prepared commercially (Wagner and Folkers 1964) by modifications of Reichstein (1933) procedure as illustrated in Figure 1: D-glucose (I) is catalytically or electrolytically reduced to D-sorbitol (II) which is transformed to L-sorbose (III) by bacterial oxidation; di-o-isopropyline-L-sorbose (IV) is prepared in order to protect the alcohol groups during oxidation with permanganate; acid hydrolysis of the iso-propylidene groups yields 2-keto-L-gulonic acid (V) which is simultaneously isomerized and lactonized to produce L-ascorbic acid (VI). The synthetic compound is identical with the crystalline substances isolated from the cortex of adrenals or from oranges (Szent-Gyorgy 1938) and lemons (Waught and King 1931).

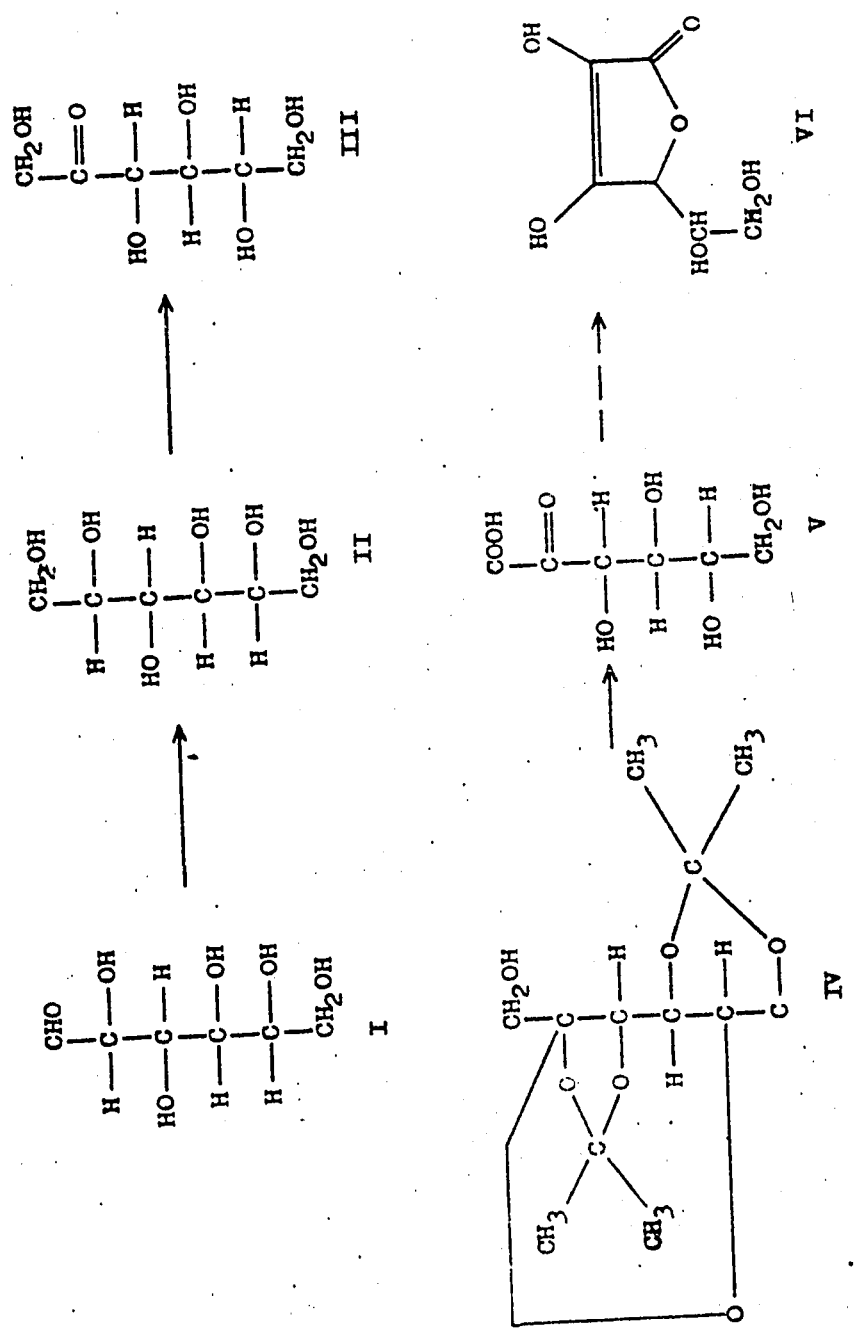
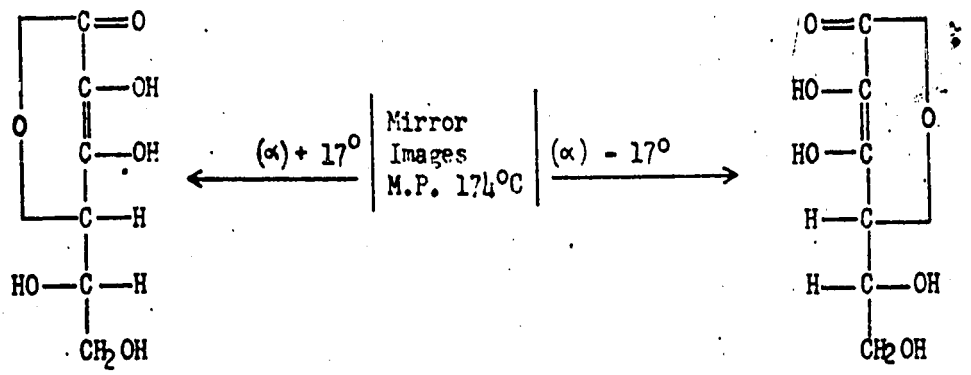


Figure 1. Synthesis of L-ascorbic acid

Since there are two asymetric carbons in ascorbic acid, there can be four optically active isomers of which only two are enantimorphic (mirror image) pairs; the structure of these isomers written according to the conventional Fischer form is given in Figure 2. Both L-ascorbic acid and D-isoascorbic acid have the γ-lactone to the right of the carbon chain, but differ only in the optical activity due to the second hydroxyl group.

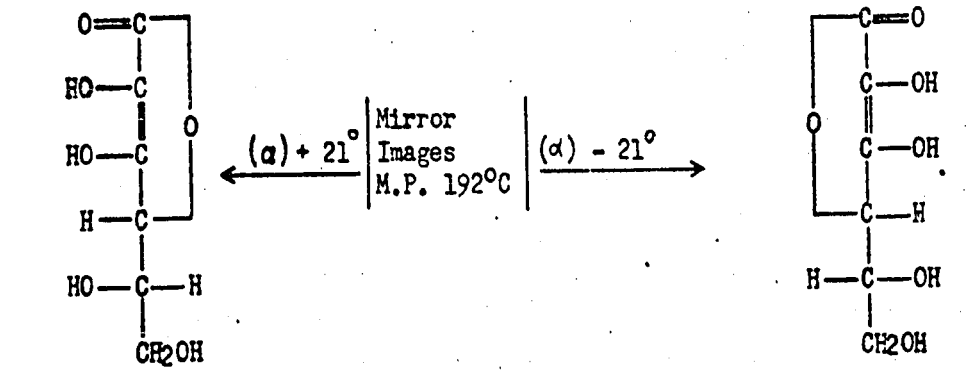
D-ascorbic acid and L-isoascorbic acid do not present any commercial interest, but D-isoascorbic acid (also named D-araboascorbic acid or erythorbic acid) has been used interchangeably with L-ascorbic acid as an antioxidant in the food industry. However, the antiscorbutic activity of D-isoascorbic acid has been reported to vary from 0 to 5 per cent that of L-ascorbic acid (Wang et al. 1962). Because D-isoascorbic acid can be easily synthesized from D-glucose as a cultural metabolite of Penicillia (Yagi et al. 1967, Shimizu et al. 1967), it is available at a lower cost than L-ascorbic acid. The market for D-isoascorbic acid in Canada is considerable since it was estimated at 500,000 lbs. per year in 1966-1967; there has even been speculation lately about the possibility of using D-isoascorbic acid as an antioxidant for L-ascorbic acid in fruit drinks (Private communications from a manufacturer).

The quantities of D-isoascorbic acid used as an antioxidant will vary with different types of food products (see Part II) and in certain cases, depending on nutritional habits significant quantities of D-isoascorbic could be ingested. For metabolic studies involving L-ascorbic acid and D-isoascorbic acid it is thus necessary to have analytical methods that not only differentiate both isomers but that are also unaffected by other interfering substances.



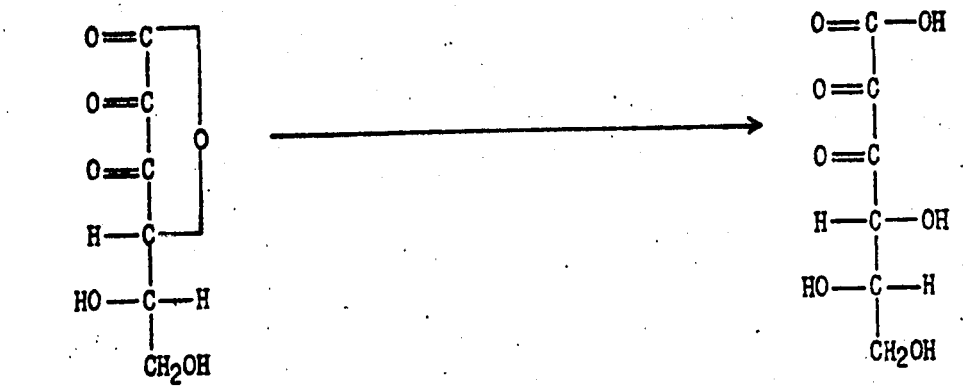
L-ISOASCORBIC ACID

D-ISOASCORBIC ACID



L-ASCORBIC ACID

D-ASCORBIC ACID



DEHYDRO-L-ASCORBIC ACID

DIKETOGULONIC ACID

Figure 2. Isomers and oxidation products of L-ascorbic acid

The analytical methods used to determine L-ascorbic acid

An enormous amount of litterature has been published regarding the determinations of vitamin C. A quotation from "Methods of Vitamin assay; 3rd Ed. 1966 by the Association of Vitamin Chemists Inc. page 289, will best summarize the problem: "The situation regarding chemical analyses remains dynamic. The complex relationship between the compounds possessing vitamin C activity, as well as the chemical similarity of these compounds to others which are inactive, has made the existence of a single, simple and specific method impossible. This has led to a proliferation of methodology which has continued to the present".

In general the vitamin C methodology can be divided as follows: (a) oxido-reduction methods, (b) reaction of ascorbic acid with diazotized compounds and with amines, (c) reaction of the ketonic groups of dehydroascorbic acid and diketogulonic acid with 2,4-dinitrophenylhydrazine.

(a) Oxido-reduction methods

In these methods, advantage is taken of the chemical characteristics of the dienol group on carbon 2 and 3 of ascorbic acid; the two hydrogen atoms of this group can easily be oxidized to yield dehydroascorbic acid. When dehydroascorbic acid is present in the solution to be analyzed, a true total vitamin C value cannot be obtained unless the dehydro form is previously reduced.

The classical L-ascorbic acid assay is that of Tillmans (1930) in which the vitamin is titrated to a pink end point with 2,6-dichloroindophenol. In the oxidized form, 2,6-dichloroindophenol is blue in

neutral or alkaline solution and pink in an acid solution; in the process of reduction by ascorbic acid, 2,6-dichlorindophenol becomes colorless and dehydroascorbic acid is formed. The mechanism of the reaction is shown in Figure 3.

Visual titration in acid solution requires a large enough concentration of vitamin C so that the pink end point can be obtained by an amount of dye significantly above the reagent blank; titration of 1 mg of L-ascorbic acid in 5 ml of solution will yield reproducible results, but it is difficult, even for an experienced analyst, to obtain accurate results with concentrations lower than 0.2 mg per 10 ml. For this reason, this method can be used mainly for the assay of L-ascorbic acid in fruits, fruit juices and pharmaceuticals.

The dye is decolorized by other reducing substances such as ferrous salts, -SH compounds (e.g. cysteine, glutathione), reductic acid, reductones (Lugg 1942) and the isomers of ascorbic acid. Evelyn (1938) has reported that interfering substances often account for more than 90.7 per cent of the total indophenol reducing capacity of urine.

Attempts have been made to eliminate interference of some of these substances by using sample blanks. Formaldehyde was used for this purpose because at pH of 3.5 it condensed with ascorbic acid but not appreciably with ferrous salts or reductones (Lugg, 1942; Robinson and Stoz, 1945; Kuusi, 1960; Pelletier, 1967). Mapson (1943) found that, at a pH of 2.0, ascorbic acid was rapidly removed by condensation and reductones were slowly removed but at a linear rate; by extrapolation it was possible to determine the amount of 2,6-dichloroindophenol reduced by reductones before condensation with formaldehyde. According to Lugg (1942),

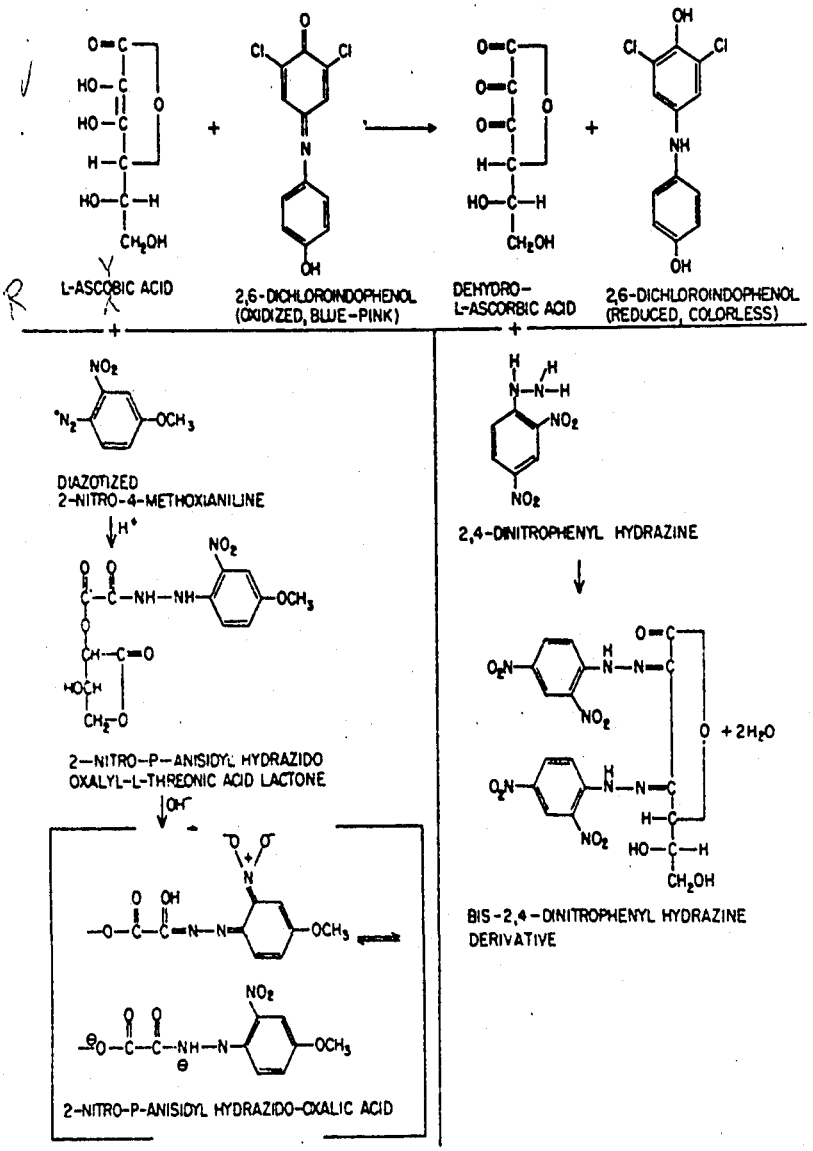


Figure 3. Chemical reactions of vitamin C

the interferences of sulfites, thiosulfates, cysteine, H_2S, H_2S-treated pyruric acid, etc. could be eliminated because these rapidly condensed with formaldehyde at pH 1.5 while ascorbic acid condensed very slowly. Thus the use of formaldehyde condensation for obtaining blanks in the titration of ascorbic acid is limited to certain applications and is time consuming. Pelletier and Morrison (1966) made use of air oxidation in presence of ethylenediamine tetraacetic acid to oxidize ferrous and stannous salts, but not ascorbic acid which could be subsequently titrated with 2,6-dichloroindophenol. Owen and Iggo (1956) recommended the use of p-chloromercuribenzoic acid to suppress the interference the sulfhydryl type of reducing compounds, but they also stated that although satisfactory for the determination of ascorbic acid in biological materials, the method was less accurate than the formaldehyde procedure when applied to urine because p-chloromercuribenzoic acid abolished only a small part of the interfering substances.

The end point in visual titrations is often masked by the colour of extracts and is difficult to determine. One way to circumvent this is to extract the reduced dye with chloroform (McHenry and Graham, 1935) and ethyl ether (Nadkarni 1965); these methods, however, require several titrations and a large quantity of sample. A simple approach proposed by Pelletier and Morrison (1965), consists in detecting the end point by the increase in optical density observed at 545 mμ.

Several photometric methods based on the decrease in optical density of 2,6-dichloroindophenol due to ascorbic acid have been proposed: Mindlin and Butler (1938); Evelyn et al. (1938): Bessey (1938): Hughes (1956), and Howard and Constable (1966). Bessey (1938) showed that a pH of 3.4 was

optimal because at low pH values, the dye was reduced spontaneously by H ions and at a pH above 4, other reducing substances reacted more readily with 2,6-dichloroindophenol. By taking colorimeter readings at successive intervals after the reactants are mixed, one can correct by extrapolating for the presence of interfering substances that react more slowly than ascorbic acid, but not of those that react more rapidly. Such procedures are too time consuming for the measurement of a large quantity of samples. Hughes (1956) on the other hand found a pH of 2-3 more suitable than 3-4 and measured the color intensity of the dye within 30 seconds after its addition; later Hughes (1964) made use of cation exchange resin (Zeo-Karb 225) to remove yellow pigments and about 30 percent of the original capacity of the urine to reduce the dye and he assumed that this decrease was due to removal of interfering substances. Personal experience with Hughes' method indicates that, below a pH of 3, 2,6-dichloroindophenol is not stable enough to obtain accurate results. Another variant of this photometric method consisted in measuring excess 2,6-dichloroindophenol extracted in xylene after reduction of the dye (Pepkowitz 1943).

An empirical method for evaluating the vitamin C status of individuals was based on the time required for decolorization of 2,6-dichloroindophenol applied intradermally (Rotter 1933), but the test was considered too variable to have a significant clinical value (Holland et al. 1947). Recently a lingual vitamin C test, based on the time required for decolorization of 2,6-dichloroindophenol placed on the tongue, has been found suitable for evaluating the vitamin C status of individuals (Cheraskin and Ringsdorf 1968).

Barakat et al. (1955) have described the titration of L-ascorbic acid with N-bromosuccinimide: after ascorbic acid has been totally oxidized to dehydroascorbic acid, a second selective oxidation of KI liberated iodine which produced a visual end point with starch added as an internal indicator. They claimed that reductones, reductic acid and iron salts caused no interference with this titration. Evered (1960) used N-bromosuccinimide for titrating L-ascorbic acid, but detected the end point by extracting liberated iodine in organic solvent. Hardesty (1964) modified the latter procedures for potentiometric titration by using platinum electrodes with a polarizing current and titrating to the point of maximum inflection.

Other oxidation systems for determining L-ascorbic acid were based on the reduction of ferric ions and the determination of the resulting ferrous ions. Sullivan and Clark (1955), determined the ferrous ions as the red-orange, α,α'-dipyridyl complex and found that in the determination of L-ascorbic acid in urine other reducing substances could be inhibited by the presence of orthophosphate and a high degree of acidity (pH 1-2); Maickel (1960) found the Sullivan and Clark procedure applicable to the determination of ascorbic acid in animal tissues; Varm (1965) used a similar system but determined the ferrous ion after formation of a colored complex with bathophenanthroline; Przyborowski (1966) titrated the ferrous ions with ethylenediamine tetraacetic acid in presence of xylenol orange but applied his procedure only to pure L-ascorbic acid and pharmaceutical preparations. Kum-Tatt and Leong (1964) determined L-ascorbic acid in urine by reduction of mercuric chloride to mercurous chloride at a pH of 3.5-5.0; the insoluble mercurous salt was separated

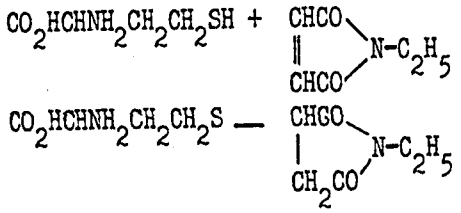
by centrifugation, dissolved with potassium iodide solution and back titrated with standard sodium thiosulfate. They claimed that ferrous salts did not interfere, but did not rule out that other reducing substances were not interfering.

Perrin and Perrin (1946) have successfully used the polarograph for the electrolysis (electrooxidation) of L-ascorbic acid between two electrodes, one of which consisted of fine drops of mercury falling from a capillary placed in the solution: when gradually increasing voltage was applied to the electrodes and the resulting current-voltage curve was plotted, a typical wave (a step) was obtained for L-ascorbic acid. The addition of a small amount of copper salt to destroy L-ascorbic acid provided a blank. Cysteine was reported not to interfere. Applicability of this method to biological materials other than milk has not been reported.

Besides being subject to interference from other reducing substances, methods based on oxido-reduction will determine only ascorbic acid and not dehydroascorbic acid which may be present in the samples to be analysed. Various means have been suggested for converting dehydroascorbic acid to ascorbic acid. Reduction of dehydroascorbic acid with hydrogen sulfide is possible (Bessey 1938) but such a procedure is laborious and one must ensure removal of excess reductant before estimating the ascorbic acid formed; furthermore, hydrogen sulfide is known to react with certain quinones, aldehydes, keto acids (e.g. pyruvic acid) etc. (Smyte and King 1942) and produce powerful reducing substances, some of which might be of a sulfhydryl nature (King 1941).

Stewart and Sharp (1945) have used Escherichia coli and Staphylococcus albus to reduce dehydroascorbic acid to ascorbic acid; although they have shown their procedure to be applicable to urine and blood plasma, it is too laborious for the analysis of many samples.

Dehydroascorbic acid can be easily reduced by homocysteine at a pH of about 7.0. Hughes (1956) used homocysteine in presence of boric acid in order to prevent the reduction of dehydroascorbic acid and permit the reduction of the dehydro forms of interfering substances such as alloxan, reductic acid, glucoreductones (Lugg 1947); this provided a blank for the determination of dehydroascorbic acid after homocysteine treatment without boric acid. Howard and Constable (1966) used a modification of Hughes' procedure for determining vitamin C in urine. Lento et al. (1963) also reduced dehydroascorbic acid to ascorbic acid with homocysteine for their polarographic determinations, and excess homocysteine was removed by treatment with N-ethylmaleimide as follows:



Hewitt and Dickes (1961) found the reduction with homocysteine suitable for estimating dehydroascorbic acid and ascorbic acid in plant tissues: their method consisted of measuring the absorbance of ascorbic acid at 265 mμ and subtracting the absorbance obtained after oxidation with ascorbic acid oxidase.

One might think that the use of ascorbic acid oxidase to oxidize ascorbic acid would provide suitable blanks for the chemical determination of ascorbic acid. Cucumber juice and squash are good sources of ascorbic acid oxidase but the enzyme is not specific (Stewart and Sharp, 1945) and will oxidize all the ascorbic acid stereoisomers in addition to glucoreductones, reductic acid, etc. (Stark and Dawson, 1963).

(b) Reaction of ascorbic acid with diazotized compounds and amines

Schmall et al. (1953), 1954) introduced a method based on the coupling of a diazonium cation (2-nitro-4-methoxyaniline) with ascorbic acid at the site of the keto group and tautomerism to form a phenylhydrazide of the 4-oxalate of D-threonic lactone (Figure 3). Although the method does not respond to 2,3-diketogulonic acid, and sulfurous acid, it is limited because it does not determine dehydroascorbic acid and requires as much as 0.5 mg ascorbic acid per 5 ml of the solution to be analysed; the interference of reductones and reductic acid however can be removed by the formaldehyde condensation method of Robinson and Stotz (1945). Davidek and Davidkova (1959) have reported that the method of Schmall et al. (1954) gave low values in presence of flavanoids. Weeks and Deutch (1965) increased the sensitivity to 15 µg ascorbic acid per ml by using diazotized p-nitroaniline, but found that cysteine interfered. Recently, Michaelsson and Michaelsson (1967) have used the zinc chloride double salt of diazotized 4-nitroaniline 2,5-dimethoxy-aniline (Brentamine, Fast Black K salt) for the determination of ascorbic acid but not dehydroascorbic acid in blood plasma.

Deutch and Weeks (1965) introduced a fluorometric assay for vitamin C which is based on the reaction of dehydroascorbic acid with o-phenylenediamine to give a fluorescent quinoxaline. They have applied their procedure to pharmaceuticals, beverages and milk base infant formulas but not to biological materials. It is not known if there is interference from diketogulonic acid a product of further oxidation of dehydroascorbic acid. The method is limited because not less than 15 µg of ascorbic acid per ml of solution is required for assay.

(c) Reaction of the ketonic groups of dehydroascorbic acid and diketogulonic acid with 2,4-dinitrophenylhydrazine.

Roe and Kuether (1943) described a method based on the reaction of the ketonic group of dehydroascorbic acid or diketogulonic acid with 2,4-dinitrophenylhydrazine to yield red-colored osazones (Figure 3). This bis-2,4-dinitrophenylhydrazine derivative yields a highly stable color that absorbs maximally at 500-550 mu when treated with 85 per cent H_2SO_4. The relative specificity of this dinitrophenylhydrazine method is based on the following principles (Roe 1961):

(1) The characteristic color with 2,4-dinitrophenylhydrazine is formed only with 5-carbon and 6-carbon sugarlike compounds; triose sugars for example do couple with 2,4-dinitrophenylhydrazine but uncouple when treated with 85 per cent sulfuric acid (Hove, 1965).

(2) The rate of reaction of sugars is much slower than that of dehydroascorbic acid and their interference is less marked at 37°C;

(3) During the reaction with 2,4-dinitrophenylhydrazine, a little thiourea provides a weakly reducing medium which prevents decomposition

of dehydroascorbic acid and diketogulonic acid and discoloration of the 2,4-dinitrophenylhydrazine solution by any oxidation products that might form.

(4) Ascorbic acid must be oxidized to dehydroascorbic acid before any coupling can occur; Norit (an activated charcoal) is the best oxidizing agent since it acts also as a clarifying agent.

In order to differentiate ascorbic acid, dehydroascorbic acid and diketogulonic acid, Roe et al. (1948) used hydrogen sulfide to convert dehydroascorbic acid to ascorbic acid:

(a) since hydrogen sulfide did not reduce diketogulonic acid, the latter could be determined with 2,4-dinitrophenylhydrazine;

(b) ascorbic acid, dehydroascorbic acid and diketogulonic acid were determined after oxidation with bromine;

(c) dehydroascorbic acid and diketogulonic acid were determined in a solution that was neither reduced, nor oxidized.

For this differentiation, incubation with 2-4-dinitrophenylhydrazine was prolonged to 6 hours instead of 3 hours, because the rate of reaction of dehydroascorbic acid was less rapid than that of diketogulonic acid. Although this procedure can differentiate ascorbic acid, dehydroascorbic acid and diketogulonic acid, it has undesirable features: the use of different blanks for ascorbic acid, dehydroascorbic acid and diketogulonic acid; reduction with hydrogen sulfide and removal of excess hydrogen sulfide; incubation during 6 hours; and addition of 5 ml sulfuric acid drop by drop in an ice bath. Because the method is too time consuming, it is not practical for doing numerous assays in one day.

Several attempts have been made to simplify the procedure of Roe and Kuether (1943). Bolin and Book (1947) introduced the use of 2,6-dichloroindophenol instead of Norit to oxidize ascorbic acid: this modification was found suitable for the determination of vitamin C in blood (Hausman-Lench and Lewis 1961), in urine (Polk, Flanagan and Van Loon 1960) in rat liver extracts (Schwartz and Williams 1955). Lowri (1945), in a microprocedure for plasma, serum or blood, used copper sulfate to oxidize ascorbic acid. Meyer et al. (1952) found that activated charcoals sometimes retain a significant amount of ascorbic acid or dehydroascorbic acid and used bromine to oxidize ascorbic acid from adrenal glands and removed excess bromine with aniline.

Shaffert and Kingsley (1955) carried out the coupling reaction with 2,4-dinitrophenylhydrazine during 5 minutes at 100°C instead of 3 hours at 37°C. This modification has been strongly criticized by Roe (1961) since it could result in erroneously high values due to sugarlike compounds.

Several authors have suggested other acids in order to avoid the tedious addition of 85 per cent sulfuric acid. Glacial acetic acid (Bolomey and Kemmerer 1946, 1947) resulted in a considerable drop in sensitivity, and Schwartz and Williams (1955) used a mixture (2:3) of 85 per cent phosphoric acid and concentrated hydrochloric acid in order to obtain a two fold increase in sensitivity over glacial acetic acid. Hausman-Lench and Lewis (1961) found the hydrochloric acid fumes objectionable and preferred a (1:1) concentrated hydrochloric acid solution since it did not decrease the sensitivity.

Chromatographic separation and measurement of the 2,4-dinitrophenyl osazones have been used as a mean of eliminating interfering substances (Szoke 1960; Mapson 1961 and Vuilleumier and Nobile 1962), but such procedures are so elaborate that the daily analysis of several samples is not readily accomplished.

Extractions of vitamin C from the samples to be analysed.

Several extracting solutions have been used, depending on the chemical method of analysis, in order to stabilize vitamin C and avoid interference from other materials: these extracting agents consist in a variety of acid solutions (Chapman et al. 1951) but one of the most popular is metaphosphoric acid. Farmer and Abt (1936) confirmed the results of other workers that ascorbic acid was more stable in 2 per cent metaphosphoric acid than in 5 or 10 per cent trichloroacetic acid: they found no loss to occur in plasma filtrates deproteinized with metaphosphoric acid. On the other hand, the methods of Roe and Kuether (1943) and Lowry et al. (1945) made use of trichloroacetic acid. This acid had the advantage of preventing the adsorption of vitamin C on charcoal (Roe and Kuether, 1943); however, in order to differentiate ascorbic acid, dehydroascorbic acid, and diketogulonic acid. Roe et al. (1948) used metaphosphoric acid in presence of stannous chloride. Dehydroascorbic acid, however, cannot be determined in whole blood because of the oxidation of ascorbic acid to dehydroascorbic acid during acid extraction (Borsook et al. 1936).

Differential determination of D-isoascorbic acid and L-ascorbic acid.

Because of the close similarity of L-ascorbic acid and D-isoascorbic acid (Figure 2), it was not surprising not to find in the literature a

specific chemical analytical method capable of differentiating both isomers. There are, however, a few chromatographic methods for the determination of the isomers: a review of some of these methods has been given by DeRitter (1965). The sensitivity of these methods is below that required for the assay of biological materials. For example, Fabianek and Herp (1967) using the paper chromatographic method of Miki et al. (1962) and the thin layer procedure of Brenner et al. (1964) obtained satisfactory results with pure solutions, but no yield with tissue extracts. Weeks and Deutch (1967) chromatographed the two isomers on silicic acid-impregnated glass fiber paper and determined each with the microfluorometric method of Deutch and Weeks (1965); but this is not sensitive enough for the assay of the biological specimens from humans since a concentration of at least 1.8 µg per 20 µl is required for the assay. A paper chromatographic method for separating L-ascorbic acid from D-isoascorbic acid has recently been published by Pazarincevic and Damanski (1968), but such a method is only semiquantitative. The method of Vuilleumier (1967) for separating the 2,4-dinitrophenylhydrazine derivatives of the two isomers also lacks the sensitivity required for biological materials.

The need of better methods for determining vitamin C and D-isoascorbic acid.

After reviewing this tremendous amount of literature on the determination of vitamin C and D-isoascorbic acid, it certainly appeared obvious that the situation regarding the chemical analysis of vitamin C was still dynamic and that no satisfactory analytical method existed

for differentiating vitamin C and D-isoascorbic acid in biological materials. Because studies on the metabolism of vitamin C and D-isoascorbic acid would require a large number of determinations of these compounds, and in view of the lability of these isomers, methods capable of handling this load were required.

In view of determining the purity of the D-isoascorbic acid used in these studies, chromatographic procedures were modified in order to separate one part of L-ascorbic acid from 100 parts of D-isoascorbic acid.

By using the reaction 2,4-dinitrophenylhydrazine with dehydroascorbic acid and other chemical reactions with vitamin C in various sequences, a highly specific, accurate, reproducible and fairly rapid method was developed for the quantitative differentiation of ascorbic acid, dehydroascorbic acid and diketogulonic acid in biological materials; in addition, the quantitative differentiation of the D-isoascorbic acid forms from the L-ascorbic acid was accomplished by making use of their different rates of reaction with 2,4-dinitrophenylhydrazine.

MATERIALS AND METHODS

A. Chromatographic separation of D-isoascorbic acid and L-ascorbic acid.

Materials

Apparatus. A Eastman Chromatogram Developing apparatus was used for developing the chromatograms.

Reagents. A silicic-acid impregnated glass fiber sheet, 7" x 8", (Applied Science Laboratories, Inc., State College, Pennsylvania 16801) was placed in a 8" x 12" stainless steel pan and immersed with occasional mixing for 17 minutes in 100 ml of 6 per cent metaphosphoric acid (6 g of metaphosphoric acid pellets dissolved to 100 ml with distilled water). The soaked sheet was suspended in the draft of a fume hood for 2 hours and 15 minutes and subsequently dried 15 hours over an asbestos sheet placed on the top shelf of a Fisher Isotherm Oven set at 92.5°C.

L-ascorbic acid (obtained from Nutritional Biochemicals Corporation) and D-isoascorbic acid (supplied by Hoffman-Laroche, Inc., Montreal) were dissolved in 3 per cent metaphosphoric acid.

The developing solvent (Brenner et al. 1964) consisted of the following mixture: acetonitrile, 33 ml; butyronitrile, 16.5 ml; distilled water, 1.0 ml.

The spraying reagent which consisted of 3.5 per cent molybdophosphoric acid in isopropanol was obtained from Brinkmann Instruments Inc.

Methods

Two spots were applied at 23 mm from the bottom of the silicic-acid impregnated glass fiber sheet at a distance of 20 mm from each other.

The spots were dried by suspending the sheet in a hood for 10 minutes. The chromatogram was developed until the solvent reached the top of the paper (about 18 minutes). After being dried in the draft of a hood for about 10 minutes, the sheet was sprayed lightly with 3.5 per cent molybdophosphoric acid.

B. Determination of vitamin C.

Materials

Apparatus. A Coleman Jr. II Model 6/20 spectrophotometer and/or a Spectronic 20 with a 17 mm light path vacuum cuvette was used to measure the per cent transmittance.

A water bath was maintained at 32°C with the Haake-Thermostat Unitherm. A cold water bath was maintained below 5°C with ice.

Glass columns (450 mm by 10 mm) with Teflon stopcocks were used for the ion exchange chromatography of urine samples except those from subjects receiving supplements of L-ascorbic acid. A small pledget of glass wool was inserted into the bottom of each column and covered with 1 cm of 60/80 mesh glass beads. About 10 cm^3 of Amberlite IR-120 analytical grade were packed into the column. Thirty ml of 2 N NaOH was passed through and followed by distilled water until the effluent was about neutral. Thirty ml of 2 N HCL was then passed through, and the resin washed until neutral. The NaOH-HCl treatment was repeated for each determination.

Reagents. Solutions were diluted with distilled water unless otherwise indicated and were filtered if necessary.

Boric acid 5 per cent (W/V) was stored at room temperature.

The following solutions were made every 2 weeks and stored in a refrigerator: stock metaphosphoric acid solution, 50 per cent (W/V); dipotassium hydrogen phosphate, 45 per cent (W/V); 2,4-dinitrophenylhydrazine, 4 per cent (W/V) in 9 N sulfuric acid; and 2,6-dichloroindophenol solution by dissolving 125 mg of sodium 2,6-dichloroindophenol in 100 ml of warm distilled water, filtering, and making to 500 ml with washings.

The following solutions were prepared daily: 2, 2.66, and 4 per cent metaphosphoric acid by diluting 4, 5.32 and 8 ml. respectively, of stock solution (50 per cent) to 100 ml; L-ascorbic acid standard, 6 μg per ml of 2 per cent metaphosphoric acid; DL-homocysteine, 6.25 mg per ml; thiourea-phosphoric acid solution, 5 per cent (W/V) and 64 per cent respectively, phosphoric-nitric acid mixture (2:1); and Antifoam FG-10 emulsion (Dow Corning Corporation) diluted (0.1:20).

Methods

Preparation of Samples. The urine samples were collected over enough metaphosphoric acid pellets to make a 4 per cent solution after diluting with distilled water one volume of urine to about 2 volumes. The collected samples were kept at about 5°C and assayed within 24 hours or frozen and assayed after thawing at room temperature. Samples from human subjects on a vitamin C intake of less than 100 mg per day were treated as follows: 5 ml of urine containing 4 per cent metaphosphoric acid was added to a column of IRA-120 (H^+); the effluent was collected into a 25 ml volumetric flask containing 1.2 ml of 25 per cent metaphosphoric acid, and the volume was completed to 25 ml with the washings from the column (5 ml portions of distilled water).

The heparinized blood, plasma and/or serum were assayed as soon as possible after being collected. Five ml of specimen was added slowly and with agitation to 15 ml of 2.66 per cent metaphosphoric acid. These were thoroughly mixed (at that point, the blood had precipitated and darkened). After centrifugation, the supernatant fluid was removed with a pipette and filtered.

Tissues from guinea pigs were kept on dry ice after removal. The samples were blended for 20 seconds with a solution of 4 per cent metaphosphoric acid at about 5°C and diluted with distilled water to obtain 2 per cent metaphosphoric acid. One or two drops of non-diluted Antifoam FG-10 were added to prevent foaming of certain samples. The optimal final dilution was 3 to 7 µg of vitamin C per ml.

Total vitamin C assay (ascorbic and dehydroascorbic acid).

All determinations were done in duplicate. Two ml of sample or standard solutions were pipetted into 150 mm by 20 mm test tubes labelled 1 and 2. All mixing operations in the subsequent steps were made by vortex.

Homocysteine solution (0.2 ml) was added to all tubes marked 2 and boric acid (0.5 ml) to all tubes labelled 1; the solutions were mixed very gently after each addition.

The following steps were performed for in duplicate. Drops of 2,6-dichloroindophenol were added rapidly with gentle mixing to tube 1 until a purple-pink colour was obtained, and the same number of drops of distilled water was added in test tube 2; 0.5 ml dipotassium hydrogen phosphate solution was added with mixing to tubes 1 and 2, and 0.2 ml homocysteine solution to tube 1. These tubes were allowed to stand for 30 minutes while other tubes were processed in the same way. Drops of distilled water were

added to some standards or samples in order to correct for variations in the amounts of 2,6-dichloroindophenol.

After 30 minutes, one duplicate determination was processed in the following order: 0.5 ml boric acid solution was added to tube 2 and mixed; 1 ml of thiourea-phosphoric acid solution was added with thorough mixing to tubes 1 and 2, and was followed with 0.5 ml 2,4-dinitrophenylhydrazine solution added in the same way. These tubes were immediately placed in a water bath at 32°C for exactly 80 minutes.

The tubes were cooled for at least 5 minutes in a cold water bath at less than 5°C; 5 ml of phosphoric-nitric acid mixture was added to the cooled test tubes while bubbling nitrogen moderately. The nitrogen bubbling was continued for about 2 minutes. At this point 4 to 8 tubes were processed at a time. One or 2 drops of diluted Antifoam FG-10 were added in case of excessive foaming. The tubes were allowed to cool for about 5 more minutes and were kept at room temperature for about 45 minutes. The per cent transmittance, with the respective blanks set at 100, was measured at 520 mμ after rinsing the vacuum cuvette with about 1 ml of the solution to be read.

Calculations were as follows:

$$\mu g \text{ of vitamin C per ml of sample} = 6 \times \frac{\text{net absorbance (tube 1 - tube 2) of sample}}{\text{net absorbance (tube 1 - tube 2) of standard}}$$

Ascorbic acid assay

The procedure was as above for tube 1. Tube 1' was treated as tube 1 except that distilled water was added in place of 2,6-dichloroindophenol.

Calculations were as follows:

$$\mu g \text{ ascorbic acid per ml of sample} = 6 \times \frac{\text{net absorbance (tube 1 - tube 1')} \text{ of sample}}{\text{net absorbance (tube 1 - tube 1')} \text{ of standard}}$$

C. Differential determination of L-ascorbic acid (AA) and D-isoascorbic acid (IAA).

Materials and methods were essentially as described under B. Determination of vitamin C, except that duplicate determinations were made at 32° and 52°C.

Calculations were made as follows:

[1] μg AA per ml sample = $6[f(c/d)-e]/[b(c/d)-a]$

[2] μg IAA per ml sample = $6[e-(ag/6)]/c$

Where a-f refer to absorbances; a, 6 μg AA at 52°C; b, 6 μg AA at 32°C; c, 6 μg IAA at 52°C; d, 6 μg IAA at 32°C; e, sample at 52°C; f, sample at 32°C; and q is the result obtained by Eq. (1).

RESULTS

A. Chromatographic separation of D-isoascorbic acid(IAA) and L-ascorbic acid (A A).

A typical chromatogram of AA and IAA is shown in Figure 4. Rf values for AA varied from 0.38 - 0.40 and those of IAA from 0.50 - 0.56 depending on the concentrations. The intensities and size of the bluish spots depended on the concentration. Two µg of AA and IAA gave weak but distinct spots that were very well separated. IAA spots at 200 µg were large and elongated. Addition of 20 µg AA to 200 µg IAA produced a compact spot well separated from IAA. Addition of 2 µg AA to 200 µg IAA produced a less intense spot that was also separated from IAA.

B. Determination of vitamin C.

Mixtures of 6 µg L-ascorbic acid (AA) per ml with sufficient glucoreductones (Lugg 1947) to yield a pseudovitamin C content of 10.8 µg per ml by the method of Roe and Kuether (1943) yielded complete recoveries of true vitamin C. D-glucose and lactose in quantities 500 times larger than AA, and citric, glyoxylic, oxalic, and pyruvic acids in quantities 20 times larger than AA did not interfere. Nicotinic and caffeine in concentrations 66 times larger than AA were also without interference.

Samples of urine from subject on regular diets were passed through columns of IRA-120 (H^+) as described in the method's section by a technique very similar to that of Hughes (1964) who used Zeo-Karb 225 as a cation exchange resin. As shown in Table I, different dilutions of urine gave essentially the same values and complete recoveries of added AA or dehydroascorbic acid (DHAA). Twenty-four hour urine specimens

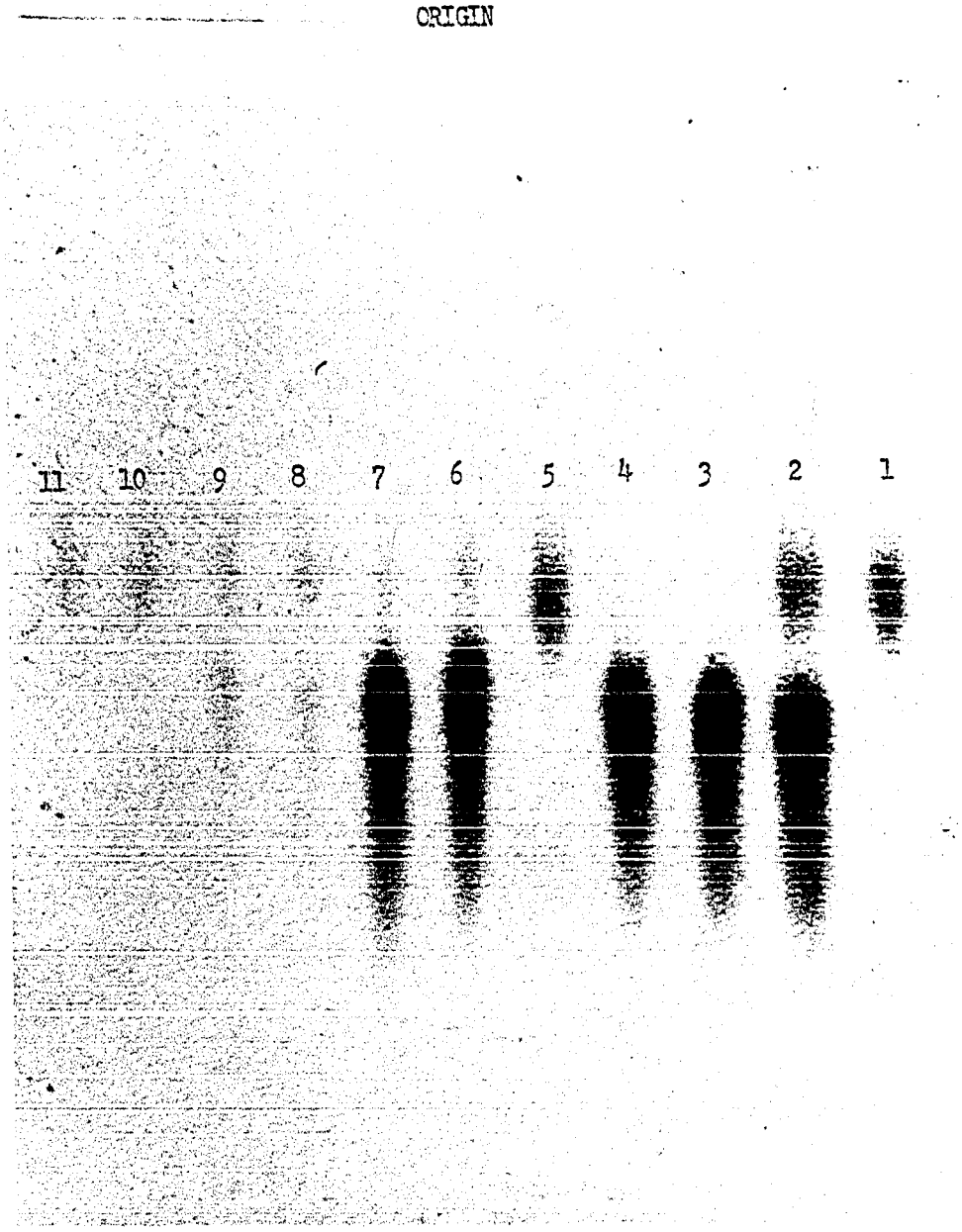


Figure 4. Chromatogram obtained in silicic acid impregnated glass fiber paper. The spots contained L-ascorbic acid (AA) or D-isoascorbic acid (IAA) as follows: No 1 and 5 (20 µg AA), No 10 and 11 (2 µg AA), No 3 and 4 (200 µg IAA), No 2 (200 µg IAA + 20 µg AA), No 6 and 7 (200 µg IAA + 2 µg AA) and No 8 and 9 (2 µg AA + 2 µg IAA).

Table I

Vitamin C in urine and recoveries of

L-ascorbic acid and dehydroascorbic acid

Sample	Dilution	mg % (present)	mg % (added)	Per cent recoveries (added)
1	5:45	2.59	4.00	98
1	15:35	2.56	1.33	95
2	5:20	1.97	--	--
2 (oxidized)	5:20	--	(1.97)*	103

*In the form of dehydroascorbic acid obtained by the oxidation of L-ascorbic acid.

from a subject on an intake of 2 g vitamin C per day were assayed for AA and total vitamin C (AA + DHAA) and as shown in Table II, gave coefficients of variation less than 4 per cent.

The method was compared with two widely used methods based on the 2,4-dinitrophenylhydrazine (DNPH) reaction to determine vitamin C in sheep sera. The results are reported in Table III. By using blanks where DNPH was added after incubation in the proposed method, values comparable to the other methods were obtained, but with the regular blank (tube 2, p. 26), smaller values of true vitamin C were obtained. As shown in Table IV, recoveries of AA and DHAA added to various sera were satisfactory.

No difficulty was encountered in the determination of vitamin C in the heart, kidneys, brain, spleen, or liver of guinea pigs. Mean recoveries of 4.5 µg L-ascorbic acid added per ml of liver extracts in 6 different days were 103.1% with a coefficient of variation of 2.4 per cent. Assays of a frozen liver extract on 7 different days indicated a slight loss due to freezing and thawing and gave a coefficient of variation of 6.6 per cent.

C. Differential determination of L-ascorbic acid (AA) and D-isoascorbic acid (IAA).

The time course for osazone formation from 2,4-dinitrophenylhydrazine and oxidized AA and IAA, as reflected by the increase in absorbance of osazones at 520 mµ, is illustrated in Figure 5 (32°C), and Figure 6 (52°C). The production of osazones occured much more rapidly from AA containing systems than from corresponding systems containing IAA. The osazone formation with IAA was maximal after 2 hours at 52°C, but only

Table II

Variation of assays done on different days for L-ascorbic acid (AA) and vitamin C in 24 hours urine specimens

Day	Basal excretion (mg)		Saturation excretion* (mg)	
	AA	Vit. C	AA	Vit. C
1	--	--	1320	1452
2	--	--	1272	1352
3	27.4	29.7	1264	1374
4	28.4	30.7	1340	1456
5	26.9	28.6	1380	1464
6	25.8	28.7	1348	1444
Mean	27.1	29.4	1320	1423
C.V. (%)	4.0	3.3	3.4	3.4

* 2 g ascorbic acid per day in divided doses.

Table III

Vitamin C in sheep serum (mg/100 ml)

Method	L-ascorbic acid	Vitamin C	
		specific	non-specific
Roe & Kuether (1943)	---	---	0.48
Lowry et al (1945)	---	---	0.48
Proposed	0.20	0.25	0.47*

*Using the same type of blanks as in the above methods.

Table IV

Recoveries of ascorbic acid (AA) and dehydroascorbic acid (DHAA) from sera

Serum	mg % added (AA or DHAA)	Per cent recoveries	
		AA	DHAA
Sheep	0.36	103.3	96.6
"	0.72	96.5	103.5
"	1.08	103.5	101.1
Rabbit	1.33	102.0	98.9
Pig	2.0	97.0	--

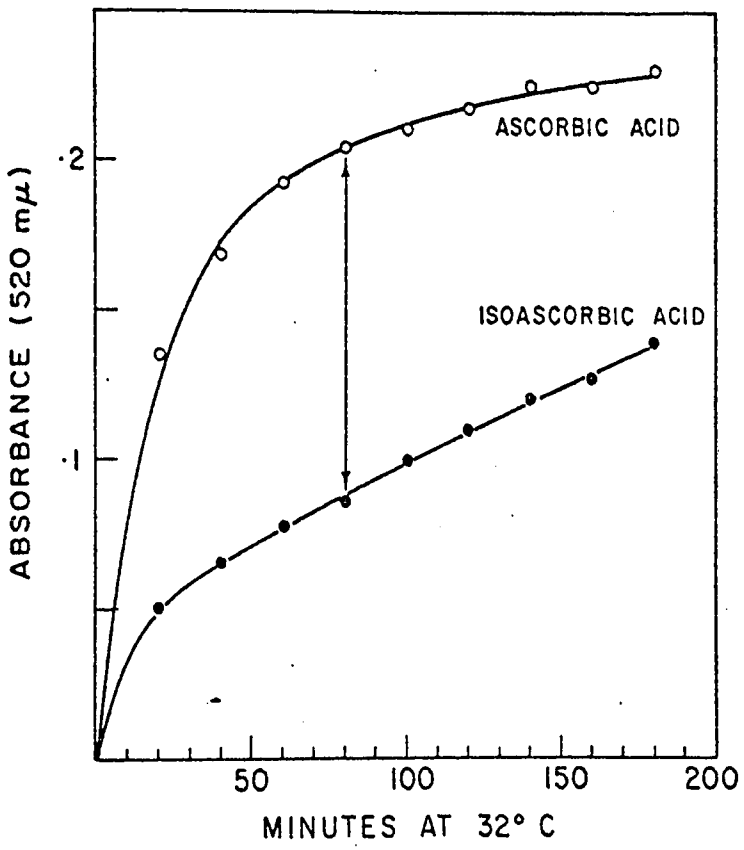


Figure 5. Effect of incubation at 32°C on osazone formation from L-ascorbic acid and D-isoascorbic acid (6 μg/ml). (o) L-ascorbic acid; (•) D-isoascorbic acid.

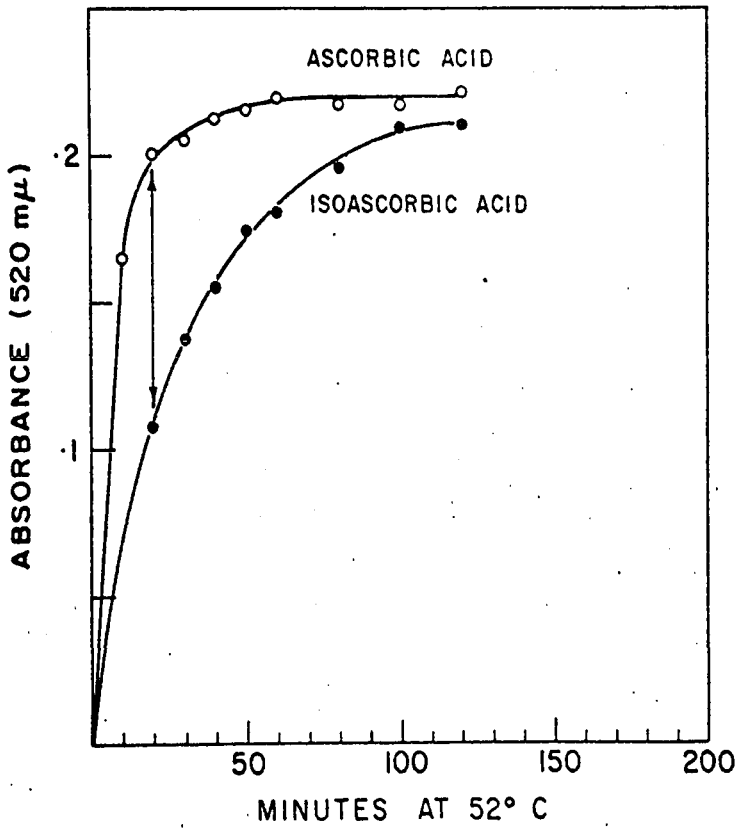


Figure 6. Effect of incubation at 52°C on osazone formation from L-ascorbic acid and D-isoascorbic acid (6 μg/ml). (o) ascorbic acid; (●) isoascorbic acid.

half completed after the same time at 32°C. The maximal difference between the two isomers ocurred after 80 minutes at 32°C.

Absorbance measurements for various levels of AA and IAA at 32°C and 52°C for 80 minutes, as shown in Figure 7, were linear for concentrations up to about 8 μg per ml of assay solution.

Recoveries on three different days of mixtures of AA and IAA added to extracts of guinea pig organs are given in Table V. The values for AA were reproducible and indicated a high recovery whereas those for IAA were more variable, particularly for brain and spleen extracts.

Table V

Typical recoveries of mixtures of L-ascorbic acid (AA) and D-isoascorbic acid (IAA) from guinea pig organs

Organ	AA (μg ± S.D.*/ml extract)		IAA (μg S.D.*/ml extract)	
	Added	Recovered	Added	Recovered
Liver	1.2	1.12 ± 0.08	4.8	4.88 ± 0.17
"	1.8	1.77 ± 0.14	4.2	4.25 ± 0.20
"	2.4	2.33 ± 0.04	3.6	3.72 ± 0.10
"	3.0	2.92 ± 0.06	3.0	3.15 ± 0.09
"	3.6	3.51 ± 0.09	2.4	2.56 ± 0.12
"	4.2	4.26 ± 0.11	1.8	1.81 ± 0.17
"	4.8	4.79 ± 0.07	1.2	1.27 ± 0.13
Spleen	3.59†	3.41 ± 0.11	4.0	4.16 ± 0.70
Adrenals	2.31†	2.29 ± 0.16	4.0	4.38 ± 0.39
Kidneys	3.25†	3.31 ± 0.09	4.0	4.12 ± 0.46
Brain	7.51†	7.30 ± 0.24	4.0	3.99 ± 0.94

* S.D. calculated for 3 assays on different days.

† Amount found prior to addition of IAA.

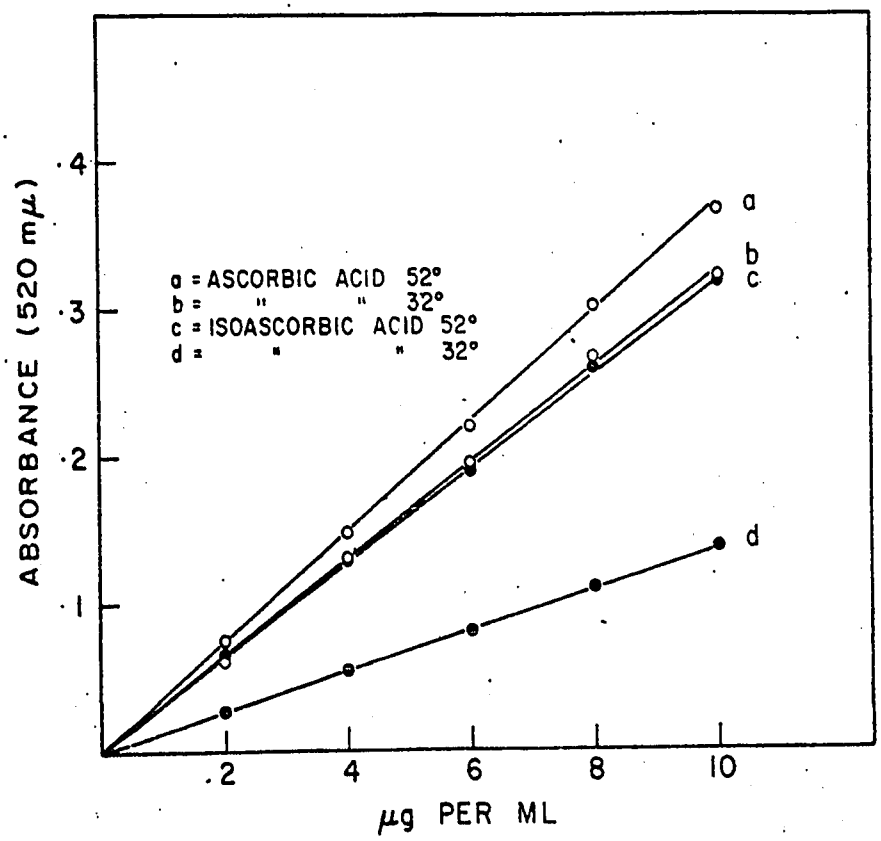


Figure 7. Standard curves for L-ascorbic acid and D-isoascorbic acid at 32 and 52°C.

DISCUSSION

A. Chromatographic separation of D-isoascorbic acid (IAA) and L-ascorbic acid (AA).

Although chromatographic separations of the IAA speciman did not yield quantitative results, they indicated that much less than 1 per cent of the sample could be present as AA. Thus, the IAA speciman, because of its high purity, could be used in the development of an analytical method for differentiating AA and IAA, and in studies on the metabolism and antiscorbutic activity of IAA.

B. Determination of vitamin C.

Rationale of test system

2,4-dinitrophenylhydrazine (DNPH) couples with dehydroascorbic acid (DHAA) and diketogulonic acid (DKGA) to yield osazones. In sequence procedure 1 (tube 1, p. 26), AA and DHAA give rise to osazones because 2,6-dichloroindophenol oxidizes AA to DHAA and homocysteine at pH 7.1 does not reduce DHAA to AA in presence of boric acid (Hughes, 1956). In procedure 2 (tube 2, p. 26), neither AA nor DHAA form osazones because there is no oxidation, and homocysteine at pH 7.1 converts DHAA to AA before the addition of boric acid. In procedure 1', DHAA but not AA gives rise to osazone because there is no oxidation, but homocysteine does not reduce DHAA to AA in presence of boric acid. DKGA is not affected by homocysteine (Howard and Constable 1966) and yields osazones in procedures 1, 2 and 1'. Interfering substances such as reductones (R) are reduced by homocysteine in presence or absence of boric acid but they still yield a certain amount of osazones which is the same in tubes 1, 2 and 1'. Consequently tube 1 minus tube 2 equals AA plus DHAA and tube 1 minus tube 1' equals AA.

2,6-Dichloroindophenol was preferred to Norit (Roe and Kuether 1943) for the oxidation of AA because it was simpler to use (Schwartz and William 1955; Hausman-Lench and Lewis 1961); a deviation of ± 2 drops in the end point was acceptable.

The optimal range of pH (about 7 to 7.2) for reduction of DHAA with homocysteine was readily obtained by the addition of 0.5 ml of 45 per cent K_2HPO_4. The amount of homocysteine and time for reduction of DHAA were in excess of minimal requirements.

Thiourea provided a mildly reducing medium to prevent coloration of DNPH solutions by oxidants (Roe and Kuether 1943) and prevented ascorbic acid oxidation in the blanks. Phosphoric acid prevented DNPH precipitation and was preferred to other acids because slight variations in the amount added had no significant effects. The amount of DNPH was selected from a noncritical range; mixing was necessary during the DNPH addition to prevent its precipitation. The coupling reaction could be completed rapidly at 100°C (Schaffert and Kingsley 1955), but this was avoided because interference from sugars was more marked (Roe 1961). Although these interferences were cancelled by the blanks, a temperature of 32°C was selected to keep them to a minimum. The coupling reaction was about 85 per cent complete after 1 hour and 20 minutes at 32°C, and Beer's law was obeyed. This was more convenient than the 3 to 6 hours required after Norit treatment (Roe et al. 1948).

The nitric acid-phosphoric acid mixture used for the color development eliminated the tedious dropwise addition of sulfuric acid (Roe and Kuether 1943) and increased the sensitivity by a factor of 1.5. The mixture was added while cooling and while bubbling nitrogen in order to prevent discoloration by nitrous oxides. Contamination of the glassware

with organic matter could also result in the formation of nitrous oxides. These oxides are readily produced when nitric acid is diluted with water. Glacial acetic acid (Bolomey and Kemmerer 1946), and phosphoric acid plus hydrochloric acid mixtures (Schwartz and Williams 1955) were not considered because they had not improved the sensitivity obtained with sulfuric acid. Measurements of the per cent transmittance 30 or 60 minutes after withdrawal from the cold bath were not significantly different.

Advantages of the proposed method

The originality of the proposed method comes from the fact that various chemical properties of ascorbic acid, dehydroascorbic acid and diketogulonic acid have been used for the differential determination of each of these compounds in presence of interfering substances. The procedure is much more simple and practical than the widely used procedure of Roe et al. (1948): it uses homocysteine instead of hydrogen sulfide as a reducing agent, and 2,6-dichloroindophenol instead of charcoal as an oxidizing agent; it develops the osazones color with a nitric-phosphoric acid mixture instead of sulfuric acid which must be added drop by drop with constant mixing in a bath of crushed ice; it requires only one blank instead of three and only one hour and 20 minutes incubation instead of six hours. The greater dilution of samples, because of the reaction with homocysteine, is compensated by the greater intensity of the color developed. The proposed method is more specific because results are not affected by reductones and similar substances. Because of its accuracy and precision, the method is suitable for nutritional and metabolic studies on vitamin C.

C. Differential determination of L-ascorbic acid (AA) and D-isoascorbic acid (IAA).

The two basic requirements for the differentiation of AA and IAA were, firstly, a large difference in the absorbance due to each isomer at one temperature and secondly, a minimal difference in the absorbance due to each isomer at a second temperature; a greater maximal difference was produced at 32°C (80 minutes) than at 52°C (20 minutes) but the second requirement was achieved more conveniently at 52°C (80 minutes) than at 32°C (several hours). For these reasons, two temperatures (32 and 52°C) at a constant time (80 minutes) were selected instead of only one temperature at two different times (Garmon and Reilly 1962; Guttman 1966).

The derivation of proportional equations based on reaction kinetics has been thoroughly described by Garmon and Reilly (1962) and Guttman (1966), but it is possible to arrive at a similar equation by a simple rationale. At any given concentration the absorbance values in Figure 7 can be interrelated to give the following equations:

[3] $d(c/d)-c = 0$

[4] $b(c/d)-a = \chi$

[5] $(b+d)(c/d)-(a+c) = \chi$

The factor c/d is the same for any concentration of IAA standard. It is evident that by multiplying the 32°C absorbance by c/d and substracting the 52°C absorbance, the absorbances due to IAA cancel out but these due to AA give a value χ which is the same wether AA is alone or mixed with IAA. Furthermore the values χ give a linear relationship with various concentrations of AA. Consequently, the difference obtained by the expression $f(c/d)-e$ of Equation [1] of the calculations is due only to AA in the

sample. As shown in the numerator of Equation [2] of the calculations, the absorbance due to AA at 52°C can be calculated from the concentration q found by Equation [1], and can be subtracted from the total absorbance at 52°C to yield the net absorbance due to IAA.

The main advantage of the present method over paper chromatographic methods is that it is sensitive enough to be applied to the assay of biological specimens. Fabianek and Herp (1967) had no sucess with two chromatographic methods for the assay of guinea pig organs. Weeks and Deutsch (1967) have separated IAA from AA on silicic acid impregnated glass fiber paper and have applied their method to foods, but since the minimum amount required is about 2 µg per 22 µl spot, its application to biological specimens would be restricted to the assay of adrenals extracted in about 1 ml of solution.

Recoveries by the present method were more accurate and reproducible for AA than for IAA. The IAA assays of organs from guinea pigs receiving no IAA gave negative values in most cases and only insignificant traces in the other. The assay values of added IAA in the presence of AA indicated that the method was suitable to determine the incorporation of IAA by the organs of guinea pigs.

REFERENCES

Barakat, M.Z., M.F.A. El-Wahab and M.M. El-Sadi. 1955. Action of N-Bromosuccinimide on ascorbic acid. New titrimetric method for estimation of vitamin C. Anal. Chem. 27: 536-540.

Bessey, O.A. 1938. A method for the determination of small quantities of ascorbic acid and dehydroascorbic acid in turbid and colored solutions in the presence of other reducing substances. J. Biol. Chem. 126: 771-784.

Bolin, D.W. and L. Book. 1947. Oxidation of ascorbic acid to dehydroascorbic acid. Science, 106: 451.

Bolomey, R.A. and A.R. Kemmerer. 1946. The determination of ascorbic acid; a simplified method. J. Biol. Chem. 165: 377-378.

Bolomey, R.A. and A.R. Kemmerer. 1947. Spectrophotometric studies of the Roe method for the determination of dehydroascorbic acid. J. Biol. Chem. 167: 781-785.

Borsook, H., H.W. Davenport, C.E.R. Jeffreys and R.C. Warner. 1937. Oxidation of ascorbic acid and its reduction in vitro and in vivo. J. Biol. Chem. 117: 237-279.

Brenner, G.S., D.F. Hindley, L.M. Perkins and S. Webber. 1964. Isomerization of the ascorbic acids. J. Org. Chem. 29: 2389-2392.

Chapman, D.G., O. Rochon and J.A. Campbell. 1951. Estimation of ascorbic acid in pharmaceuticals (with particular reference to interfering substances). Anal. Chem. 23: 1113-1115.

Cheraskin, E. and W.M. Ringsdorf. 1968. A lingual vitamin C test. Int. J. for Vitamin Res. 38: 114-126.

Davidek, J. and Davidkova. 1959. EnfluB der flavonoide auf die kolorimetrische bestimmung der L-ascarabinsaure mit 4-methoxy-2-nitralin. Sonderdruck aus, "Die Nahrung" 3. Heft 5/6: 515-519.

De Ritter, E. 1965. Chromatographic and extraction techniques for vitamin C assay. J. Ass. Off. Agr. Chem. 48: 985-991.

Deutsch, M.J. and C.E. Weeks. 1965. Microfluorometric assay for vitamin C. J. Ass. Off. Agr. Chem. 48: 1248-1256.

Evelyn, K.A., H.T. Malloy and C. Rosen. 1938. The determination of ascorbic acid with photoelectric colorimeter. J. Biol. Chem. 126: 645-654.

Evered, D.F. 1960. The determination of ascorbic acid in highly colored solutions with N-bromosuccinimide. Analyst 85: 515-517.

Fabianek, J. and A. Herp. 1967. Antiscorbutic activity of D-arabo-ascorbic acid. Proc. Soc. Exptl. Biol. Med. 126: 462-465.

Farmer, C.J. and A.F. Abt. 1936. Determination of reduced ascorbic acid in small amounts of blood. Proc. Soc. Exptl. Biol. Med. 34: 146-150.

Garmon, R.J. and C.N. Reilly. 1962. Kinetic analysis of mixtures by the method of proportional equations. Anal. Chem. 34: 600-606.

Guttman, D.E. 1966. Analysis of steroids in mixtures using the kinetics of blue tetrazolium reduction. J. Pharm. Sci. 55: 919-922.

Hardesty, W.S. 1964. Potentiometric determination of vitamin C in highly colored products. J. Ass. Off. Agr. Chem. 47: 754-755.

Hausman-Lench, E.R. and G.J. Lewis. 1961. Determination of ascorbic acid in blood. Chemist-Analyst 50: 18-19.

Hewitt, E.J. and G.J. Dickes. 1961. Spectrophotometric measurements on ascorbic acid and their use for the estimation of ascorbic acid and dehydroascorbic acid in plant tissue. Biochem. J. 78: 384-391.

Holland, A.J., J.C. Canniff and M. Bruger. 1947. The evaluation of the vitamin C status of human subjects. J. Lab. Clin. Med. 32: 124-129.

Hove, E.L. 1965. Conference on vitamin C methodology: a summary report. J. Ass. Off. Agr. Chem. 48: 991-996.

Howard, A.N. and B.J. Constable. 1966. The use of homocysteine in the estimation of ascorbic acid in urine. Clin. Chim. Acta. 13: 387-392.

Hughes, R.E. 1956. The use of homocysteine in the estimation of dehydroascorbic acid. Biochem. J. 64: 203-208.

Hughes, R.E. 1964. Use of cation-exchange resin in the determination of urinary ascorbic acid. Analyst 89: 618-620.

King, C.V. 1941. Chemical methods for the determination of vitamin C. Ind. Eng. Chem. Anal. Ed. 13: 225-227.

Kum-Tatt, L. and P.C. Leong. 1964. A new method for the determination of ascorbic acid in urine. Clin. Chem. 10: 575-580.

Kuusi, T. 1960. Reductone interference in the assay of ascorbic acid. A comparative study of different methods of analysis. Suomen Kemistilehti, 33: 139-143.

Lento, H.G., C.E. Daugherty and A.E. Denton. 1963. Polarographic determination of total ascorbic acid in foods. Agr. and Food Chem. 11: 22-26.

Lugg, J.W.H. 1942. The use of formaldehyde and 2,6-dichlorophenol indophenol in the estimation of ascorbic acid and dehydroascorbic acid. Australian J. Exptl. Biol. Med. Sci. 20: 273-285.

Linkswiler, H. 1958. The effect of ingestion of ascorbic acid and dehydroascorbic acid upon the blood levels of these two components in human subjects. J. Nutr. 64: 43-54.

Lowry, O.H., J.A. Lopez and O.A.J. Bessey. 1945. The determination of ascorbic acid in small amounts of blood serum. J. Biol. Chem. 160: 609-615.

Maickel, R.P. 1960. A rapid procedure for the determination of adrenal ascorbic acid. Application of the Sullivan and Clarke method to tissue. Anal. Biochem. 1: 498-501.

Mapson, L.W. 1943. Vitamins methods VI. The estimation of ascorbic acid in the presence of reductones and allied substances. J. Soc. Chem. Ind. (London) 67: 223-232.

Mapson, L.W. 1961. A note on the estimation of dehydro-L-ascorbic acid in plant tissues by the Roe and Kuether procedure. Biochem. J. 80: 359-461.

McHenry, E.W. and M. Graham. 1935. Observations on the estimation of ascorbic acid by titration. Biochemical J. 29: 2013-2019.

Meyer, K.H., C.H. Haselbach and R.A. Boissonnas. 1952. Dosage rapide de l'acide ascorbique des surrénales du rat. Helvetica Chemica Acta 35: 1781-1783.

Michaelsson, G. and M. Michaelsson. 1967. A new diazo method for the determination of ascorbic acid in blood plasma. Scand. J. Clin. Lab. Inv. 20: 97-103.

Miki, T., N. Kikuchi and Y. Shashi. 1962. Paper chromatographic estimation of L-ascorbic acid and D-araboascorbic acid. J. Vitaminology 8: 279-284.

Mindlin, R.L. and A.B. Butler. 1938. The determination of ascorbic acid in plasma. J. Biol. Chem. 122: 673-686.

Nadkarni, B.Y. 1965. Determination of ascorbic acid in colored extracts: a new modification of the indophenol technique. Mikrochim. Acta 1: 21-27.

Owen, J.A. and B. Iggo. 1956. Use of P-chloromercuribenzoic acid in the determination of ascorbic acid with 2,6-dichloroindophenol. Biochem. J. 62: 675-680.

Pazarincevic, F. and M. Damanski. 1968. Séparation et dosage de l'acide ascorbique et de l'acide isoascorbique. Qualitas Plantarum et Materiae Vegetales 16: 210-214.

Pelletier, O. and A.B. Morrison. 1966. Determination of ascorbic acid in presence of ferrous and stannous salts. J. Ass. Off. Anal. Chem. 49: 800-803.

Pelletier, O. and A.B. Morrison. 1965. Content and stability of ascorbic acid in fruit drinks. J. Am. Diet Ass. 47: 401-404.

Pelletier, O. 1967. Determination of ascorbic acid in enriched evaporated milk. J. Ass. Off. Anal. Chem. 50: 817-820.

Pepkowitz, L.P. 1943. The rapid determination of ascorbic acid by the adaptation of the Stotz's method to plant materials. J. Biol. Chem. 151: 405-412.

Perrin, D.R. and D.D. Perrin. 1946. The polarographic estimation of ascorbic acid in milk. The N.Z. J. of Science and Technology 28A: 266-270.

Polk, A., T.L. Flanagan and E.J. Van Loon. 1960. Rapid method for determination of ascorbic acid in urine. Clin. Chem. 6: 558-563.

Przyborowski, L. 1966. Complexometric determination of ascorbic acid. Dissertationes Pharmaceuticae et Pharmacologicae, 18: 505-509.

Reichstein, T.A., A. Grusser and R. Oppenauer. 1933. Syntheses of D- and L-ascorbic acid (vitamin C). Helv. Chim. Acta 16: 1019-1033.

Robinson, W.B. and E.J. Stoz. 1945. The indophenol-xylene extraction method for ascorbic acid and modifications for interfering substances. J. Biol. Chem. 160: 217-225.

Roe, J.H. 1961. Appraisal of methods for the determination of L-ascorbic acid. Ann. N.Y. Acad. Sci. 92: 277-283.

Roe, J.H. and C.A. Kuether. 1943. The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid. J. Biol. Chem. 147: 399-407.

Roe, J.H., M.B. Mills, M.J. Oesterling and C.M. Damron. 1948. The determination of diketo-l-gulonic acid, dehydroascorbic acid, and L-ascorbic acid in the same tissue extract by 2,4-dinitrophenylhydrazine method. J. Biol. Chem. 174: 201-208.

Rotter, H. 1937. Determination of vitamin C in living organism. Nature, 139: 717.

Schaffert, R.R. and G.R. Kinsgley. 1955. A rapid, simple method for the determination of reduced, dehydro, and total ascorbic acid in biological materials. J. Biol. Chem. 212: 59-68.

Schmall, M., C.W. Pifer and E.G. Wollish. 1953. Determination of ascorbic acid by a new colorimetric reaction. Anal. Chem. 25: 1486-1490.

Schmall, M., C.W. Pifer, E.G. Wollish, R. Duschinsky and H. Gainer. 1954. Colorimetric determination of ascorbic acid. New developments concerning the reaction with diazotized 4-methoxy-2-nitro-aniline. 26: 1521-1530.

Schwartz, M.A. and J.N. Williams Jr. 1955. New Procedure for ascorbic acid analysis by the osazone method. Proc. Soc. Exptl. Biol. Med. 88: 136-138.

Shimizu, K., T. Nishiyama, T. Inoue, N. Takano, M. Mikita, M. Yamazaki, T. Azuma and S. Osana. 1967. Studies on erythorbic acid production by fermentation. Part II. Erythorbic acid production by jar fermentation. Agr. Biol. Chem. 31: 346-352.

Smyte, C.V. and C.G. King. 1942. A study of ascorbic acid synthesis by animal tissues in vitro. J. Biol. Chem. 142: 529-541.

Stark, G.R. and R. Dawson. 1963. Ascorbic acid oxidase. In: The enzymes 8: 297-311; Boyer, P.P., H. Lardy and K. Myrbäch, editors, Academic Press.

Stewart, Jr. A.P. and P.F. Sharp. 1945. Determination of vitamin C in presence of reducing substances. Selective oxidation-reduction method. Ind. and Eng. Chem. Anal. Ed. 17: 373-376.

Sullivan, M.X. and H.C.N. Clarke. 1955. A highly specific method for ascorbic acid. J. Ass. Off. Agr. Chem. 38: 514-518.

Szent-Györgyi, A. 1928. Observations on the function of peroxidase systems and the chemistry of the adrenal cortex; description of a new carbohydrate derivative. Biochem. J. 22: 1387-1409.

Szöke, K. 1960. Ein Beitrag zur quantitativen bestimmung von vitamin C in wärmebelian delten substances. Sonderdruch aus, Die Nahrung 9: 825-831.

Tillmans, J. 1930. The antiscorbutic vitamin. Z. Untersuch Lebensm, 60: 34-44.

Vann, L.S. 1965. A rapid method for the determination of ascorbic acid in urine by ferric reduction. Clin. Chem. 11: 979-985.

Vuilleumier, J.P. and S. Nobile. 1962. Assay of vitamin C in compound feeds. World Poultry Congr. Proc. 12th. Sidney. 238-241.

Vuilleumier, J. 1967. Analytische Probleme bie der bestimmung von vitamin C in zusammenhang mit ernährungserhebungen. Internationale Zeitschrift für Vitaminforschung Seharatabzug. 37: 504-514.

Wagner, A.F. and K. Folkers. 1964. L-ascorbic acid. In: Vitamins and Coenzymes pp. 308-329. Intersciences Publishers.

Wang, M.M., K.H. Fisher and M.L. Doods. 1962. Comprehensive metabolic response to erythorbic acid and ascorbic acid by the human. J. Nutr. 77: 443-447.

Waught, W.A. and C.G. King. 1932. Isolation and Identification of vitamin C. J. Biol. Chem. 97: 325-331.

Weeks, C.E. and M.J. Deutsch. 1965. Assay of ascorbic acid in Pharmaceuticals: a modified colorimetric procedure using P-nitroaniline. J. Ass. Off. Agr. Chem. 48: 1245-1248.

Weeks, C.E. and M.J. Deutsch. 1967. L-ascorbic acid and D-isoascorbic acid. Quantitative separation and assay. J. Ass. Off. Anal. Chem. 50: 793-798.

Yagi, J., T. Yamashita, K. Kato, Y. Takagi and H. Sahai. 1967. Studies on erythorbic acid production by fermentation. Part I. Erythorbic acid producing strain and cultural conditions. Agr. Biol. Chem. 31: 340-346.

PART II.

THE ANTISCORBUTIC ACTIVITY OF D-ISOASCORBIC ACID*

* Part of the results reported in this section has been published as indicated in the following references:

1 Pelletier, O. 1969. Differential determination of D-isoascorbic acid and L-ascorbic acid in guinea pig organs. Can. J. Biochem. 47: 449-453.

2 Pelletier, O. and C. Godin, 1969. Vitamin C activity of D-isoascorbic acid for the guinea pig. Can. J. Physiol. Pharmacol. 47: 985-991.

3 Pelletier, O. 1969. Turnover rates of D-isoascorbic acid and L-ascorbic acid in guinea pig organs. Can. J. Physiol. Pharmacol. 47: 993-997.

INTRODUCTION

The spectacular role of vitamin C in the prevention and cure of scurvy

Since ancient times, scurvy made spectacular ravages specially among explorers and armies. Written accounts of this disease go back to the time of the crusades in the 13th century. In 1498, when Vasco da Gama sailed around Cape of Good Hope, scurvy claimed the lives of 100 men from a crew of 160.

The report of the second voyage of Jacques Cartier to Canada in 1535 contains a typical description of an "unknown disease" (scurvy) which started by swelling of the legs and development of purplish spots in the legs; this progressed to the hips, thighs, shoulders, arms, neck and mouth. The mouth became so infected that the gums were rotten and were losing flesh. Other symptoms were depression, general weakness and diarrhea. An autopsy on one of the dead revealed a white heart, black lungs and dark flesh on the surface. During that winter spent near Stadacona (near Quebec City), Cartier lost 25 men from a crew of 110 before he was told by the natives that the disease could be cured by an extract from the bark and needles of a tree. This, we know, provided vitamin C.

In our days, only occasional cases of scurvy are still to be seen. The spectacular way in which vitamin C is known to cure scurvy has been so impressive that the other biochemical functions of vitamin C are practically disregarded in attempting to determine the daily requirement of this vitamin for the maintenance of good health; furthermore, there is not enough concern about the possibility that an impairment of

connective tissue insufficient to provoke the general symptoms of scurvy may over prolonged periods favor the development of other diseases in certain organs or tissues.

Collagen synthesis in relation to scurvy.

Some of the characteristics of scurvy are loosening of teeth, swollen joints, petechial hemorrhages from venules, subcutaneous, articular, muscular and intestinal hemorrhages which can be attributed to defects in collagen synthesis (King 1967).

The metabolism of connective tissues and the role of vitamin C in the synthesis of collagen have been reviewed briefly by Rivers (1965) and in greater detail by Gould (1968). Collagen is synthesized by specialized cells such as fibroblasts, osteoblasts and odontoblasts. The fibroblasts form the intercellular substances (ground substance) in which are imbedded the collagenous fibers; mucoproteins and mucopolysaccharides are also involved in holding the collagen fibrils together thus forming collagenous fibers.

A collagen fibril consists of 3 polypeptide chains. These are held together by hydrogen bonds between oxygen atoms of one peptide chain and the nitrogen atom of another chain; two of the chains are called $\alpha 1$ and differ from the other $\alpha 2$ by their amino acid composition. The α chains (monomers) form left handed helices, although a right handed helix results when the three chains are linked together. The left handed α configuration arises from structure restriction due to pyrrolidine rings (hydroxyproline and proline) and is stabilized by hydrogen bonds. Proline and hydroxyproline comprise 25 per cent of the amino acid residues; some hydroxylysine is also found, but in a much smaller concentration than hydroxyproline;

glycine which is found in every third position comprises about 33 per cent of the amino acid residues and gives the 3 Å repeat of the helix. The three chains together give a 100 Å rope like repeat; another repeat of 700 Å results from covalent links of such ropes from end to end (Piez 1966)

Protocollagen (the polypeptide precursor of collagen) has half of its proline residues hydroxylated, after release from the ribosomes, by a specific enzyme, proline hydroxylase, which requires, as cofactors, O_2, vitamin C, Fe^{++} and ketoglutarate (Kivirikko and Prockop 1967a; Hutton et al. 1967); there is evidence that the same system is involved in the hydroxylation of lysine (Kivirikko and Prockop 1967b). In absence of vitamin C, protocollagen is synthesized but hydroxylation does not occur and no fibers are formed. The mechanism by which L-ascorbic acid causes the hydroxylation of proline and hydroxyproline in the polypeptide chain has not been fully elucidated. There is evidence that oxygen-18 is incorporated in hydroxyproline (Fujimoto and Tamiya 1962; Prockop et al. 1962); it seems possible although not yet demonstrated that the microsomal electron transport model (Figure 3) producing hydroxyl radicals is linked with the hydroxylation of proline (Rivers, 1953; Gould, 1968).

Apparently the role of L-ascorbic acid in collagen synthesis is not stereospecific since Robertson (1963) has reported that the impaired collagen synthesis in scorbutic guinea pigs was rectified by large doses of D-isoascorbic acid given frequently to maintain its concentration above 200 µg per g of granulation tissue. Schimizu et al. (1965) have shown that D-isoascorbic acid was as effective as L-ascorbic acid in

promoting collagen synthesis in tissue cultures when the concentration of D-isoascorbic acid in the medium was 200 µg per ml as compared to 50 µg L-ascorbic acid per ml.

The biosynthesis of L-ascorbic acid and D-isoascorbic acid

L-ascorbic acid is synthesized in a wide variety of higher plants and animal species. Notable exceptions are man, primates, guinea pigs, the fruiteating bat (Roy and Guha 1958; Burns 1959) and several species of passeriform birds (Chaudhuri and Chatterjee, 1969). Because of this metabolic insufficiency, L-ascorbic acid is a vitamin for these species and is necessary for the prevention of scurvy (Waugh and King 1932) caused by a defect in the synthesis of collagen (Wolbach and Howe 1926).

In animals that synthesize L-ascorbic acid, the biosynthetic pathway starts from D-glucose (I) (or D-galactose) as illustrated in Figure 1. In rats, the pathway is believed to proceed through D-glucuronic acid (II) which is reversibly reduced to L-gulonic acid (III) which in turn is cyclized to L-gulono-y-lactone (IV); L-gulono-y-lactone (IV) in presence of L-gulono-lactone oxidase forms 2-keto-L-gulono-y-lactone (V) which then isomerizes to L-ascorbic acid, (VI) (Wagner and Folkers 1964). Another pathway in animals consists in the conversion of D-glucuronic acid (II) to D-glucuronolactone (VII) which in presence of D-glucuronolactone reductase forms L-gulono-y-lactone (IV), (Burns and Evans 1956; Chatterjee et al. 1960). In mammals, the liver is the site of vitamin C synthesis, while in some birds synthesis of vitamin C occurs in the kidney, whereas in others it is produced in the liver (Chaudhuri and Chatterjee, 1969).

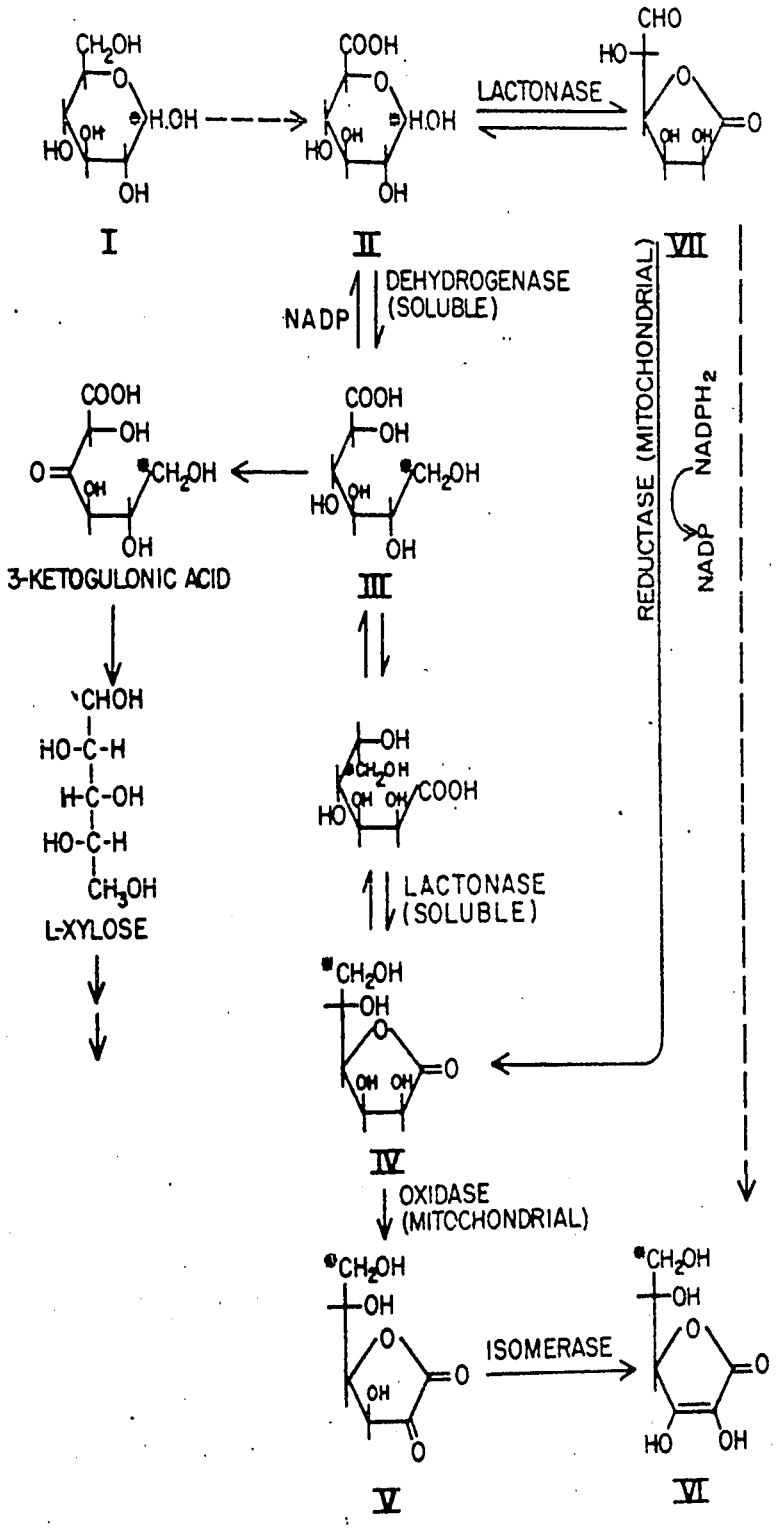
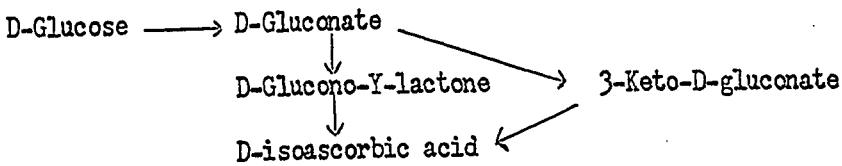


Figure 1. Biosynthesis of L-ascorbic acid

In the animal species that are not producing L-ascorbic acid, the enzymes D-glucuronolactone reductase and L-gulonic acid oxidase are believed to be missing due to a genetic defect (Chaudhuri and Chatterjee 1969), causing a so-called "inborn error of metabolism". However, since there is evidence that D-glucurono-y-lactone can produce L-ascorbic acid in man (Baker et al. 1962) one should wonder if D-glucuronolactone reductase is really missing in man.

Numerous analogs have been tested for their vitamin C activity (Zilva 1935). To be active, the analogs must possess the 5-atom lactone ring containing a dienolic group to the right of the carbon chain written according to the Fischer convention; for this reason, D-ascorbic acid and L-isoascorbic acid (Figure 2, Part I) have no antiscorbutic activity (Smith 1946) while that of D-isoascorbic acid has been reported to vary from 0 to 5 per cent that of L-ascorbic acid (Wang et al. 1962).

D-isoascorbic acid can be synthesized from D-glucose, D-gluconate and sucrose by some species of Penicillium; the following pathway has been suggested (Takahashi et al. 1960):



Yagi et al. (1967) tested microorganisms isolated from soil and found that only Penicillium but no other genera produced D-isoascorbic acid. Developing this process for commercial production, Shimizu et al. (1967) obtained a 45 per cent yield from the glucose supplied and purified D-isoascorbic acid by absorption on a weakly basic anion exchange resin (Amberlite IR-45) followed by elution with 1 N HCl using a multibed extraction system.

L-ascorbic acid and D-isoascorbic acid as antioxidants in foods

Because of their remarkable oxido-reduction properties and commercial availability, L-ascorbic acid and D-isoascorbic acid have been found useful as antioxidants for foods and beverages. They function as antioxidants in food systems by being oxidized to the dehydro forms and preventing the oxidation of other food constituents; furthermore, they shift the redox potential of the system to the reducing range by providing H ions which can reduce undesirable oxidation products (Borenstein 1965).

The antioxidant properties of L-ascorbic acid and D-isoascorbic acid have been reviewed by Yourga et al. (1944): these acids can prevent darkening and flavour changes in beer, control rancidity in oil emulsions such as mayonnaise and salad dressing, control discoloration and undesirable flavour changes in a variety of glass and tin-packed foods naturally low in vitamin C, delay the development of off flavours in orange juice, and prevent the development of oxidized flavours in milk.

Esselsen et al. (1945) reported that D-isoascorbic acid is oxidized more rapidly than L-ascorbic acid in food products and that its addition to tomato and grape juice prevented the loss of L-ascorbic acid occurring during storage; however, their assays, 2,6-dichloroindophenol titration and the 25-day weight response bioassay method using guinea pigs exhausted of L-ascorbic acid (Zilva 1935), did not differentiate between the activity due to the presence of dehydro-L-ascorbic acid, dehydro-D-isoascorbic acid and D-isoascorbic acid. The addition of D-isoascorbic acid was required to maintain the flavor and color of tomato juice but was not required in grape juice. Yourga (1948) reported that high vacuum (20 in. of mercury) eliminated zonal grey-brown off colors at the top portion of

glass jars containing carrots; L-ascorbic acid and D-isoascorbic acid (0.01 to 0.1 per cent) were found to be as efficient as 10 in. mercury vacuum and improved the color of the product throughout the jar.

The presence of L-ascorbic acid and D-isoascorbic acid prevented browning and flavor loss of easily oxidized fruits, e.g. peaches, apples, plums, apricots, pears and white fleshed cherries when cut for use in baking, freezing or canning (Tressler and Du Bois 1944). The color deterioration of canned cherries can be controlled by addition of D-isoascorbic acid or L-ascorbic acid to the syrup (Kunz and Robinson 1958). Hope (1961) reported that, when apples halves were canned without removing the oxygen from the headspace, the can contained 10-12 volumes per cent of oxygen; the addition of L-ascorbic acid (300 mg per lb. of fruit) reduced headspace oxygen, increased residual ascorbic acid, controlled browning and prevented corrosion of the can. Reyes and Luh (1962) studied the effect of L-ascorbic acid and D-isoascorbic acid as antioxidant in frozen sliced freestone peaches under thawing conditions; they found that both isomers inhibited the formation of orthoquinones by polyphenolase and the darkening resulting from the polymerization of these orthoquinones.

L-ascorbic acid and D-isoascorbic acid can be used as antioxidant with cured meat products. According to Watts et al. (1955), the denaturation of oxyhemoglobin or oxymyoglobin of fresh meat bring about oxidation of the heme iron to give brown methemoglobin or metmyoglobin; the formation of cured meat pink-red color involves a reduction of both metmyoglobin and nitrite to form nitric oxide hemochromogen; sulfhydryl groups freed during denaturation of muscle proteins are important to

bring this reduction. Reducing agents such as L-ascorbic acid and D-isoascorbic acid improved the development of cured meat color and provided protection against off flavours and light fading (Mill et al. 1958).

Unsaturated fats in fish react with atmospheric oxygen to form peroxide intermediates which produce rancidity and discolouration of the flesh of fish; treatment of the fish flesh with L-ascorbic acid or D-isoascorbic acid solutions (dipping or glazing) reduced the formation of peroxides and enhanced the retention of natural flavor and color (Tarr, 1948).

Thus there is a wide variety of food and beverages in which L-ascorbic acid and D-isoascorbic acid can be used as antioxidants. The levels will vary with the type of product, e.g. 47 mg per 100 g cured meat products, 30-50 mg per 100 g frozen fruits, 10-50 mg per 100 ml soft drinks, 15-75 mg per liter of beer and the amount resulting from dipping fish in 0.25 to 2 per cent solutions (Report from R.O. Read, 1967. Division of Standards, Additives and Pesticides, Food and Drug Directorate, Ottawa).

Metabolism of L-ascorbic acid

The known pathways of L-ascorbic acid metabolism are shown in Figure 2. L-ascorbic acid can be easily oxidized to dehydroascorbic acid. The reaction is reversible and the dehydro form has as much antiscorbutic activity as the reduced form (Borsook et al. 1937; Linkswiler 1958). L-ascorbic acid is apparently oxidized to dehydroascorbic acid in two stages, the first stage being a semiquinone like

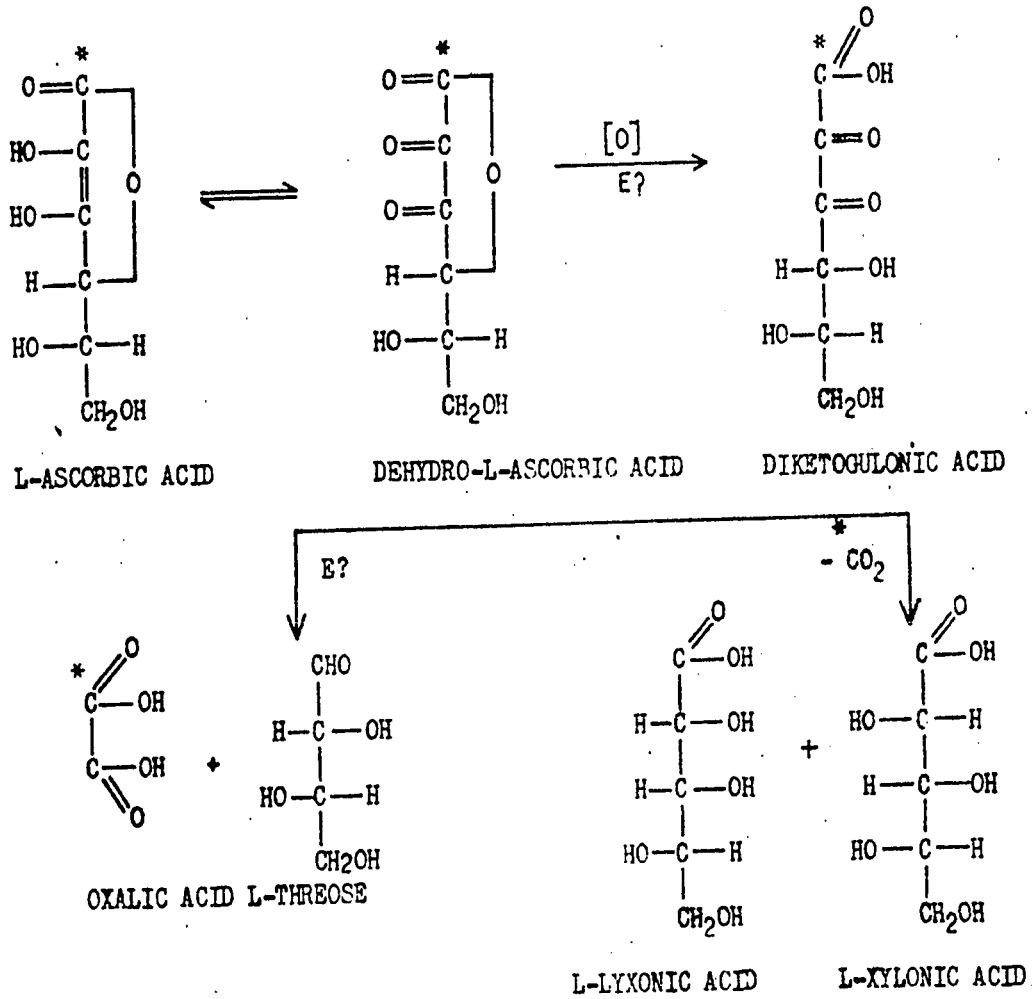


Figure 2. Catabolism of L-ascorbic acid

derivative (monodehydroascorbic acid); evidence for monodehydroascorbic acid has been obtained from aqueous solutions standing at room temperature (Levandoski et al. 1964) and evidence for the participation of a free radical in ascorbic acid oxidation has been obtained by paramagnetic resonance spectroscopy (Yamazaki et al. 1960).

The ease with which L-ascorbic acid may be oxidized and reduced would suggest that it may play a role in reactions involved in electron transfer in the cell; there is indeed some favorable evidence that this happens in animal tissue, since an ascorbic acid dependent NADH oxidation has been demonstrated in rat liver and kidney microsome (Staudinger et al. 1961). The proposed scheme is shown in Figure 3. Devine and Rivers (1969) have demonstrated that the monodehydroascorbic acid-transhydrogenase involved in this scheme was present in adrenal gland and maternal placental tissue.

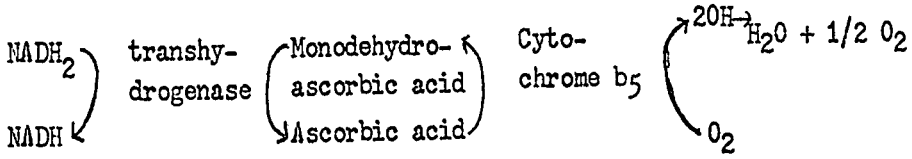


Figure 3. Microsomal electron transfer model.

The presence of low levels of dehydroascorbic acid in plasma (Clemetson and Anderson, 1966) is not surprising in view of the presence of a dehydroascorbic acid reductase in erythrocytes (Hughes and Kilpatrick, 1964) and in various tissues (Hughes, 1964; Grimble and Hughes 1967).

Although guinea pigs, like man, have a requirement for L-ascorbic acid, they metabolize it in a different way; for example, in guinea pigs, the main route of metabolism of ascorbic acid involves extensive oxidation

of its entire carbon chain to CO_2 and a smaller portion of the vitamin is excreted in the urine as ascorbic acid, diketogulonic acid and oxalic acid (Burns et al. 1951; Burns et al. 1956; Dayton et al. 1966). In man, however, oxalic acid represents a major metabolite of L-ascorbic acid (Hellman and Burns 1958; Baker et al. 1962, 1966; Atkins et al. 1964). The data regarding the degradation of L-ascorbic acid to CO_2 in man are conflicting: Hellman and Burns (1958) detected no CO_2 after intravenous administration of uniformly labelled ascorbic acid. Abt et al. (1963) reported that after oral administration of L-ascorbic acid-1-C^{14} to 4 human subjects, 18 to 28 per cent of the dose was expired as $C^{14}O_2$ during 7 days and the major part of this was expired during the first day. Baker et al. (1962, 1966) found that less than 1 per cent of orally administered L-ascorbic acid-1-C^{14} was converted to $C^{14}O_2$, but when they administered a solution containing 18 per cent of the labelled vitamin C as dehydroascorbic acid, about 6 per cent of the total dose could be recovered as labelled CO_2 24 hours after administration; furthermore, when dehydroascorbic acid comprised about 50 per cent of the dose and diketogulonic acid 30 per cent, about 36 per cent was recovered as labelled CO_2 in 24 hours. Because of these results, Baker et al. (1966) concluded that impurities or decomposition products of L-ascorbic acid could explain the significant conversion of L-ascorbic acid-1-C^{14} to CO_2 which had been reported by other workers. Von Schuchling and Abt (1965) indicated that they were well aware of the unstability of L-ascorbic acid, and using radioactively pure L-ascorbic acid, they confirmed their previous findings with another human subject. Following the ingestion of L-ascorbic acid-1-C^{14} they found an appreciable amount of $C^{14}O_2$ expired within the first hour (rate of more than 2 per cent of the dose per hour);

thereafter, a precipitous lowering of $C^{14}O_2$ to less than 0.4 per cent per hour occurred and after the first day it fell to less than 0.1 per cent of the amount ingested. In view of these conflicting results, it is uncertain that, under certain experimental conditions, L-ascorbic acid cannot yield a significant amount of CO_2.

The mechanism by which L-ascorbic acid is oxidized to CO_2 "in vivo" is still obscure. Chan et al. (1958) reported that homogenates of guinea pigs liver can decarboxylate dehydroascorbic acid with the formation of L-xylose; furthermore, rat kidneys can oxidize L-ascorbic acid to dehydroascorbic acid and produce L-xylonic acid and L-lyxonic acid after decarboxylation (Kanfer et al. 1959); the breakdown product L-threonic acid has not yet been demonstrated.

There is a wide difference between the half life of L-ascorbic acid in guinea pigs and in man; it has been estimated to be about 4 days for the guinea pigs (Burns et al. 1956) and about 16 days for humans (Hellman and Burns 1958). In accord with this, guinea pigs will develop signs of scurvy 15 days after being deprived of vitamin C (Schow 1966), as compared to 17 weeks with humans (M.R.C. Report, 1948); furthermore, the amount of vitamin C per Kg body weight per day necessary to prevent scurvy is about 2 mg for guinea pigs (Dunker et al. 1942) and about 0.15 mg for humans (M.R.C. Report, 1948).

Metabolism and antiscorbutic activity of D-isoascorbic acid

Literature on the metabolism of D-isoascorbic acid is rather scarce. Ikeuchi (1954) found that in vitamin C deficient human subjects, very little of injected L-ascorbic acid was excreted while the urinary excretion of ingested D-isoascorbic acid was about 10 to 15 times higher during the

first six hours; by measuring both the reduced and dehydro form, they found that the six-hour urinary excretion of both forms amounted to about 5 per cent of the L-ascorbic acid dose and more than 50 per cent of the D-isoascorbic acid dose.

As indicated by blood levels, D-isoascorbic acid given orally is rapidly absorbed by humans in a manner similar to L-ascorbic acid (Kadin and Osadca 1959; Wang et al. 1962). Using an analytical method that did not differentiate between the two isomers, Wang et al. (1962) found that 50-70 per cent of 100 to 300 mg doses of D-isoascorbic acid was filtered through the glomeruli and not reabsorbed to any appreciable extent by the tubular cells of the kidneys. Rivers et al. (1963), also using a non-specific analytical method, found that when human subjects partially depleted of L-ascorbic acid were given supplements of L-ascorbic acid or D-isoascorbic acid, the white blood cell levels responded to L-ascorbic acid but not to D-isoascorbic acid; they concluded that the uptake or tissue fixation of L-ascorbic acid is structurally specific for the L-configuration at carbon 5. Kadin and Osadca (1959), using paper chromatographic techniques, measured the two isomers in the urine 4 hours after a 300 mg oral dose of D-isoascorbic acid was given to subjects previously saturated with L-ascorbic acid and suggested that there was no significant deposition of D-isoascorbic acid in the tissues since there was no apparent displacement of L-ascorbic acid.

Zilva (1935) administered intravenously 45 to 50 mg of D-isoascorbic acid to guinea pigs depleted of vitamin C, and after 24 hours found a significant incorporation of D-isoascorbic acid, as determined by the 2,6-dichloroindophenol titration, in the small intestine, large intestine,

liver, adrenals and "carcass"; the amount of L-ascorbic found in animals which had received about the same amount of L-ascorbic acid was significantly higher than that obtained with the animals treated with D-isoascorbic acid. The amounts excreted in the urine were not significantly different.

Several authors have published results indicating that D-isoascorbic acid has about one twentieth the activity of L-ascorbic acid (Dalmer and Moll 1933; Demole 1934; Zilva 1935; Yourga et al. 1944), but Reiff and Free (1959), using a diet consisting of a rabbit ration reported that as much as 250 mg D-isoascorbic acid per day did not support the growth of guinea pigs; D-isoascorbic acid had no therapeutic effect per se and tended only to slow down the development of acute deficiency by sparing L-ascorbic acid. In view of this controversy, Fabianek and Herp (1967) tested the antiscorbutic activity of D-isoascorbic acid in guinea pigs previously depleted of vitamin C for one week; they found that oral doses of D-isoascorbic acid varying from 10 to 200 mg apparently replaced the antiscorbutic activity of L-ascorbic acid during 115 days of oral dosage; they found incorporation of an "ascorbic acid" in the liver, spleen and adrenals of the guinea pigs, but due to the lack of a suitable method, did not determine if the "ascorbic acid" was L-ascorbic acid or D-isoascorbic acid. Hughes and Hurley (1969), subsequent to the publication (Pelletier 1969) of some of the results presented in this thesis, reported that D-isoascorbic acid, administered orally to guinea pigs at a dosage of 1.5 mg per 100 g of body weight, was not retained by the tissues while D-isoascorbic acid administered intramuscularly produced a significant deposition of D-isoascorbic acid in tissues although to a lesser extent than that produced by

the same doses of L-ascorbic acid; they concluded that the gastrointestinal membranes were not equally permeable to both isomers. On the contrary, Martin (1961) had reported that two hours after injection of 20 and 40 mg D-isoascorbic acid or its dehydro form to rats, there was no increase in the level of ascorbic acid like material in the brain. There was on the other hand a significant increase after injection of 20 mg of dehydro-L-ascorbic acid, although practically none was obtained after L-ascorbic acid injection. Dehydro-L-ascorbic acid being non-ionic and more lipoid soluble was able to cross the cellular barriers more readily than L-ascorbic acid and was then reduced to L-ascorbic acid, but this transport and storage mechanism was thought to be structurally specific for L-ascorbic acid; this would explain the low antiscorbutic activity of D-isoascorbic acid. According to Martin, the kidneys' ability to oxidize ascorbic acid would facilitate the penetration of vitamin C through cellular barriers in an un-ionized form.

Statement of the Problem

Because D-isoascorbic acid can be synthesized from glucose at a lower cost than L-ascorbic acid, it is not surprising that it is becoming more widely used as an antioxidant in the food industry; in fact, a private report from a manufacturer indicated a market of over 500,000 lbs per year in Canada. Supposing that 25 per cent of the Canadian population consumes half of this amount, this would result in a daily intake of about 70 mg which could satisfy the vitamin C requirements if it was L-ascorbic acid.

The purpose of this work is to find out what happens to D-isoascorbic acid given orally to guinea pigs and humans, to determine if this

isomer is retained by the body and if it can displace L-ascorbic acid or affect its metabolism; what is its half life in guinea pig organs as compared to L-ascorbic acid and finally to determine if D-isoascorbic acid has antiscorbutic activity per se and what is its mode of action.

MATERIALS AND METHODS

A. Uptake of D-isoascorbic acid (IAA) by guinea pig organs

Synthetic diet

The composition of the basal diet was (in percentage): vitamin-free casein 30; cane sugar, 20; corn starch, 20; corn oil, 7.4; non-nutritive fiber, 15; salt mix (U.S.P. XIV), 4; potassium acetate, 2.5; magnesium oxide, 0.5; plus a vitamin mixture excluding ascorbic acid. The vitamin mix supplied per 100 g of diet: 350 mg choline chloride, 200 mg L-inositol, 20 mg niacin, 10 mg p-aminobenzoic acid, 8 mg calcium pantothenate, 3 mg riboflavin, 2 mg thiamine, 1 mg folic acid, 0.1 mg biotin, 12 µg vitamin B_{12}, 20 µg menadione, 158 U.S.P. units of vitamin D and 979 U.S.P. units of vitamin A.

Experiment

Male guinea pigs weighing approximately 300-350 g were fed the synthetic diet with 0.1 per cent vitamin C for 1 week, and only animals showing satisfactory growth were used for this study.

To determine the uptake of IAA, eight guinea pigs were given ad libitum the basal diet with 2 per cent IAA and 0.1 per cent AA together for 1 week, while a control group of eight received the same diet with only 0.1 per cent AA. The guinea pigs were given twenty times more IAA than AA on the basis that IAA had one twentieth the activity of AA.

To test the retention of IAA when followed by AA and the effect of presaturation with IAA on subsequent uptake of AA, three groups of nine guinea pigs were first depleted while receiving the basal diet devoid of vitamin C for 1 week; during the following week, 2 per cent IAA was added to the diets of groups 1 and 2 while group 3 remained on the deficient

diet; for the last week, group 2 was transferred to the diet containing 2 per cent IAA plus 0.1 per cent AA, and group 1 and 3 to the diet with 0.1 per cent AA.

The guinea pigs were anesthetized with pentobarbital and bled by heart puncture before removal of the brain, heart, liver, spleen, kidneys, and adrenals. The excised organs were immediately weighed, wrapped in parafilm, and placed on dry ice. The frozen tissues were extracted within the next 24 hours by blending with cold 4 per cent metaphosphoric acid to which 1-2 drops of antifoam FG-10 emulsion (Dow Corning) were added. Extracts were diluted to contain about 6 µg AA plus IAA per ml of 2 per cent metaphosphoric acid, filtered, and frozen until essayed.

The purity of D-isoascorbic acid was determined by chromatography on silicic-acid impregnated glass fiber sheets as described in Part I. The quantitative differentiation of oxidized AA and IAA was based on their different rates of osazone formation with 2,4-dinitrophenylhydrazine by the procedure described in Part I-C.

B. Vitamin C activity of D-isoascorbic acid (IAA) for the guinea pig Diets

The synthetic diet used was that described under Part II-A. The Rockland rabbit ration was prepared from ground whole wheat, suncured alfalfa meal, soybean oil meal, and 1 per cent calcium carbonate; declared average analyses indicated 17.4 per cent protein, 65.8 per cent carbohydrates, 1.6 per cent fat and 14.9 per cent fiber. The vitamin content of the two diets is summarized in Table I. The vitamin C content of the diets was determined by the 2,4-dinitrophenylhydrazine procedure described in Part I-B.

TABLE I

Vitamins content per 100 g of diet

	Rockland Rabbit ration*	synthetic diet
Choline, mg	712-748	350 (choline chloride)
L-inositol, mg	36	200
Niacin, mg	3.52	20
P-aminobenzoic acid, mg	93	10
Pantothenic acid, µg	1211-1700	8000 (calcium salt)
Riboflavin, µg	424-722	3000
Thiamine, µg	220-265	2000
Folic acid, µg	-	1000
Biotin, µg	-	100
Vitamin B_{12}, µg	-	12
Vitamin D, U.S.P. units	21	158
Vitamin A, U.S.P. units	450-1100	979
Menadione, µg	-	20
Vitamin C, mg	0.37†	0.14†

* Representative content declared by the manufacturer.

† Actual assays (mean of 3 assays for the synthetic diet).

Experiment 1

A group of 21 male guinea pigs weighing an average of 225 g were fed the synthetic diet and a similar group was fed the rabbit ration. During the first 8 days only of that period, the guinea pigs were given orally 10 mg AA daily in order to build up their vitamin C levels to comparable levels. Vitamin C was removed from the diet during 17 days, then each group was subdivided into 3 sub-groups of 7 animals and each animal was kept in an individual cage. Every day during two months, a dose of 2 mg AA freshly dissolved in 0.2 ml distilled water was given orally to each guinea pig of sub-group 1 and 2, and similarly 40 mg IAA to every guinea pig of sub-group 3. Sub-group 1 and 3 were fed ad libitum and sub-group 2 was pair fed with 3. Water was given ad libitum at all times. The food intake was measured daily and the body weight every 2-3 days. For two 12-hour periods during the last week of the experiment, the guinea pigs were placed in metabolic cages and their urine was collected in glass vials containing 1 ml of 50 per cent meta-phosphoric acid. The urine samples were diluted to 25 ml, filtered and frozen for a few days before being analysed.

Another group of 7 guinea pigs weighing also an average of about 225 g was fed ad libitum with the synthetic diet and received 10 mg AA daily for 8 days; after 17 days of depletion the 7 guinea pigs were killed in order to determine the degree of vitamin C depletion in their organs before the AA and IAA treatments. The organs of 6 other guinea pigs similarly depleted for 19 days were also analysed.

The organs of these animals as well as those of the guinea pigs treated with AA or IAA were removed and prepared for analysis as pre-

viously described in A. The only modification to the analytical procedure was the use of standard curves instead of a single level of standard. Absorbances were measured for the AA standards at 2, 4, 6 and 8 µg per ml, and for mixtures of IAA and AA containing the following amounts in µg per ml: 2 + 4, 3 + 3, 4 + 2, 5 + 1. The net absorbance due to IAA standards was calculated by subtracting from the total absorbance the absorbance contributed by AA in the mixture as read from the AA curve. Calculations were made as described previously except that the absorbance due to 5 µg was used as read from the curve; in cases where the extended line of the AA standard curve was above the origin, this difference was subtracted from the absorbances of the 5 µg AA standard and of the unknown samples. The IAA standard curves in presence of AA were slightly higher than in absence of AA; the recoveries of AA and IAA added to extracts of guinea pigs' organs were not significantly better with these modifications than those obtained with the method described previously (Part I-B).

Experiment 2

The following experiment was designed to determine if there was any difference in the maintenance of vitamin C activity after discontinuing IAA and AA treatments of guinea pigs.

A group of 34 guinea pigs weighing an average of 203 g were fed the synthetic diet ad libitum. During 8 days, 10 mg L-ascorbic acid was administered orally each day to all animals so that they would reach comparable vitamin C levels. During the following 19 days the guinea pigs were given the synthetic diet without L-ascorbic acid in order to deplete their vitamin C stores. The guinea pigs were then divided into two sub-groups: 0.1 per cent AA was included in the diet of the first sub-group

while 2 per cent IAA was included into the diet of the second sub-group, and these diets were given ad libitum for 16 days in order to build up the AA or IAA levels of the guinea pigs. Three guinea pigs from each sub-group were killed and analyses of their organs were done as described above. The diet devoid of AA or IAA was given again to the surviving animals during 27 days. During the experiment the body weight of each animal was measured every 2-3 days.

C. Turnover of D-isoascorbic acid (IAA) and L-ascorbic acid (AA) in guinea pigs' organs

Experiment 1

A group (A) of 19 guinea pigs and a group (B) of 23, weighing an average of 370 g were fed during one week with the synthetic diet (described in Part II-A) containing 2 per cent AA in order to build up their vitamin C to comparable levels. After 18 days of depletion, 0.1 per cent AA was incorporated into the diet of group A and 2 per cent IAA into the diet of group B. The weight of each animal was recorded every 2-4 days. After receiving AA or IAA for 9 days, the guinea pigs were again given the deficient diets during 4 days. Animals from each group were killed on days 0, 1, 2 and 4, after AA and IAA were removed from the diet. The AA and IAA content in the organs of the animals was determined by the 2,4-dinitrophenylhydrazine procedure described in Part 1-B.

Experiment 2

Another group of 88 guinea pigs, weighing an average of 240 g was fed with the synthetic diet (described in Part II-A) containing 0.1 per cent vitamin C during one month before being injected intraperitoneally with 2.75 μc L-ascorbic acid-1-C^{14} (New England Nuclear Corp.) plus 5 mg

AA dissolved in 0.2 ml of distilled water. Twenty-four hours after the injection the group was subdivided according to weight into 13 sub-groups of 6 or 7 guinea pigs. One sub-group was killed, and the remaining sub-groups received the deficient diets ad libitum as follows: 4 without AA or IAA, 4 with 0.1 per cent AA added, and 4 with 2 per cent IAA. The guinea pigs from one sub-group on each diet were killed 1, 3, 6 and 10 days after receiving the diet. The organs of the animals were removed and extracts were prepared for liquid scintillation counting in the same way as described previously for chemical analyses. Ten ml of filtered extract were mixed with 13 ml of toluene and Triton ^{R} X-100 (7:6) containing 0.215 per cent PPO (2,5-diphenyloxazole) and 0.0054 per cent POPOP [1, 4-bis-2(5-phenyloxazolyl)] in 20-ml screw cap vials according to the method of Patterson and Greene (1965). The gels were counted with a Model 314 EX-2 Packard Tricarb liquid scintillation spectrophotometer. The optimal gain of 50 per cent was used for counting all samples with the green channel set at 80-1000 and the red channel at 80-300. A quenching curve of per cent efficiency against the ratio red channel/green channel was prepared by adding toluene-C^{14} to extracts from the organs of guinea pigs not injected with L-ascorbic acid-1-C^{14}. The efficiency varied from 52.5 per cent to 60.5 per cent and mean recoveries of added toluene-C^{14} was 99.8 per cent with a standard deviation of 2.5 per cent.

D. Urinary excretion of orally administered D-isoascorbic acid (IAA) to humans

Ten human volunteers (five smokers and five non-smokers) members of the Food and Drug Directorate in Ottawa participated in this study. The experiment consisted in comparing the urinary excretion of D-isoascorbic

acid (IAA) with that of L-ascorbic acid (AA) before and after saturation with vitamin C. Lunches and dinners were controlled by making use of T.V. dinners and by excluding the following items from the diets: oranges, grapefruits or their juices; apple juice; fruits, fruit juices and drinks; tomatoes or their juice; "instant breakfasts"; potatoes other than those in T.V. dinners; potato chips.

At 4 PM on the day before the urinary excretion of AA was to be measured, the subject took a dose of 75 mg AA and started to make use of the controlled diet in order to have a comparable vitamin C intake. At 8:30 AM, on the next day, a dose of 75 mg AA was given to each subject; their urine was collected until 4 PM in brown glass bottles containing metaphosphoric acid pellets in sufficient quantity to make a 4 per cent solution and was kept cool (about 10°C). Samples of urine were diluted on the same day and part of the samples were kept frozen in polyethylene bottles until assayed within about 3 days by the 2,4-dinitrophenylhydrazine procedure described in Part 1-B. At 4 PM on the same day another dose of 75 mg AA was given and the urine collected for vitamin analysis until 8:30 AM on the next day. The same procedure was then repeated by giving doses of IAA (at 8:30 AM and 4 PM in place of AA).

During 5 days, doses of 1 g AA were given to all volunteers at 8:30 AM and 4:00 PM in order to build up their vitamin C to saturated levels. During the following 4 days, the doses of AA were discontinued in order to allow elimination of excess vitamin C.

The urinary excretion of 75 mg doses of AA and IAA was again studied as described above, and on a subsequent day the urinary excretion of the same amounts of AA and IAA given together was studied.

RESULTS

A. Uptake of D-isoascorbic acid (IAA) by guinea pig organs

As shown in Table II, feeding guinea pigs a diet containing mixtures of AA and IAA resulted in significant losses of AA and gains of IAA as compared with the organs of control guinea pigs receiving only AA. The total amount of AA plus IAA in the adrenals, hearts and kidneys of the experimental animals approximated the total AA of control animals, but was less in the livers ($P < 0.05$) and spleens ($P < 0.01$). In the experimental group, the brains, spleens, and livers contained less IAA and AA, the hearts and kidneys about equal amounts of AA and IAA, and the adrenals more IAA than AA. In the control group, all organs except the brain showed an absence of IAA; a trace of IAA found in the brain was within experimental error.

While the results reported in Table III show a more extensive incorporation of IAA in organs of guinea pigs due to 1 week of IAA treatment preceding 1 week of AA and IAA (group 2), they also demonstrate a rapid loss of incorporated IAA during 1 week of AA treatment (group 1). In general, the total AA and IAA in the organs of these two groups approximated the total AA of controls (group 3) to which AA was given during 1 week after the depletion period.

B. Vitamin C activity of D-isoascorbic acid (IAA) for the guinea pig

Experiment 1

As shown in Figure 4, the food intake of guinea pigs fed ad libitum with the synthetic diet and receiving AA increased stadily after the depletion period until the last 3 weeks when it remained nearly constant; at all times the animals given the diet ad libitum and receiving

Table II

Average uptake of D-isoascorbic Acid (IAA) in guinea pigs fed a diet containing 2.0% IAA with 0.1% L-ascorbic acid (AA) for one week as compared to guinea pigs (controls) receiving only 0.1% AA.

	Experimental		Control	
Organ	IAA (mg. %)*	AA (mg. %)	IAA (mg. %)	AA (mg. %)
Liver	6.6 ± 0.8	11.4 ± 0.8	ND†	25.1 ± 1.5
Brain	6.2 ± 0.9	16.8 ± 0.6	1.4 ± 0.5	22.5 ± 0.7
Spleen	10.1 ± 1.8	16.7 ± 0.8	ND	39.0 ± 4.3
Adrenals	73.5 ± 4.1	50.4 ± 5.1	ND	134.8 ± 8.3
Heart	2.8 ± 0.5	2.0 ± 0.3	ND	5.1 ± 0.4
Kidneys	5.4 ± 0.8	4.4 ± 0.3	ND	9.6 ± 0.5

* Mean value and S.E. for 8 guinea pigs.

† None detectable

TABLE III

Retention of L-ascorbic acid (AA) and D-isoascorbic acid (IAA) due to feeding IAA alone or mixed with AA to depleted guinea pigs

Organ	Component retained†	Dietary treatments* Group I (IAA; AA)**	Group II (IAA; AA + IAA)**	Group III (Nil; AA)**
Liver	IAA	0.5 ± 0.4	5.8 ± 0.4	ND‡
	AA	14.5 ± 2.6	6.6 ± 0.7	15.0 ± 1.5
Brain	IAA	2.7 ± 1.0	10.2 ± 1.0	0.2 ± 0.2
	AA	15.2 ± 2.4	8.8 ± 0.8	17.0 ± 1.8
Spleen	IAA	0.6 ± 0.4	14.7 ± 2.0	0.4 ± 0.4
	AA	28.2 ± 3.6	14.6 ± 2.0	28.1 ± 1.5
Adrenals	IAA	7.9 ± 2.6	56.8 ± 5.6	3.5 ± 1.9
	AA	59.0 ± 0.7	28.0 ± 4.7	66.7 ± 9.1
Heart	IAA	0.1 ± 0.1	0.9 ± 0.3	0.1 ± 0.1
	AA	3.3 ± 0.6	2.3 ± 0.4	4.8 ± 0.9
Kidneys	IAA	0.3 ± 0.3	2.4 ± 1.0	ND‡
	AA	4.9 ± 1.0	3.6 ± 1.0	5.2 ± 0.6

* All groups were first fed the basal diet without AA or IAA for one week. The other two treatments and their sequence were as indicated under each group and lasted one week each.

† Mean retentions in mg % ± S.E. for 9 guinea pigs in group I and III, and 6 guinea pigs for group II. Three animals in group II refused feed and were discarded.

** AA (0.1%) and/or IAA (2.0%) were added to the basal diet.

‡ None detectable.

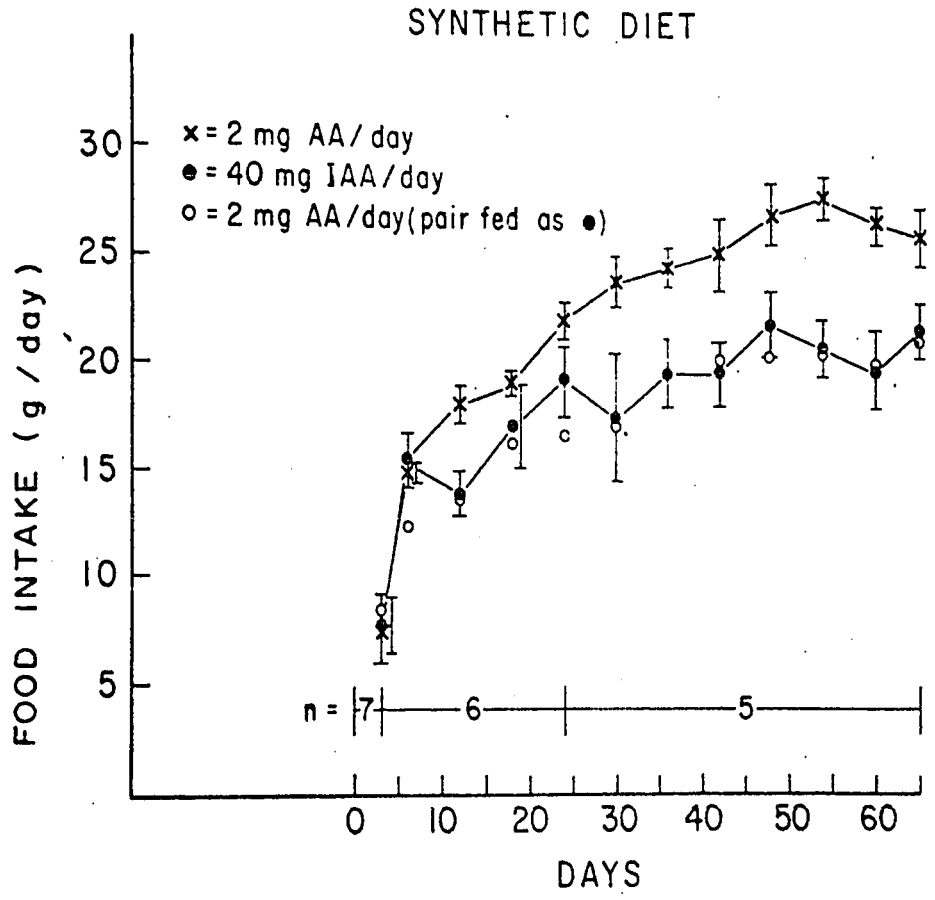


Figure 4. Daily intake (g) of synthetic diet (mean ± S.E.) during L-ascorbic acid (AA) and D-isoascorbic acid (IAA) treatment of depleted guinea pigs.

IAA consumed less food than those receiving AA. When the guinea pigs were fed the rabbit ration ad libitum and AA (Figure 5), there was a large increase in their food intake during the first 3 weeks after the depletion period, a decrease during the next 2 weeks and a slight increase during the last 4 weeks; the IAA treated animals consumed less food furing the first month of treatment.

The weight curves (Figure 6) of the animals fed with the synthetic diet show that this group started to lose weight 12 days after AA was removed from diet. All sub-groups started to gain weight rapidly 2 days after being given AA or IAA. After one week of treatment, the guinea pigs receiving AA and pair fed with those receiving IAA showed a one week retardation in growth but afterwards gained weight consistently in a manner parallel to the IAA sub-group. After 3 weeks of treatment and for the remainder of the experiment, the guinea pigs receiving AA and diet ad libitum grew at a greater rate than the IAA treated animals. One of the guinea pigs receiving IAA, and one pair fed control died in the first week of treatment, and another pair fed guinea pig died in the third week.

The guinea pigs fed with the rabbit ration (Figure 7) also started to lose weight after 12 days of depletion. The weight gain of the guinea pigs fed ad libitum and treated with AA (sub-group 1) increased steadily for about 3 weeks after which it underwent a drastic decline. The weight response of the IAA treated animals (sub-group 2) was delayed for a few days but then gave an increase parallel to sub-group 1. The AA treated animals pair fed with sub-group 2 gained about the same weight for the first month of treatment, but then grew more slowly during the last month.

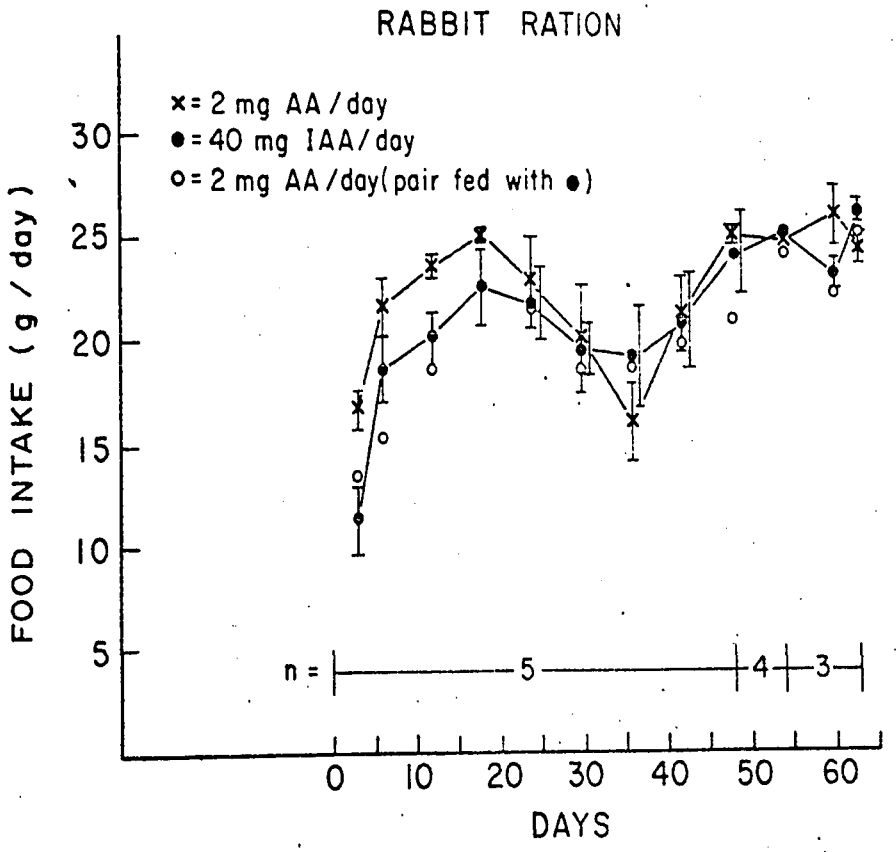


Figure 5. Daily intake (g) of rabbit ration (mean ± S.E.) during L-ascorbic acid (AA) and D-isoascorbic acid (IAA) treatment of depleted guinea pigs.

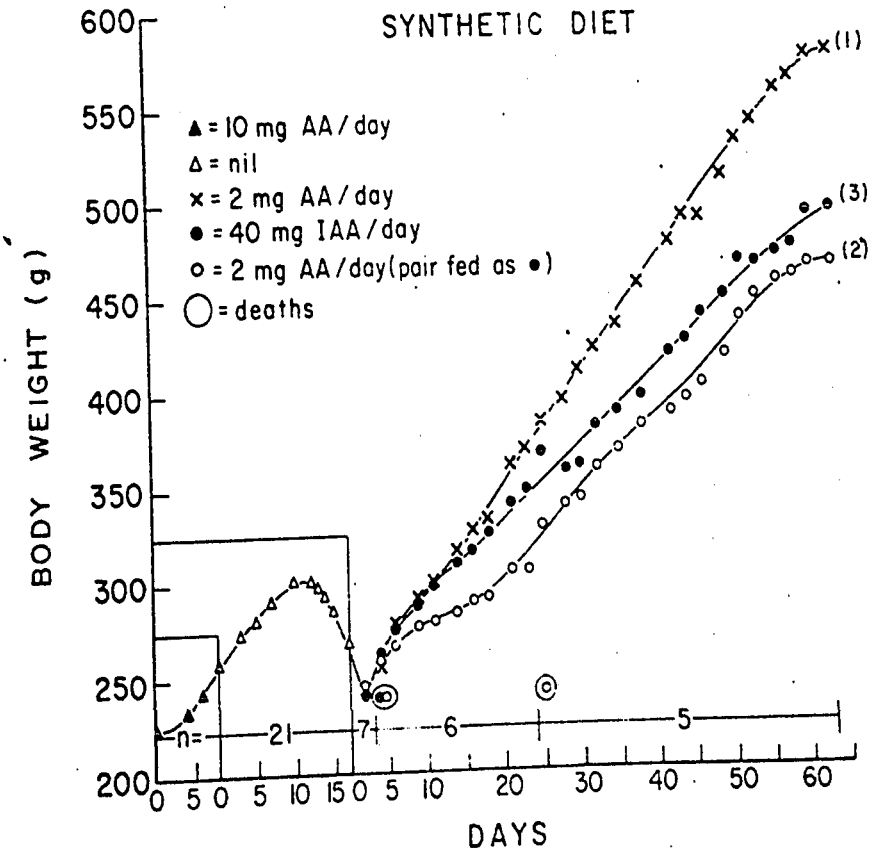


Figure 6. Mean body weight (g) of guinea pigs fed with the synthetic diet during depletion and following treatment with L-ascorbic acid (AA) and D-isoascorbic acid (IAA).

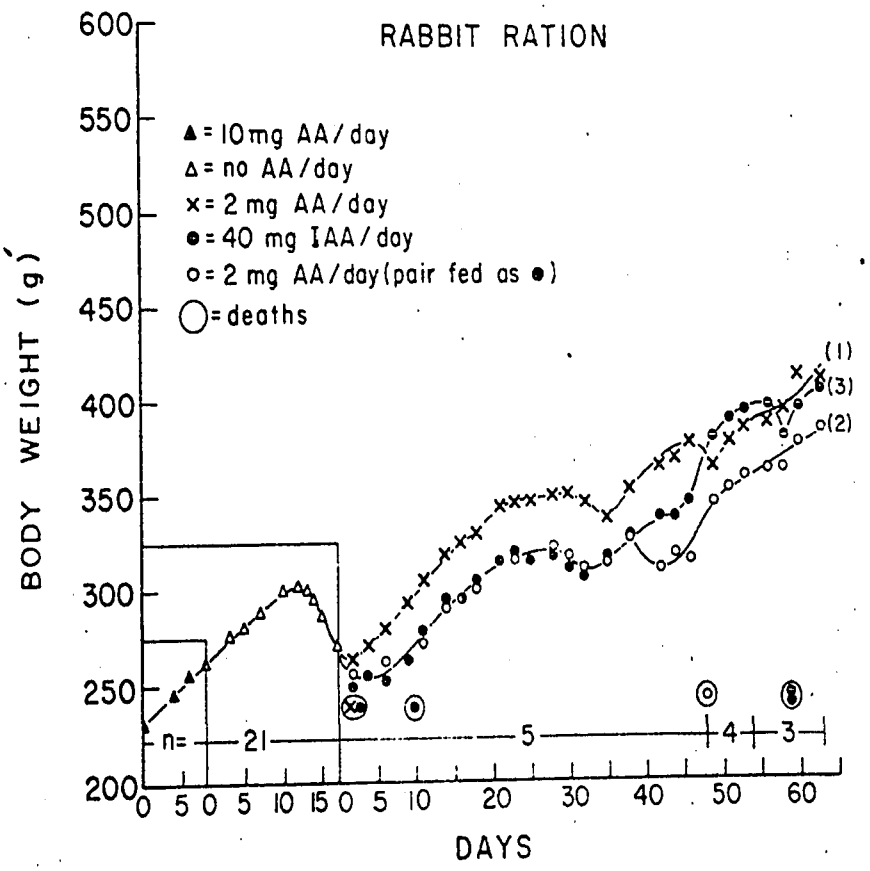


Figure 7. Mean body weight (g) of guinea pigs fed a rabbit ration during depletion and following treatment with L-ascorbic acid (AA) and D-isoascorbic acid (IAA).

Two of the guinea pigs treated with IAA died in the first two weeks of treatment and two more in the last week, while one of the animals treated with AA fed ad libitum died in the first week and one pair fed died after seven weeks.

The urinary excretion of AA, IAA and diketogulonic acid (DKG) during 2 twelve-hour periods is given in Table IV. The guinea pigs receiving IAA excreted only about 1.9 per cent of the dose as IAA, 0.4 per cent as DKG (IAA) and 0.13 per cent as AA.

The organs weights (Table V) were not significantly different even when their relation to body weight was taken into consideration.

As shown in Table VI, the AA content of organs of AA treated animals was similar for both diets given ad libitum and there was no significant difference due to the diet or its restriction. The IAA treated animals had an IAA content smaller than the AA content of their pair fed controls receiving AA. The hearts of the IAA treated animals contained no IAA, but traces of AA, and the kidneys practically no IAA but some AA. Traces of AA were also found in the liver and brain.

In Table VII, the vitamin C content in the organs of young scorbutic guinea pigs deprived of vitamin C by being fed the synthetic diet for 17 and 19 days is compared to that of guinea pigs treated with IAA after 17 days of depletion. No attempt was made to obtain values for guinea pigs depleted longer than 19 days because scurvy was causing them to die. The signs of scurvy observed in these animals were those described previously (See section Collagen synthesis in relation to scurvy in the introduction of Part II).

TABLE IV

Percent of doses* (± S.E.) of L-ascorbic acid (AA), D-isoascorbic acid (IAA) and diketogulonic acid (DKG) in 12-hr urine excretion

Diet†	AA (1)	AA (2)	AA (3)	IAA (3)
S-A	1.5 ± 0.1	1.1 ± 0.1	0.10 ± 0.03	1.7 ± 0.3
S-B	1.6 ± 0.2	1.7 ± 0.2	0.18 ± 0.02	2.4 ± 0.6
R-A	1.2 ± 0.1	1.1 ± 0.1	0.05 ± 0.04	1.9 ± 0.2
R-B	1.5 ± 0.2	1.4 ± 0.2	0.18 ± 0.05	1.7 ± 0.8
Mean	1.5 ± 0.2	1.3 ± 0.2	0.13 ± 0.04	1.9 ± 0.5
	(DKG)	(DKG)	(DKG)	(DKG)**
S-A	1.6 ± 0.2	2.2 ± 0.4	nil	0.4 ± 0.1
S-A	1.2 ± 0.2	1.6 ± 0.1	nil	0.3 ± 0.0
R-B	2.3 ± 0.1	2.3 ± 0.1	nil	0.5 ± 0.1
R-B	1.6 ± 0.2	0.6 ± 0.2	nil	0.5 ± 0.1
Mean	1.7 ± 0.2	1.7 ± 0.2	nil	0.4 ± 0.1

* AA dose = 2 mg; IAA dose = 40 mg

† S = synthetic diet; R = rabbit ration; A and B = different days; 1 and 3 = ad libitum, and 2 pair fed as 3. Groups 1 and 3 received AA while group 2 received IAA.

** DKG (D-isoascorbic acid).

TABLE V

Mean weight (g) of organs of guinea pigs treated with L-ascorbic acid (AA) or D-isoascorbic acid (IAA) after depletion*

Diet†	B.W.	Liver	Brain	Heart	Kidneys	Adrenals**
S1	548	21.8	2.8	1.5	3.6	0.36
S2	493	21.0	2.8	1.4	3.7	0.30
S3	463	19.6	2.8	1.4	3.6	0.31
R1	423	18.3	2.8	1.3	3.4	0.25
R2	406	17.2	2.7	1.3	3.1	0.28
R3	373	17.4	2.6	1.2	3.1	0.24

* Depleted 17 days and treated for 2 months with 2 mg AA or 40 mg IAA.

** No statistically significant difference between 2 and 3.

†S = synthetic diet; R = rabbit ration; 1 and 3 = ad libitum, and 2 = pair fed as 3. Groups 1 and 2 received AA while group B received IAA.

Table VI

L-ascorbic acid (AA)* and D-isoascorbic acid (IAA*) of guinea pigs treated orally with AA or IAA after depletion†

Diet**	Liver	Brain	Heart	Kidneys	Adrenals
S-1	2.9 ± 0.3	5.5 ± 1.0	0.8 ± 0.1	1.5 ± 0.1	19.2 ± 2.1
R-1	3.1 ± 0.5	6.7 ± 0.4	1.0 ± 0.1	1.5 ± 0.2	25.9 ± 2.7
S-2	3.3 ± 0.6	6.8 ± 0.4	1.1 ± 0.2	2.0 ± 0.2	24.1 ± 2.8
R-2	3.0 ± 0.2	6.3 ± 0.3	1.0 ± 0.1	1.5 ± 0.2	24.4 ± 1.6
S-3	0.2 ± 0.1	0.5 ± 0.1	0.1 ± 0.1	0.8 ± 0.1	1.6 ± 0.2
S-3‡	(1.0 ± 0.6)	(2.8 ± 0.3)	(0)	(0.1 ± 0.1)	(9.8 ± 1.4)
R-3	0.4 ± 0.3	0.7 ± 0.1	0.4 ± 0.2	1.3 ± 0.2	1.0 ± 0.2
R-3‡	(1.2 ± 0.2)	(3.0 ± 0.3)	(0)	(0)	(13.3 ± 1.1)

*mg % AA ± S.E. and (IAA ± S.E.) including dehydro forms.

†Depleted 17 days and treated for 2 months with 2 mg AA or 40 mg IAA.

**S = synthetic diet; R = rabbit ration; 1 and 3 = ad libitum and 2 = pair fed as 3. Groups 1 and 2 received AA while group 3 received IAA.

‡ Values between parenthesis refer to IAA.

TABLE VII

Comparison of vitamin C (mg %) found in young guinea pigs after depletion* and after treatment with D-isoascorbic acid (IAA)

Organ	Depletion period		IAA treatment†
	17 days	19 days	2 months
Liver	0.79	0.46	0.17
Brain	5.69	5.01	0.53
Heart	0.16	0.23	0.08
Kidneys	0.54	0.39	0.83
Adrenals	2.35	1.09	0.001

* All the depleted guinea pigs showed various degrees of scurvy revealed by hemorrhages in the muscles around the knees of the hind legs.

† After 17 days of depletion.

Experiment 2

The weight curves of Figure 8 demonstrate that 2 per cent IAA incorporated to the synthetic diet was as effective as 0.1 per cent AA in restoring the growth of vitamin C depleted guinea pigs. When IAA and AA were removed from the diet, the animals previously treated with IAA lost weight and died much more rapidly than those previously treated with AA. As shown in Table VIII, the average IAA content of the liver and spleen of 3 guinea pigs after 16 days of IAA treatment was lower than the AA content in the same organs of AA treated animals, but the IAA or AA content of other organs were comparable. After the IAA treatment traces of AA were found in all organs except the kidneys.

C. Turnover of D-isoascorbic acid (IAA) and L-ascorbic acid (AA) in guinea pigs organs

Experiment 1

Table IX shows that the guinea pigs continued to gain weight for about two weeks after they received the deficient diet. The subsequent loss of weight continued for the remaining 4 days of depletion and for 2 days during treatment with 0.1 per cent AA or 2 per cent IAA in the diet. Although not statistically significant the weight gain was greater after IAA than after AA treatment.

The rate of loss of AA or IAA from the organs of guinea pigs after withdrawal of the isomers from the diet is presented in Figure 9. Initially, except for the liver, there was more IAA in the organs after IAA treatment than AA after AA treatment; in all organs the rate of loss of IAA was much more rapid than that of AA, and, after 4 days the IAA content was much lower than the AA content in all organs except the

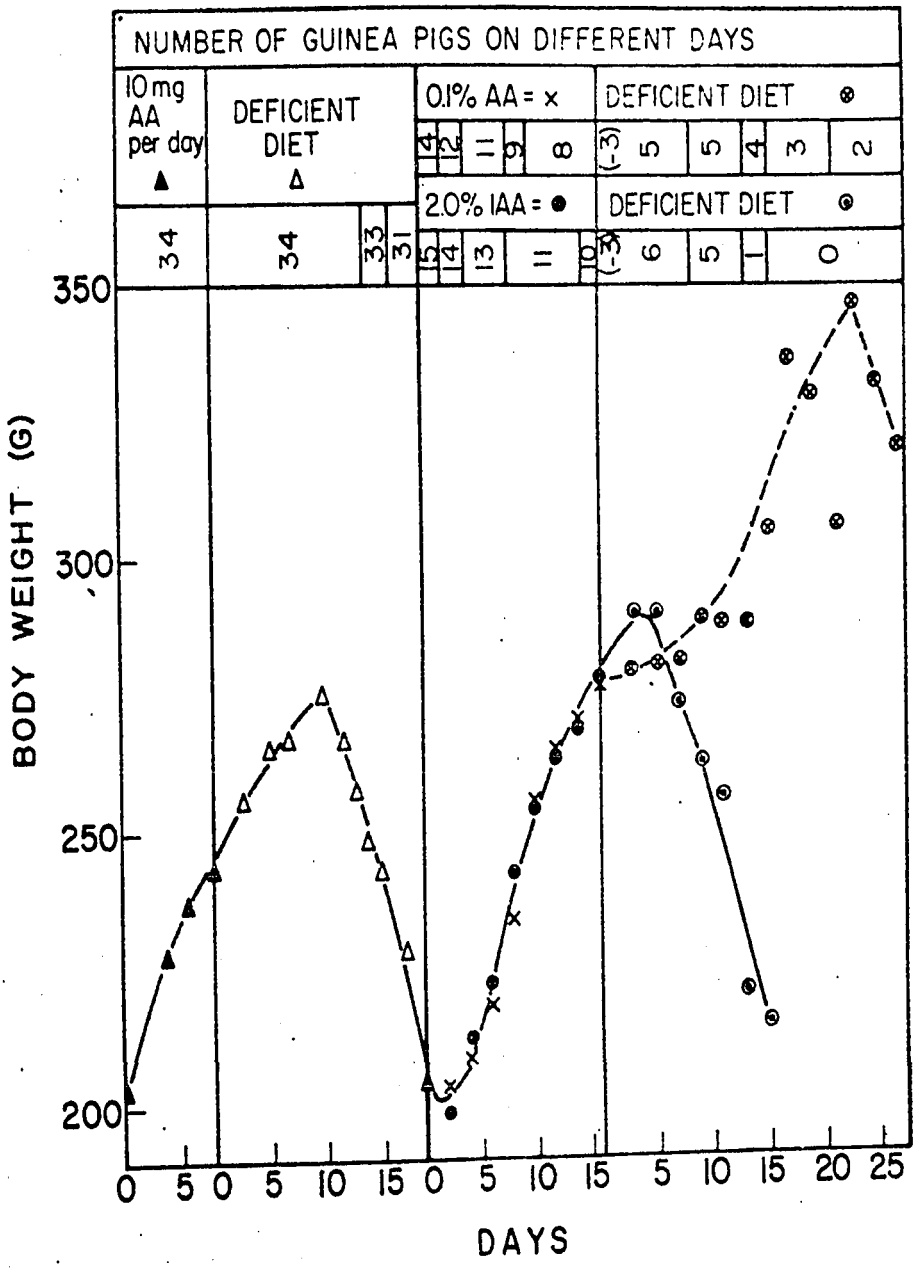


Figure 8. Mean body weight (g) of guinea pigs fed a synthetic diet during depletion, subsequently treated with L-ascorbic acid (AA) and D-isoascorbic acid (IAA) and finally depleted again.

Table VIII

L-ascorbic acid (AA)* and D-isoascorbic acid (IAA)*, of guinea pigs treated with 0.1% AA or 2% IAA in diet after depletion.

	AA diet	IAA	
Organ	AA	AA	IAA
Liver	23.1	1.2	8.8
Brain	22.1	4.7	22.1
Heart	7.9	0.2	6.1
Spleen	41.3	3.3	28.0
Adrenals	95.9	5.3	102.2
Kidneys	11.6	0	10.1

* AA and IAA in mg%.

Table IX

Body weight* of guinea pigs depleted of vitamin C during 18 days and treated with 0.1% L-ascorbic acid (AA) or 2% D-isoascorbic acid (IAA) in diet for 9 days

	Deficient diet			0.1% AA	2% IAA
Day	Group A	Group B	Day	Group A	Group B
0	369 ± 8	360 ± 10	18	398 ± 14	387 ± 13
2	384 ± 10	373 ± 11	20	384 ± 15	389 ± 12
7	403 ± 12	392 ± 12	22	387 ± 15	396 ± 12
11	421 ± 12	412 ± 13	24	386 ± 15	394 ± 13
14	423 ± 13	416 ± 14	27	405 ± 16	412 ± 14
17	408 ± 14	400 ± 13			

* Mean body weight (g) and S.E. for 19 guinea pigs (Group A) and 23 guinea pigs (Group B).

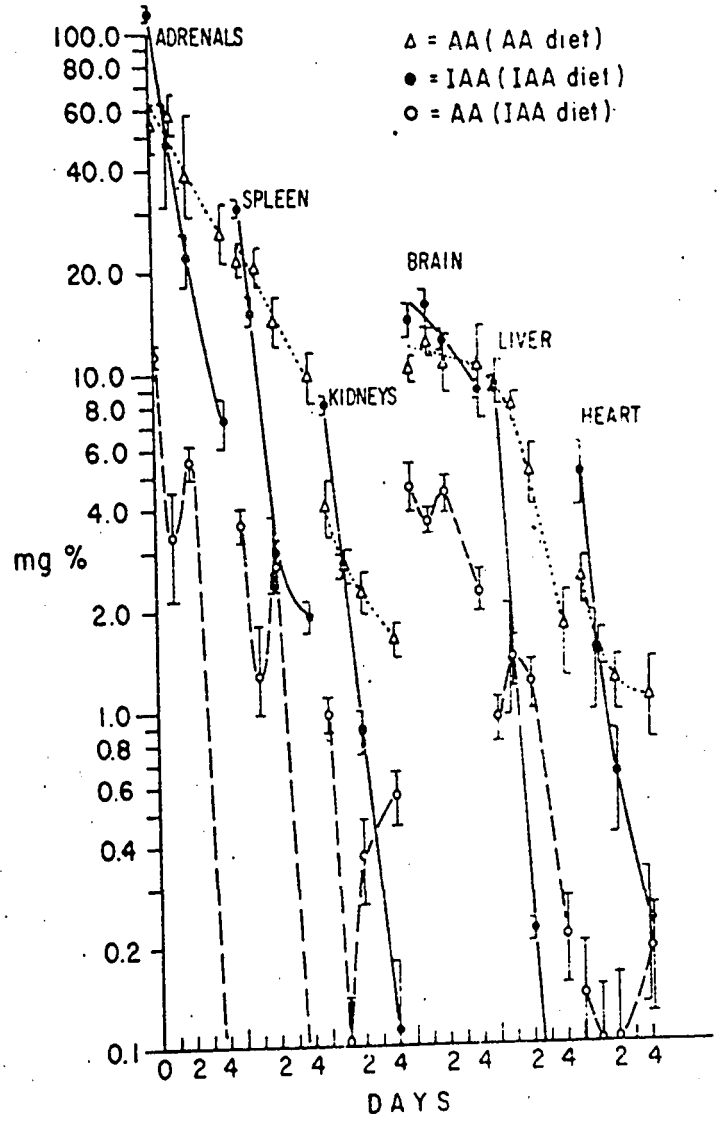


Figure 9. L-ascorbic acid (AA) and D-isoascorbic acid (IAA) depletion in guinea pigs. Vertical lines represent the standard error of the mean.

brain. A small amount of AA found in the organs of IAA treated animals did not vary according to a regular pattern.

Half-lives calculated from Figure 9 are shown in Table X. The longest half-lives were found in the brain, and that of AA was 4 times longer than that of IAA. In other organs, half-lives of AA varied from 2 to 3 days and were 4 to 5 times longer than that of IAA.

Experiment 2

The loss of L-ascorbic acid-1-C^{14} from the organs of guinea pigs (Figure 10 and Figure 11) was less for the guinea pigs fed with the deficient diet than for those receiving AA or IAA. Except for the brain, the turnover of L-ascorbic acid-1-C^{14} was increased more by IAA than by AA.

Half-lives for L-ascorbic acid-1-C^{14} were calculated graphically from Figure 10 and Figure 11 and are presented in Table XI. Because of the slow uptake of L-ascorbic acid-1-C^{14} in the brain, its half-life value was calculated from day 6 to 10 only. For the animals on the deficient diet, the half-life in the brain was about four times longer than in the other organs where it ranged from 2 to 4 days and increased with the degree of depletion. After treatment with AA, the half-life of L-ascorbic acid-1-C^{14} in the brain was shortened to less than one half that obtained with the deficient diet; in the other organs half-lives ranged from 2 to 3 days, i.e. were generally shorter, especially in the 6 to 10 days period. Following the IAA treatment, the half-life of L-ascorbic acid-1-C^{14} in the brain was about two thirds of that obtained with the deficient diet; for the other organs the half-lives were shorter than from day 0 to day 3, but were comparable to that of the AA treated animals from day 3 to day 10.

TABLE X

Half-life (days) of L-ascorbic acid (AA) and D-isoascorbic acid (IAA) in guinea pigs organs*

Organ	AA	IAA
Brain	20.0	4.5
Liver	2.2	0.5
Heart	2.0	0.5
Kidneys	2.5	0.6
Adrenals	3.2	0.7
Spleen	3.0	0.6

*Guinea pigs received diets containing 0.1% AA or 2% IAA for 9 days after being depleted of vitamin C for 18 days.

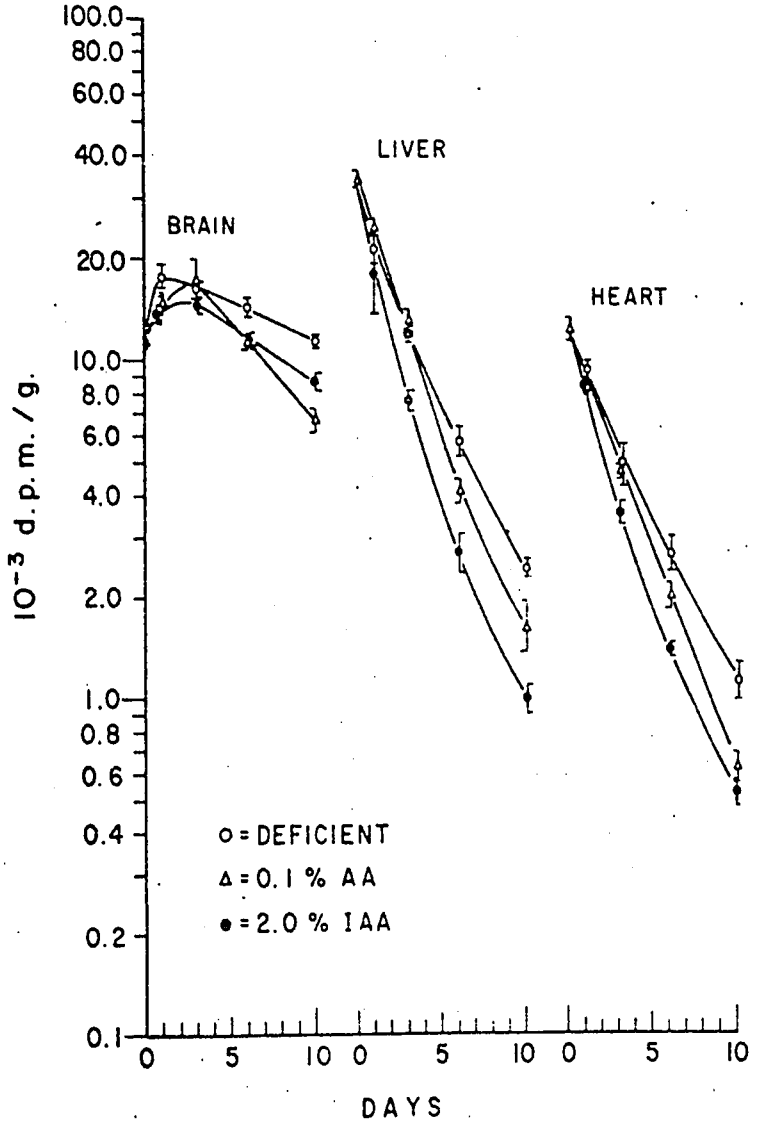


Figure 10. The effect of feeding a deficient diet, a diet containing 0.1 per cent L-ascorbic acid (AA), and a diet containing 2 per cent D-isoascorbic acid (IAA) on the loss of L-ascorbic-1-C^{14} acid from the brain, liver, and heart of guinea pigs. Vertical lines represent the standard error of the mean.

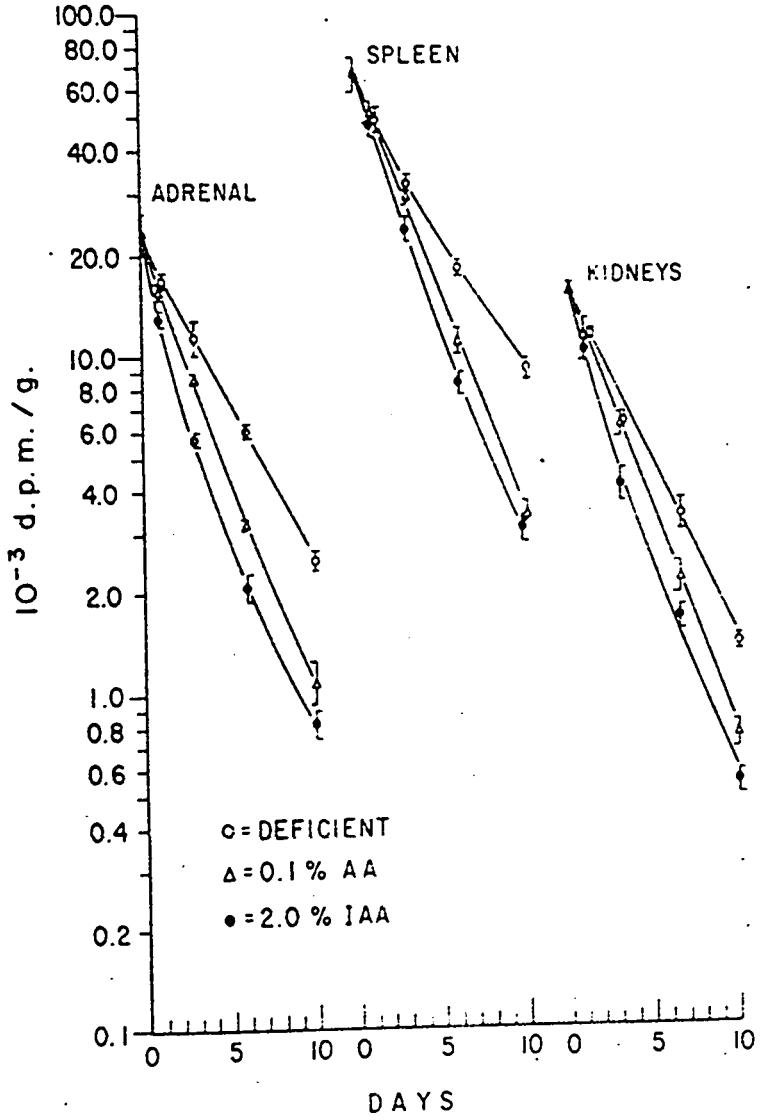


Figure 11. The effect of feeding a deficient diet, a diet containing 0.1 per cent L-ascorbic acid (AA), and a diet containing 2 per cent D-isoascorbic acid (IAA) on the loss of L-ascorbic acid-1-C^{14} acid from the adrenals, spleen, and kidneys of guinea pigs. Vertical lines represent the standard error of the mean.

TABLE XI

Half-life of L-ascorbic acid-1-C^{14}* in guinea pigs organs

Days†	Brain	Liver	Heart	Kidneys	Adrenals	Spleen
Deficient diet						
0-3	-	1.8	2.5	2.8	2.9	2.8
3-6	-	2.8	3.0	3.2	3.2	3.6
6-10	13.5	4.0	3.2	3.2	3.2	4.1
Diet + 0.1% AA						
0-3	-	2.0	2.2	2.3	2.0	2.2
3-6	-	2.0	2.4	2.1	2.1	2.4
6-10	5.3	3.0	2.4	2.6	2.3	2.4
Diet + 2% IAA						
0-3	-	1.2	1.7	1.6	1.2	2.0
3-6	-	1.9	2.1	2.3	2.0	2.0
6-10	9.0	2.5	2.8	2.5	3.0	2.5

*Guinea pigs were injected intraperitoneally with 2.75 µc L-ascorbic acid-1-C^{14} and 5 mg L-ascorbic acid after having received diet containing 0.1% AA for one month.

†Day 0 of treatments without AA, with 0.1% AA or 2% D-iso-ascorbic acid (IAA) was 24 hours after injection.

D. Urinary excretion of orally administered D-isoascorbic acid (IAA) by humans

The urinary excretion of L-ascorbic acid (AA) and D-isoascorbic acid (IAA) by humans is summarized in Table XII. Before saturation with AA, the smokers excreted significantly less of the two 75 mg doses of AA and of the two 75 mg doses of IAA than non-smokers: smokers excreted 5 and 9 per cent of two consecutive doses of AA as compared to 33 and 45 per cent of IAA, while the corresponding percentage for the non-smokers were 27 and 35 (AA), and 43 and 83 (IAA). The amount excreted by both groups from the same doses increased during the postsaturation period, but the smokers still excreted less AA and IAA than the non-smokers although the difference was statistically significant only after the second dose of IAA (4 PM to 8:30 AM urine). Smokers excreted 22 and 30 per cent of two consecutive doses of AA as compared to 49 to 59 per cent of IAA, while the corresponding percentage for the non-smokers were 41 and 45 (AA), and 49 and 89 (IAA). When AA and IAA were given together, the amounts excreted were not significantly different from the amounts excreted when the compounds were given alone.

Table XII

Urinary excretion (mg ± S.E.) of L-ascorbic acid (AA) and D-isoascorbic acid (IAA) by smokers (S) and nonsmokers (NS) after oral administration of 75 mg in AM and PM.

Isomer	Group	8:30 AM to 4 PM		4 PM to 8:30 AM		The two periods	
		AA	IAA	AA	IAA	AA	IAA
Before saturation							
AA	S	3.6 ± 0.8**		6.6 ± 1.8†		10.2 ± 2.5†	
AA	NS	18.9 ± 5.2**		26.5 ± 6.2†		45.4 ±10.7†	
IAA	S	3.8 ± 0.5	24.8 ± 3.7	3.2 ± 0.8‡	34.1 ± 5.0*	4.0 ± 1.2**	58.9 ± 7.8‡
IAA	NS	13.2 ± 3.2	31.9 ± 4.5	10.1 ± 2.8‡	62.4 ± 4.0*	23.3 ± 6.0**	94.4 ± 7.0‡
After saturation							
AA	S	16.2 ± 4.3		22.3 ± 5.5		38.5 ± 7.2‡	
AA	NS	31.0 ± 6.9		33.5 ± 5.2		64.4 ± 8.2‡	
IAA	S	12.3 ± 4.0	36.6 ± 8.1	8.2 ± 1.3‡	44.3 ± 3.9†	20.5 ± 4.0**	81.0 ± 10.8
IAA	NS	20.8 ± 3.4	36.8 ± 1.4	16.7 ± 3.7‡	66.4 ± 5.7†	37.5 ± 5.1**	103.1 ± 5.6
AA + IAA	S	11.6 ± 3.0**	32.7 ± 4.3	21.3 ± 4.8*	53.1 ± 5.7	33.0 ± 6.5**	85.8 ± 9.8
AA + IAA	NS	24.5 ± 3.4**	42.4 ± 4.4	40.1 ± 5.9*	58.3 ± 2.7	64.6 ± 8.1**	99.8 ± 5.6

*=P < 0.001; † = P < 0.01; **= P < 0.025; ‡ = P < 0.05 for the difference between S and NS.

DISCUSSION

A. Uptake of D-isoascorbic acid (IAA) by guinea pig organs

The D-isoascorbic acid (IAA) assays of organs from guinea pigs receiving no IAA gave negative values in most cases and only insignificant traces in the others. The results obtained with guinea pigs fed IAA demonstrated that IAA was taken up by organs where it replaced a corresponding amount of AA. The IAA retained could in turn be replaced by L-ascorbic acid (AA) and turned over at least as rapidly as AA for which a half-life of 3-4 days has been reported.

Several authors have reported the antiscorbutic activity of IAA to be 1/40 to 1/20 that of AA (Wang et al. 1962). Reiff and Free (1959) using a Rockland rabbit ration devoid of vitamin C reported that daily supplements of 250 mg IAA did not support growth of guinea pigs but tended only to slow down development of acute scurvy; since 0.3 mg AA plus 100 mg IAA could maintain growth for more than 3 weeks, but 0.3 mg AA alone could not, they concluded that IAA had a sparing effect. Their work led to the general belief that IAA was not retained by tissues and that it exerted its antiscorbutic activity by sparing the AA present.

Recently, Fabianek and Herp (1967) have demonstrated that administration of only 10 mg IAA with a deficient diet to guinea pigs previously depleted for 1 week prevented scurvy for as long as 115 days. Although they were unable to determine the nature of the "ascorbic acid" present in organs after the administration of IAA, they interpreted their results as indicating that IAA could replace vitamin C. Since the

demonstration of IAA incorporation by the present study supported this hypothesis it appeared worthwhile to study the antiscorbutic activity of IAA.

B. Vitamin C activity of D-isoascorbic acid (IAA) for the guinea pig

Experiment 1

Although chromatographic separations of the D-isoascorbic acid (IAA) specimen did not yield quantitative results, (Part I-A) they indicated that much less than 1 per cent of the sample could be present as AA. Under the conditions of the experiment, the IAA sample would have supplied much less than 0.04 mg of AA per day to the animals. The assays of the diets yielded only traces of vitamin C due to very weak absorbance measurements which could very well have been caused by interfering substances such as carbohydrates. The amounts of vitamin C contributed by contamination of the diets and the IAA standard could be estimated to be smaller than 0.05 mg per day for the synthetic diet, and smaller than 0.1 mg per day for the Rockland ration. These amount are insufficient to prevent scurvy and maintain growth since 0.5 mg is known to be the minimal requirement (Duncker et al. 1942).

During the 17 days depletion period, the lack of vitamin C in the diets was evident by the loss of weight after 12 days. Consequently, the rapid recovery in weight gain by the IAA treated guinea pigs after the depletion period could not be attributed to a sparing of AA since not enough AA was present in the animal nor the diets to support growth.

Treatment of guinea pigs with IAA given orally for two months after depletion of vitamin C for 17 days indicated that IAA was able to replace AA in its functions when a well balanced diet was used. The growth

response of the IAA treated animals was somewhat less than for the AA-treated animals fed ad libitum, but was in accord with a smaller food intake and corresponded to the weight gain of pair fed AA-treated animals. It would appear that the lower food intake is due to the fact that IAA has less than 1/20 the activity of AA, which is known to affect the food intake of guinea pigs (Mohan Ram 1966).

The animals on the rabbit ration and receiving AA or IAA grew poorly except for the first 3 weeks after the depletion period. This could be due to the lower and poorer protein and vitamins content of this ration as compared to the synthetic diet. The great number of deaths of guinea pigs treated with IAA was probably due to the combined effects of a poor diet plus the lower vitamin C activity of IAA, since the total content of AA plus IAA in the organs was comparable to that found for the synthetic diet (Table VI).

The total lower content of AA plus IAA in the organs of the IAA treated animals indicates also that IAA given orally as a single dose has less than 1/20 the activity of AA since 20 times more IAA was given.

Neither the kidneys nor the adrenals of the IAA treated animals were significantly enlarged as in the case of chronic hypovitaminosis and scurvy (Hughes 1965).

Except for the kidneys, the vitamin C values, shown in Table IV for the organs of young guinea pigs depleted for 17 and 19 days with the synthetic diet, were higher than those obtained for the organs of guinea pigs depleted in the same way for 17 days but subsequently treated with IAA during two months; the amounts found in the IAA-treated animals were equal or less than those found for scorbutic guinea pigs.

These results suggest that IAA does not have much sparing activity on AA and are in agreement with the previous demonstration of incorporation of IAA into guinea pigs organs (Part 2-A).

The kidneys of the IAA treated guinea pigs contained slightly more AA than those of guinea pigs depleted of vitamin C for 17 and 19 days but on the basis of the rapid turnover of AA in these organs (Part 2-C) one would have expected significantly less. This could suggest a weak isomerization of IAA into AA by the kidneys or a relatively weak synthesis of AA (Williams and Deason 1967) which would be undetectable in guinea pigs fed a deficient diet.

From experiments with humans, it had been suggested that IAA was rapidly excreted in the urine (Rivers et al. 1963). This was confirmed by the results obtained with humans (Part II-D.). However, after 12 hours, about 2 per cent of the dose was found in the urine as IAA and DKG (IAA).

Experiment 2

The incorporation of IAA into a synthetic diet indicated that IAA in a concentration 20 times larger than AA was as effective as the latter in reversing the weight loss due to vitamin C deficiency and in preventing the symptoms of scurvy in guinea pigs. Although organs of guinea pigs, except the liver and spleen, contained as much "ascorbic acid" after IAA treatment than after AA treatment, the antiscorbutic activity was maintained for a much shorter time after stopping the IAA treatment than after stopping the AA treatment. This suggested that incorporated IAA was not retained as long as AA.

C. Turnover of D-isoascorbic acid (IAA) and L-ascorbic acid (AA) in guinea pigs organs

Experiment 1

The weight data in Table IX confirm the previous experiments showing the ability of IAA to restore the growth of guinea pigs deficient in vitamin C. IAA incorporated in the diet was as much active as 20 times less AA. The greater effectiveness of dietary IAA as compared to IAA given orally is probably due to a higher intake and to the more constant availability of IAA from the diet.

The very rapid removal of IAA from the organs of guinea pigs is undoubtedly a major cause for the low antiscorbutic activity of IAA as compared to AA (Rivers et al. 1963) and for this reason greater amounts of IAA must be supplied to obtain the same effect. It thus appears that in guinea pigs, the lower activity of IAA is not due so much to its inability to be transported to the organs (Martin 1961) but rather to the ability of the organs to retain IAA. It would be of interest to determine whether this inability is due to the catabolism of IAA in the organs or to leakage of IAA from the organs.

Experiment 2

Because of C^{14} metabolites of L-ascorbic acid-1-C^{14} (CO_2 and oxalic acid) are rapidly excreted, the C^{14} remaining in the guinea pigs 24 hours after intraperitoneal injection is equivalent to the amount of L-ascorbic acid-1-C^{14} remaining in the body (Dayton and McMillan Snell 1966). It is evident from the loss of L-ascorbic acid-1-C^{14} from the organs of guinea pigs, that feeding IAA causes a higher turnover of AA i.e. a net loss of AA above that caused by a

deficient diet. Although the IAA effect was greater when the concentration of AA in the organs was high, this effect was still evident after 10 days of treatment. There was no indication that IAA had a sparing effect on incorporated AA because one would have expected the turnover of AA to be less rapid than in the animals receiving only the deficient diet.

D. Urinary excretion of orally administered D-isoascorbic acid (IAA) by humans

Kadin and Osadca (1959), using a paper chromatographic method which could differentiate between the two isomers at concentrations above physiological levels, saturated humans with AA, and on the day after discontinuing saturation doses gave 300 mg of IAA. When they found no difference in AA excreted (during the following 4 hrs) as compared to when IAA was omitted, they concluded that IAA had no displacement action. Since the turnover of AA in humans is known to be about four times longer than in guinea pigs, one would expect little turnover (about 3 mg) during 4 hr in presaturated humans, with a rate of utilization of about 20 mg per day (Baker 1967); and consequently one would expect little displacement by IAA.

On the other hand in subjects not presaturated with AA and particularly in subjects having lower vitamin C levels, incorporation of IAA would seem possible. When IAA was given to human subjects, depleted (Rivers et al. 1963) or on intakes of 10 and 25 mg AA per day (Wang et al. 1962), a greater amount of IAA was excreted as compared to the quantity of AA excreted when the same amount of AA was given in the same way;

furthermore, daily doses of IAA to depleted subjects did not increase the levels of leukocytes and plasma, while AA did (Rivers et al. 1963). Since the methods used by those workers did not differentiate between AA and IAA, their results were not conclusive.

The results reported in Table X indicate that cigarette smokers, who in general have lower vitamin C levels than non-smokers (See Part 3), excreted much less L-ascorbic acid than non-smokers during the presaturation period. The smokers also excreted significantly less IAA than non-smokers after two doses of IAA during the presaturation period, but the difference was not significant for the two doses in the postsaturation period when both groups had similar vitamin C levels. Assuming that the catabolism or loss of IAA in the postsaturation period was the same before or after saturation, it appears likely that the smokers have retained more IAA during the presaturation period. Although the uptake of IAA by human tissue cannot be ruled out, it does not appear to be significant as compared to guinea pigs, since the smokers excreted 6 times more IAA and the non-smokers 2 times more IAA after the IAA doses than AA after the AA doses. In the postsaturation period, both groups excreted about two times more IAA than AA. The percentage of IAA excreted after two doses was only 70 per cent for the non-smokers and 55 per cent for the smokers. IAA had no significant effect on the uptake of AA, because when AA and IAA were administered simultaneously, the excretion pattern of each isomer was about the same as when they were administered separately. The quantity of IAA not recovered could have been destroyed during the absorption or by some metabolic processes, and/or taken up by body tissues. It should be possi-

ble to estimate the uptake of IAA by humans by measuring the IAA content of organs following the intake of IAA or by measuring the difference in retention of a vitamin C load test after treatment with IAA or AA.

REFERENCES

Abt, A.F., S. von Schuling and T. Enns. 1963. Vitamin C requirements of man re-examined. New values based on previously unrecognized exhalatory excretory pathway of ascorbic acid. Am. J. Clin. Nutr. 12: 21-29.

Atkins, G.L., B.M. Dean, W.J. Griffin and R.W.E. Watts. 1964. Quantitative aspect of ascorbic acid metabolism in man. J. Biol. Chem. 239: 2975-2980.

Baker, E.M. 1967. Vitamin C requirements in stress. Am. J. Clin. Nutr. 20: 583-590.

Baker, E.M., J.C. Saari and B.M. Tolbert. 1966. Ascorbic acid metabolism in man. Am. J. Clin. Nutr. 19: 371-378.

Baker, E.M., H.W. Sauberlich, S.J. Wolfish, W.T. Wallace and E.E. Dean. 1962. Tracer studies of vitamin C utilization in men: metabolism of D-glucuronolactone-6-C^{14}, D-glucuronic-6-C^{14} acid and L-ascorbic acid-1-C^{14} acid. Proc. Soc. Expl. Biol. Med. 109: 737-741.

Borenstein, B. 1965. The comparative properties of ascorbic acid and erythorbic acid. Food Technology 19: 115-117.

Borsook, H., H.W. Davenport, C.E.R. Jeffreys and R.C. Warner. 1937. Oxidation of ascorbic acid and its reduction in vitro and in vivo. J. Biol. Chem. 117: 237-279.

Burns, J.J. 1959. Biosynthesis of L-ascorbic acid, basic defect in scurvy. Am. J. Med. 26: 740.

Burns, J.J., H.B. Burch and C.G. King. 1951. The metabolism of 1-C^{14}-L-ascorbic acid in guinea pigs. J. Biol. Chem. 191: 501-514.

Burns, J.J., P.G. Dayton and S. Schulenberg. 1956. Further observation on the metabolism of L-ascorbic acid in guinea pigs. J. Biol. Chem. 223: 897-905.

Burns, J.J. and C. Evans. 1956. The synthesis of L-ascorbic acid in the rat from D-glucuronolactone and L-gulonolactone. J. Biol. Chem. 223: 897-905.

Clemetson, C.A.B. and L. Andersen. 1966. Plant polyphenol as antioxidants for ascorbic acid. New York Academy of Science, 136: 339-378.

Chan, P.C., R.R. Beeker and C.G. King. 1958. Metabolic products of L-ascorbic acid. J. Biol. Chem. 231: 231-240.

Chatterjee, I.B., G.C. Chatterjee, N.C. Ghosh, J.J. Ghosh and B.C. Guha. 1960. Biological synthesis of L-ascorbic acid in animal tissues. Conversion of L-gulonolactone into L-ascorbic acid. Biochem. J. 74: 193-203.

Chaudhuri, C.R. and I.B. Chatterjee. 1969. L-ascorbic acid synthesis in birds, phylogenic trend. Science, 164: 435-436.

Dalmer, O. and T.H. Moll. 1933. The question of the antiscorbutic activities of compounds similar to ascorbic acid. Z. Physiol. Chem. 222: 116-120.

Dayton, P.G., M. McMillan-Snell and J.M. Perel. 1966. Ascorbic and dehydroascorbic acids in guinea pigs and rats. The J. of Nutr. 88: 338-343.

Demole, V. 1934. On the physiological action of ascorbic acid and some related compounds. Biochem. J. 28: 770-773.

Devine, M.M. and J.M. Rivers. 1969. Monodehydroascorbic acid transhydrogenase activity and isoenzyme concentrations in tissues of ascorbic acid deficient and control guinea pigs. J. Nutr. 97: 295-302.

Dunker, C.F., C.R. Fellers and W.B. Esselen, Jr. 1942. A Comparison of four methods for determining vitamin C with a 25-day weight response bioassay. Food Res. 7: 260-266.

Esselsen, W.B., J.J. Powers, Jr. and R. Woodward. 1945. D-isoascorbic acid as antioxidant. Ind. Eng. Chem. 37: 295-299.

Fabianek, J. and A. Herp. 1967. Antiscorbutic activity of D-iscascorbic acid. Proc. Soc. Expl. Biol. Med. 167: 462-465.

Fujimoto, D. and H. Tamiya. 1962. Incorporation of O-18 from air into hydroxyproline by chick embryo. Biochem. J. 84: 333-335.

Gould, B.S. 1968. Treatise on collagen. Volume 2, Biology of Collagen, Part A. Academic Press. London and New York.

Grimble, R.F. and R.E. Hughes. 1967. A 'dehydroascorbic acid reductase' factor in guinea-pig tissues. Separatum Experientia, 23: (362), 1-3.

Hellman, L. and J.J. Burns. 1958. Metabolism of L-ascorbic acid in man. J. Biol. Chem. 230: 923-930.

Hope, G.W. 1961. The use of antioxidants in canning apple halves. Food Technol. 15: 548-555.

Hughes, R.E. 1964. Reduction of dehydroascorbic acid by animal tissues. Nature, 203: 1068-1069.

Hughes, R.E. 1965. A note on kidney size in chronic hypovitaminosis C. Brit. J. Nutr. 19: 307-309.

Hughes, R.E. and R.J. Hurley. 1969. The uptake of D-araboascorbic (D-iso-ascorbic acid) by guinea-pigs tissues. Brit. J. Nutr. 23: 211-216.

Hughes, R.E. and G.S. Kilpatrick. 1964. The reduction of dehydroascorbic acid by hemolysates of pernicious anaemia erythrocytes. Clin. Chim. Acta. 9: 241-244.

Hutton, Jr. J.H., D.L. Tappel and S. Udenfriend. 1967. Cofactor and substrate requirement of collagen proline hydroxylase. Arch. Biochem. Biophys. 118: 231-240.

Ikeuchi, M. 1954. Biochemical studies on D-araboascorbic acid in vitamin C deficiency. J. Vitaminology, 1: 193-199.

Kadin, H. and M. Osadka. 1959. Biochemistry of erythorbic acid. Human blood levels and urinary excretion of ascorbic and erythorbic acids. Agr. Food Chem. 7: 358-360.

Kanfer, K., G. Ashwell and J.J. Burns. 1959. Formation of L-lyxonic acid from L-ascorbic acid in rat kidney. Fed. Proc. 18: 256.

King. C.G. 1967. Present knowledge of ascorbic acid (vitamin C). pp. 76-79 in: Present knowledge in nutrition, Third Ed. The Nutrition Foundation Inc. Ninety-nine Park Ave. New York, N.Y. 10016.

Kivirikko, K.I. and D.J. Prockop. 1967A. Purification and partial characterization of the enzyme for the hydroxylation of proline in protocollagen. Arch. Biochem. Biophys. 118: 611-618.

Kivirikko, K.I. and D.J. Prockop. 1967B. Enzymatic hydroxylation of proline and lysine in protocollagen. Proc. Nat. Acad. Sci. 57: 782-789.

Kunz, C.E. and W.B. Robinson. 1958. Cherry filling keeps color longer. Additions of minute amounts of isoascorbic slows color change during storage and distribution. Food Packer, Pages 17 and 32. Sept. 1968.

Levandoski, N.G., E.M. Baker and J.E. Canham. 1964. A monodehydro form of ascorbic acid to dehydroascorbic acid. Biochemistry, 3: 1465-1469.

Linkswiler, H. 1958. The effect of ingestion of ascorbic acid and dehydroascorbic acid upon the blood levels of these two components in human subjects. J. Nutr. 64: 43-54.

Martin, G.R. 1961. Studies on the tissue distribution of ascorbic acid. Ann. N.Y. Acad. Sci. 92: 141-147.

Mill, F., D.S. Ginsberg, B. Ginger, C.E. Weir and G.D. Wilson. 1958. The effect of sodium ascorbate and sodium isoascorbate on the qualities of frankfurters. Food Technology 12: 311-314.

Medical Research Council. 1948. Vitamin C requirements of human subjects. Lancet, 1: 853-858.

Mohan Ram, M. 1966. Growth rate and protein utilization in vitamin C deficiency. Ind. J. Med. Res. 54: 964-970.

Pelletier, O. 1969. Differential determination of D-isoascorbic acid and L-ascorbic acid in guinea pig organs. Can. J. Biochem. 47: 449-453.

Piez, K.A. 1966. Maturation of collagen. J. Dental Res. 45: 463-468.

Prockop, D., A. Kaplan and S. Udenfriend. 1962. Oxygen-18 studies on the conversion of proline to collagen hydroxyproline, Biochem. Biophys. Res. Commun. 9: 162.

Reiff, S.J. and A.H. Free. 1959. Nutritional studies with isoascorbic acid in the guinea pig. Agr. Food Chem. 7: 55-56.

Reyes, P. and B.S. Luh. 1962. Ascorbic acids as antioxidants for frozen freestone peaches. Food Technol. 16: 116-118.

Rivers, J.M. 1965. Ascorbic acid in metabolism of connective tissue. N.Y. State J. of Med. 65: 1235-1238.

Rivers, J.M., E.D. Huang and M.L. Doods. 1963. Human metabolism of L-ascorbic acid and erythorbic acid. J. Nutr. 81: 163-168.

Robertson, W.B. 1963. D-ascorbic acid and collagen synthesis. Biochem. Biophys. Acta 74: 137-139.

Roy, R.N. and B.C. Guha. 1958. Species difference in regard to the biosynthesis of ascorbic acid. Nature 182: 319.

Schow, S.R. 1966. A serial study of ascorbic acid depletion in the guinea pig. J. Dental Res. 45: 1232.

Schimizu, K., T. Nishiyama, T. Inoue, N. Takano, M. Mikita, M. Yamazaki, T. Azurmma and S. Osana. 1967. Studies on erythorbic acid production by fermentation. Part II. Erythorbic acid production by jar fermentation. Agr. Biol. Chem. 31: 346-352.

Schimizu, Y., D.S. McCann and M.K. Keuh. 1965. Human dermal fibroblasts in monolayer tissue culture. Effect of ascorbic analogues. J. Lab. Clin. Med. 66: 659-666.

Smith, F. 1946. Analogs of ascorbic acid in: Carbohydrate chemistry 2: 94-96. Academic Press.

Staudinger, H.J., K. Krish and S. Leonhauser. 1961. Role of ascorbic acid in microsomal electron transport and the possible relationship to hydroxylation reactions. Ann. N.Y. Acad. Sci. 92: 195-207.

Takahaski, T., M. Mitsumoto and H. Kayamori. 1960. Production of D-araboascorbic acid by Penicillium. Nature 188: 411-412.

Tarr, H.L.A. 1948. Control of ranciditis of fish flesh. II. Physical and chemical methods. J. Fish Res. Bd. Can. 7: 237-247.

Tressler, D.K. and C. DuBois. 1944. No browning of cut fruit when treated by new process. Food Industries, 16: 701, 763-765.

Von Schuchling, S.L. and A.F. Abt. 1965. Verification of L-ascorbic acid-1-C^{14} catabolism to carbon-14 dioxide in the human by liquid scintillation counting. Proc. Soc. Exp. Biol. Med. 188: 30-33.

Wagner, A.F. and K. Folkers. 1964. L-ascorbic acid. In: vitamins and coenzymes, pages 308-329. Interscience Publishers.

Wang, M.M., K.H. Fisher and M.L. Doods. 1962. Comparative metabolic response to erythorbic acid and ascorbic acid by the human. J. Nutr. 77: 443-447.

Watts, B.M., A.M. Erdman and J. Wentworth. 1955. Relation of free sulfhydryl groups to cured meat color. J. Agr. Food Chem. 3: 147-141.

Waugh, W.A. and C.G. King. 1932. The vitamin C activity of hexuronic acid from suprarenal glands. Science 76: 630.

Williams, R.J. and G. Deason. 1967. Individuality of vitamin C needs. Proc. Nat. Acad. Sci. 1638-1641.

Wolback, S.B. and P.R. Hower. 1926. Intercellular substances in experimental scorbutus. Arch. Pathol. 1: 1.

Yagi, J., T. Yamashita, K. Kato, Y. Takagi and H. Sahai. 1967. Studies on erythorbic acid production by fermentation. Part 1. Erythorbic acid producing strain and cultural conditions. Agr. Biol. Chem. 31: 340-346.

Yamazaki, I., H.S. Mason and L. Piette. 1960. Identification by electron paramagnetic resonance spectroscopy of free radicals generated from substrates by peroxidase. J. Biol. Chem. 235: 2444-2449.

Yourga, F.J. 1948. How to prevent off color in glazed carrots. Food Industries, 20: 115 and 146.

Yourga, F.J., W.B. Esselen and C.R. Fellers. 1944. Some antioxidant properties of D-isoascorbic acid and its sodium salt. Food Res. 9: 188-196.

Zilva, S.S. 1935. The behavior of L-ascorbic acid and chemically related compounds in the animal body. Antiscorbutic activity in relation to retention by the organism. Biochem. J. 29: 1612-1616.

PART III

UTILIZATION OF L-ASCORBIC ACID: EFFECT OF CIGARETTE SMOKING AND NICOTINE*

* Part of the results reported in this section has been published as indicated in the following references:

1 Pelletier, O. 1968. Smoking and vitamin C levels in humans. Am. J. Clin. Nutr. 21: 1259-1267.

2 Pelletier, O. 1970. Evaluation of the vitamin C status of cigarette smokers. Am. J. Clin. Nutr. In Press.

INTRODUCTION

Other biochemical functions of vitamin C

Although vitamin C has been reported to be involved in several biochemical processes, none of these has been as thoroughly studied as its role in collagen synthesis and the prevention of scurvy (Part II).

The involvement of vitamin C in brain and nervous functions is indicated by the mental symptoms (depression) that accompany the physical symptoms of scurvy (Pauling 1968), and by the observations of several clinicians that ascorbic acid in large doses has therapeutic value in the treatment of schizophrenia (Hoffer and Osmond 1963). In a study with 10 chronic schizophrenic patients and 8 normal persons, Van der Kamp (1966) found that the schizophrenics required much larger doses of vitamin C and a much longer time to excrete similar amounts of L-ascorbic acid in the urine. The patients metabolized 10 times more of the vitamin than normal. Earlier, Briggs (1962) had shown that a group of schizophrenics excreted significantly more dehydroascorbic and diketogulonic acid than a group of hospitalized epileptics or a group of normal persons receiving the same diet.

Noradrenaline and adrenaline are essential in the nervous system; Levin et al. (1960) demonstrated that in vitro the hydroxylation of dopamine to norepinephrine (Figure 1) by an hydroxylating enzyme (oxidase) partially purified from bovine adrenal medullary particles required L-ascorbic acid for its activity but that D-ascorbic acid, isoascorbic acid and glucoascorbic acid were also active while other enediols, SH-compounds, reduced pteridines, NADH and NADPH could not

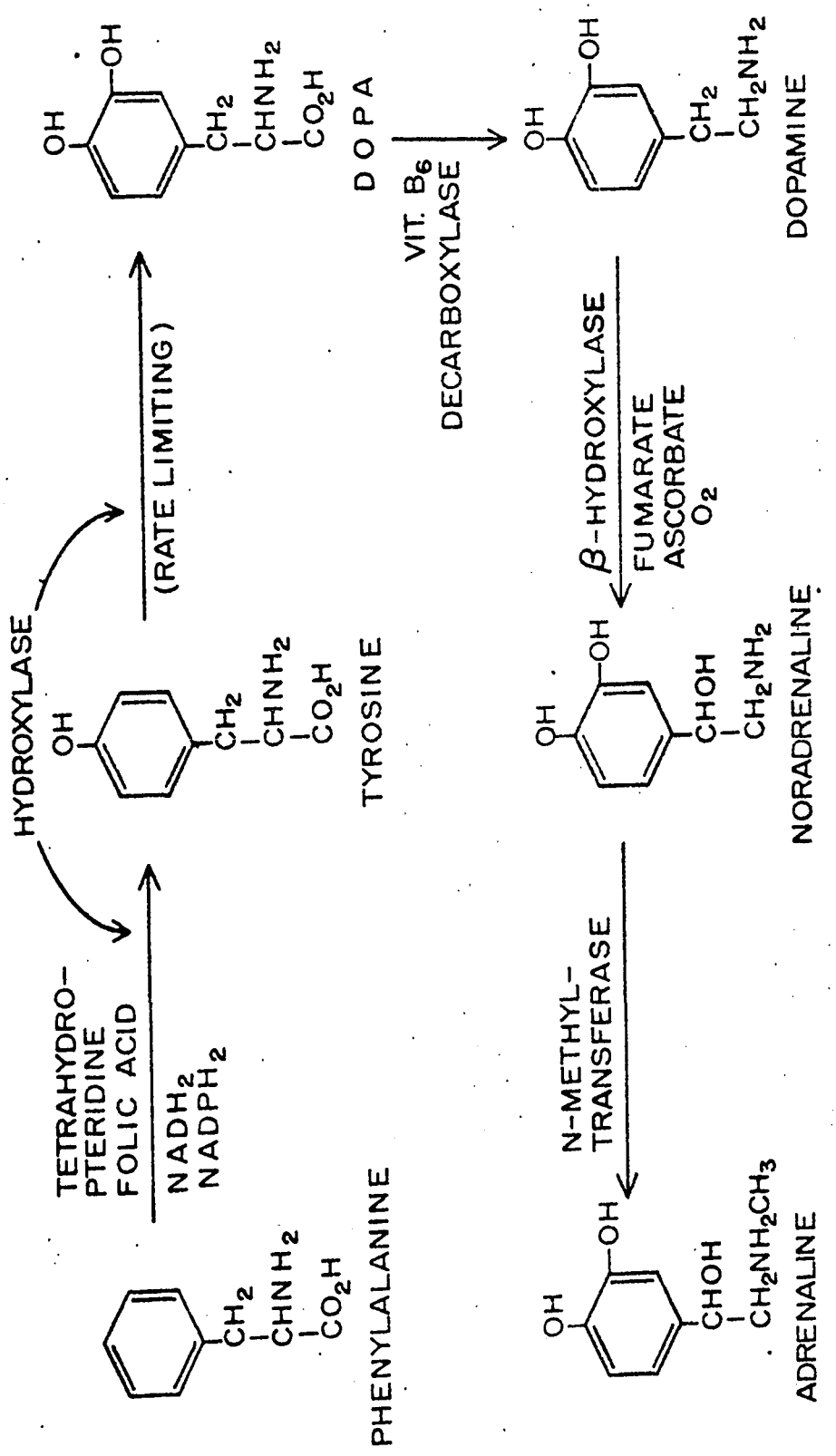


Figure 1. Noradrenaline and adrenaline synthesis.

replace ascorbate; ascorbate without the enzyme, was ineffective. Nagatsu et al. (1968) have reported that dopamine β-hydroxylase activity is unaffected by ascorbic acid deficiency. Because several laboratories had found that norepinephrine was not decreased in scorbutic guinea pigs, Kirschner (1966) suggested that ascorbic acid might just be one of several compounds that can reduce dopamine β-oxidaze in vivo and that the role of ascorbic acid might be to accelerate the rate of synthesis of norepinephrine; in fact after tyrosine depletion, vitamin C deficient guinea pigs did not replenish the heart's norepinephrine content as rapidly as non deficient guinea pigs. However, it has also been reported that the concentration of norepinephrine in hearts (Thoa et al. 1966) and irises (Sears 1969) of scorbutic guinea pigs is about half that of normal guinea pigs. Thoa et al. (1966) suggested that the uptake or binding or both, of exogenously administered H^3-norepinephrine by hearts of scorbutic guinea pigs was deficient. Sears (1969) found that irises of scorbutic guinea pigs had a reduced capacity for storing H^3-norepinephrine and suggested that the relatively high concentration of ascorbic acid in uveal tissue and aqueous humor may stabilize norepinephrine. Thus the precise role of vitamin C in the synthesis uptake and storage of norepinephrine remains to be clarified.

Weisman (1967) indicated that high concentrations of serotonin (5-hydroxytryptamine) seem to be associated with behaviour (autonomic and emotional) and with integrative functions (hypothalamus and midbrain). The first step in the synthesis of 5-hydroxytryptamine from L-tryptophan (Figure 2) i.e. hydroxylation to 5-hydroxytryptophan apparently requires a specific enzyme in the presence of copper and ascorbic acid (Franchimont and Delwaide (1966).

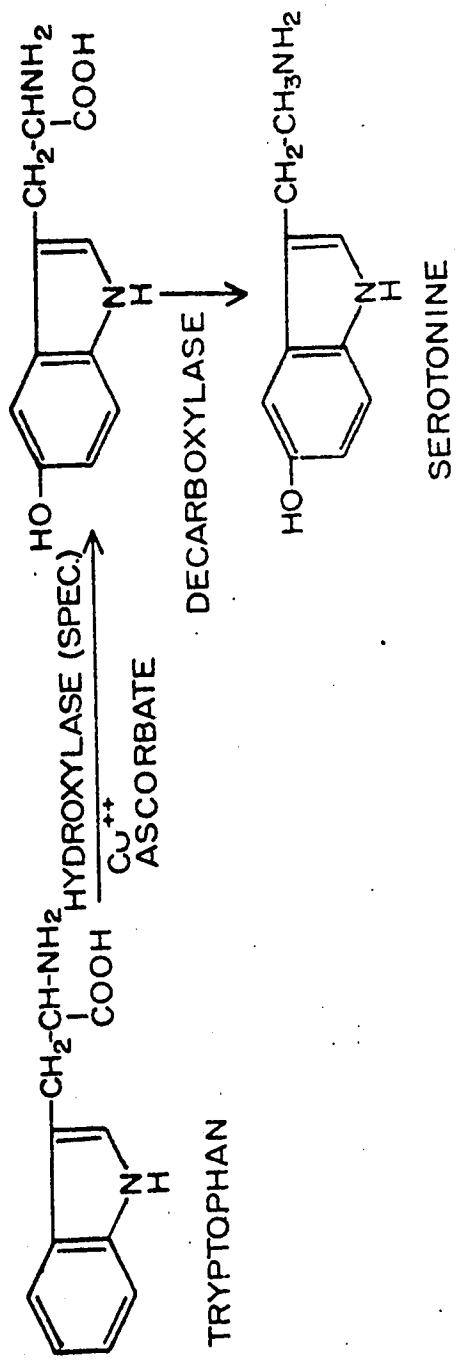


Figure 2. Serotonine synthesis.

In scorbutic guinea pigs or premature infants, too large quantities of tyrosine will result in the excretion of abnormal quantities of p-hydroxyphenylpyruvic acid and p-hydroxyphenylactic acid. Because an abnormal metabolism of tyrosine from a diet rich in proteins could cause mental retardation in premature infants, Tosberg et al. (1966) proposed that extra vitamin C be added to such diets. La Du and Zannoni (1961) have shown that L-ascorbic acid has an unusual role in tyrosine metabolism since it apparently protects p-hydroxyphenylpyruvate oxidase from inhibition by its substrate (Figure 3); this effect is produced with large quantities of tyrosine in the diet and the amount of vitamin C required to prevent this inhibition is far in excess of that necessary to prevent scurvy; furthermore this activity is shared by several biological reducing agents.

Whether L-ascorbic acid is directly involved in some of the hydroxylations required for the biosynthesis of adrenocortical hormones is mere speculation. However, vitamin C must have a functional role in the adrenals since their size more than double during scurvy (King 1967).

Increased oxygen consumption and increased thyroidal uptake of I^{131} by scorbutic guinea pigs were taken as evidence of increased thyroid activity in scurvy (Beaton et al. 1960). On the contrary, Hsu (1969) found low amounts of circulating PBI and thyroxine in scorbutic guinea pigs and suggested that scurvy either induces hypothyroidism or enhanced deiodination, or both and that vitamin C by its antioxidant properties can regulate the rate of deiodination of thyroxine.

Vitamin C plays an important role in blood formation: (1) it increases the absorption of iron from the intestinal tract (Conrad and

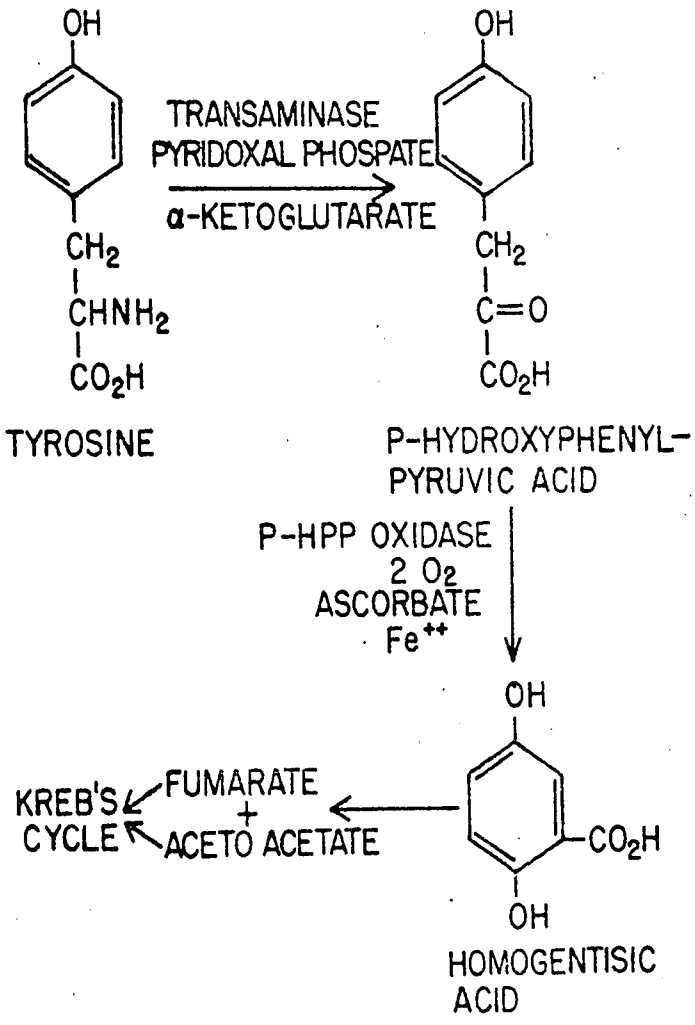


Figure 3. Tyrosine metabolism.

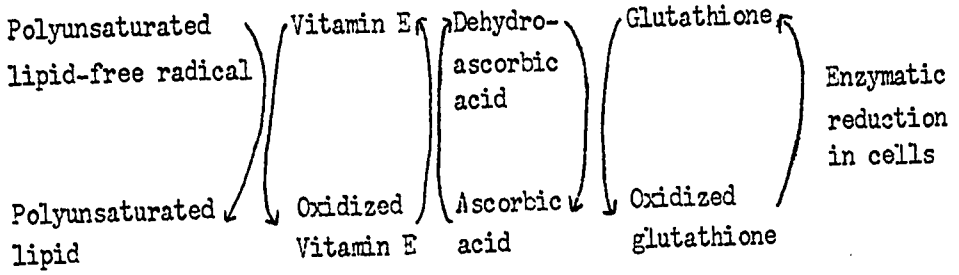
Schade 1968); (2) it increases the incorporation of plasma iron from transferrin (a protein-iron complex) into liver tissue ferritin by reducing the ferric iron of transferrin in presence of NAD and NADP (Mazur 1961); (3) it stimulates the incorporation of iron into hemoglobin (Goldbert 1963) and enhances hemoglobin synthesis (Greenbert et al. 1957).

L-ascorbic acid could enhance hemoglobin biosynthesis indirectly by its effect on the conversion of folic acid to folinic acid (Gabuzda et al. 1952). Folinic acid acts as a coenzyme for the one carbon transfer necessary for the formation of purine and pyrimidines and is thus involved in the biosynthesis of DNA, RNA and proteins (Battesti and Thuiller 1968).

The antioxidant properties of L-ascorbic acid seem to protect several vitamins, including vitamins A and E, thiamine and riboflavin (King 1967). Kendler and Perek (1968) have demonstrated that L-ascorbic acid increased the concentration of liver vitamin A in chicks receiving sufficient amounts of vitamin A but enhanced the depletion of vitamin A stores of chicks fed a diet devoid of vitamin A.

Tappel (1968) suggested that polyunsaturated lipids will more readily form free radicals, intermediates and semistable peroxides, if there is a lack of antioxidant in relation to polyunsaturated lipids. Since biological membranes are very labile to the action of peroxides and free radicals, damage to subcellular organelles might be expected; the mitochondria and endoplasmic reticulum would be most vulnerable because they are rich in polyunsaturated lipids; lysosomes could be

damaged in the same way and release powerful hydrolytic enzymes. According to Tappel, (1968) the role of vitamin C in the inhibition of lipid peroxidation could be as shown in the following scheme:



The decreased food intake of guinea pigs deficient in vitamin C was shown to decrease the mitochrondrial DNAase I (Neutral) content of such animals (Hitier and Terroine 1968). Since DNAase I is known to break DNA phosphate bonds of single and double helical chains, Hitier and Terroine (1968) speculated that a decreased resistance to viral infection could occur during scurvy. On the other hand, an increase in DNAase II (acid) of guinea pigs was caused by scurvy directly and not by the reduction in food intake (Hitier 1968); DNAase II which attacks 2 by 2 the phosphate bonds of double helical chains of DNA is readily released by labile lysosomes during scurvy; the released DNAase II could explain the serious involution of the testicles accompanied by the inhibition of spermatogenesis which occurs during scurvy; the release of free DNAase would also contribute to the development of muscular atrophy.

Vitamin C and diseases

Because vitamin C is involved in many biochemical functions, one would expect a higher incidence of some diseases in persons having low vitamin C levels.

Eposito and Valentini (1968) reported that the mean leukocyte ascorbic acid content of patients with gastrointestinal disorders was about half that of normal controls; although this deficiency could be caused by inadequate absorption, increased utilization could concomitate or even play the pathogenic role. Ressell et al. (1968) reported that the leucocyte vitamin C levels of sixty patients with gastrointestinal haemorrhages were significantly lower than those of a matched uncomplicated peptic-ulcer control group and much lower than those of a healthy control group; since no evidence of malabsorption or of increased utilization was found in these patients, poor intakes of vitamin C could have caused these lower levels.

Cheraskin et al. (1968 A,B) found a statistically significant positive correlation between a lingual vitamin C test and tooth mobility; they also found a statistically negative correlation between gingival health and plasma ascorbic acid levels, and a positive correlation between the lingual vitamin C test and gingival state (i.e. the poorer the vitamin C state, the poorer the gingival health).

Bodanski et al. (1951) found that the vitamin C levels of 43 patients with noncancerous diseases and 69 with cancerous diseases were significantly lower than those of 23 healthly persons.

According to reports by Schlegel and Pipkin (1968), recurrence of cancer of the bladder can be prevented by massive doses of vitamin C.

In atheroscelerosis, the earliest demonstrable lesion is the ground substance of the arterial intima which shows metachromasia and lipid deposits. Such lesions have been demonstrated in the arteries of vitamin C deficient guinea pigs and the intraperitoneal administration of vitamin C has prevented the development of atherosclerosis induced by cholestrol

feeding of guinea pigs (Willis, 1953). In 1955 Willis and Fishman reported that a localized depletion of ascorbic acid often existed in segments of human arteries susceptible to atherosclerosis for reasons of mechanical stress. Zaitsev et al. (1964) studied the effect of L-ascorbic supplementation on the development of experimental atherosclerosis with rabbits and found that although L-ascorbic acid did not change total blood cholesterol, it reduced cholesterol deposition in the aorta at the expense of increased deposition in the liver. Sokoloff et al. (1967) investigated the effectiveness of long term heavy dosage with L-ascorbic acid on the prevention of symptoms of induced atherosclerosis in rabbits and rats, and as a therapeutic agent in clinical atherosclerosis. Ascorbic acid treated animals had significantly lower cholesterol and triglycerides and increased lipoprotein lipase levels, and cholesterol fed rabbits receiving extra vitamin C had much less pronounced atheroma-like lesions in the vascular system. Cardiac patients responded to ascorbic therapy by an increase in lipoprotein lipase and a decrease in triglycerides levels although the changes in cholesterol levels were not very marked nor uniform. Cheraskin et al. (1968) found that non fasting serum cholesterol were significantly higher, the poorer was the vitamin C state of subjects as tested by non-fasting lingual vitamin C scores.

Coronary heart disease which is a complication of athrosclerosis (Yudkin 1969) could be adversely affected by hypovitaminosis. A study by Patterson (1941) consisted in the determination of plasma ascorbic acid in 455 consecutive admissions (adults): 56 per cent of all the patients had ascorbic acid levels below 0.5 mg. while 81 per cent of patients with coronary heart diseases were below this level.

Vitamin C absorption and metabolism

Hughes and Lewis (1965) reported that the bulk of vitamin C absorption by the guinea pig, rat and hamster occurred in the stomach, and reached a maximum in 10 minutes. However, the evidence on which this conclusion was reached was not given (Hughes and Lewis 1965). Studies done by Chinn (1938) and reported by Farmer et al. (1940), indicated that in fasting guinea pigs and rats about half of vitamin C administered by stomach tube was still present in the gastrointestinal content 6 hours after injection. In vitro studies, by Stevenson and Brush (1969) with segments of the gastrointestinal tract of guinea pigs fasted for 3 days after 4 days of depletion, indicated that the small intestine had the greatest absorption activity. Studies by Reid (1948) demonstrated that about 15 per cent of L-ascorbic acid administered intraperitoneally was secreted in the gastrointestinal tract of guinea pigs maintained on a diet devoid of the vitamin; since maximum secretion occurred about 4 hours after injection and only relatively small amounts of vitamin C were found within the caecum and large intestine in spite of the rapid transit of the contents of the stomach and small intestine, there appeared to be considerable destruction of vitamin C in the caecum.

Stewart and Booth (1964) have studied the absorption of ascorbic acid by humans; in normal subjects previously saturated with vitamin C, it took one hour before any ascorbic acid appeared in the urine following the oral administration of 11 mg. of the vitamin per kg body weight, whereas about one third of the dose was excreted in the urine following intravenous administration; maximum excretion occurred 4 hours after oral administration and there was also a one hour delay in excretion of

an additional dose given three hours after the first dose. This indicates that the delay was not due to the time required to saturate the renal tubules with ascorbic acid and to overcome the renal threshold. The liver was not likely to cause the delay in excretion since two patients with portacaval shunts did not excrete vitamin C at a faster rate. The delay was apparently not due to the time required for the gastrointestinal content to reach an absorbing area in the distal intestine since two patients with only relatively short lengths of proximal intestine remaining had an excretion pattern similar to normal persons. The results thus indicated that transport of L-ascorbic acid across the intestinal mucosa was causing the delayed urinary excretion. The efficiency of vitamin C absorption is quite high since 60 to 80 per cent of 100 mg daily intake are recovered in the urine of subjects who are practically saturated with vitamin C (Lowry 1952).

Other aspects of vitamin C metabolism have been reviewed previously (Part II).

Means of evaluating vitamin C status

The interelationship of dietary, serum, white blood cells and total body ascorbic acid has been summarized by Lowry (1952). With daily intakes of over 100 mg ascorbic acid, 60 to 80 per cent will be excreted, serum levels will be about 1 mg and tissues and white blood cells (WBC) will be saturated. With daily intakes varying from 40 to 100 mg, 20 to 60 per cent will be excreted, serum levels will vary from 0.4 to 1 mg and tissues and WBC will be moderately below ceiling levels. With daily intakes varying from 40 to 5 mg, tissue levels will vary from 50 to 25 per cent of the ceiling; in this range WBC provide an accurate status of the vitamin C status but plasma levels are too low to do so.

Crandon et al. (1940) reported that in the development of experimental scurvy in humans fed a diet deficient in vitamin C, the plasma ascorbic acid levels fell to 0 after 10 weeks of deprivation i.e. 13 weeks before the first evidence of clinical scurvy; since the WBC levels fell to 0 just before the appearance of clinical scurvy, it was concluded that WBC and not the plasma levels were a good index of the vitamin C status. Blood plasma vitamin C is at least a good indicator of the risk of scurvy since low levels occur significantly long before the appearance of scurvy. Although WBC vitamin C levels may be a good index of body stores, such a determination is not simple and different laboratories can apparently obtain quite different results (Griffith 1968); an extreme example was given by a person who visited six laboratories in Scotland and had his WBC ascorbic acid determined in each laboratory and obtained values varying widely from 15 to 54 µg per 10^8 WBC.

Several types of load tests proposed to evaluate vitamin C status have been reviewed by Youmans and Patton (1942) and Krause (1964). Most of these tests are based on the fact that excretion of a test dose does not occur to any significant degree until an individual's reserves of vitamin C have been restored. Unfortunately there is no general agreement about the size of the test dose, the mode of administration (oral, intravenous or subcutaneous), the period of collection (a few hours to several days), and most important of all about the interpretation of results. For these reasons load tests have not gained any popularity.

Derivatives of cigarette smoke

The characteristics of cigarette smoke have been reviewed in a report by the U.S. Public Health Service (1964), and by Wynder and Hoffman (1967). The effluent smoke from an average cigarette is about 500 mg. The percentage of the major constituents are: nitrogen 59; carbon dioxide 14; oxygen 13; dry matter 8; carbon monoxide 3; water 1; argon 1; C2-C6 hydrocarbons 0.5; carbonyls 0.4; methane 0.3; hydrogen cyanide 0.1; and hydrogen 0.1. Of the total materials about 60 per cent is diluting air and 16 per cent excess nitrogen. Hundreds of other identified components include organic radicals (polynuclear aromatic hydrocarbons are the best components known to trap free electrons and form radicals), peroxides (1-2 µg per cigarette) probably formed from certain olefins with molecular oxygen, β-emitting particles (mainly K-40), α- emitting elements (such as radium 226 and 228), pesticides, metallic compounds in the vaporized state, N-nitrosamines, polynuclear aromatic hydrocarbons and their alkyl derivatives. Nicotine is, among the many chemical substances in cigarette smoke, the only one known to produce acute pharmacological effects (Lucchesi et al. 1967). The nicotine content of different cigarettes may vary from 0.2 to 2.6 mg in U.S.A. (Moore and Bock, 1968) and 0.5 to 2.4 mg in Canada (Department of National Health and Welfare, Ottawa, 1968).

Physiological effects of nicotine

The physiological effects of nicotine are apparently what the cigarette smokers are after (Lucchesi et al. 1967). According to reviews by Comroe Jr. (1960, 1964) and a report by the U.S. Department of Health,

Education and Welfare (1964), nicotine can stimulate the chemoreceptors of the aortic and carotid bodies, stimulate special receptors in the pulmonary circulation and epicardium of the left ventricle and cause reflex apnea, bradycardia, systemic vasodilatation and hypotension. Nicotine can act as a central nervous system stimulant in a manner similar to amphetamine and liberate stores of adrenaline from the hypothalamus, mid-brain and medulla (Warwick and Eysenk 1963). At low concentrations, nicotine can stimulate all sympathetic ganglion cells; thus, it can cause the adrenal medulla (which in some way acts as a group of sympathetic ganglion cells) to liberate catecholamines. By its action on cells of the hypothalamus, nicotine can cause the liberation or secretion of the posterior pituitary antidiuretic hormone. Nicotine can also stimulate the secretion of 11-hydroxycorticosteroids by the adrenal cortex (Kershbaum et al. 1968). Nicotine can depress appetite; this has been attributed to (1) the increase in blood sugar due to stimulation of the sympathetic nervous system; (2) stimulation of the hypothalamus; and (3) direct effects on taste buds and mucous secretions. Smoking is also known to provide pleasure and reduce anxiety and tension.

Absorption and metabolism of nicotine

The oral cavity and respiratory tract can readily absorb nicotine (Larson et al. 1961). Absorption of nicotine from the lungs is extremely rapid since its greatest rate of excretion in the urine occurs within 15 minutes after smoking (Beckett and Triggs 1966). Because the amount of nicotine absorbed without inhaling is about one third that

absorbed with inhaling, the customary manner in which cigars and pipe smokers inhale less than cigarette smokers results in their absorbing less nicotine (Kershbaum and Bellet 1968). Nicotine administered intraperitoneally to rats was quite readily absorbed (Yamamoto et al. 1968) in a manner similar to absorption by the lungs in humans. The absorption of nicotine injected subcutaneously to rats was found to be quite rapid, but the efficiency could be decreased by lowering the pH (Travell 1960).

Nicotine-C-14 injected intraperitoneally to rats penetrated the tissues very rapidly (Yamomoto et al. 1968). The following tissue to blood ratios were obtained 10-20 minutes after injection: kidney (9), liver (7), submaxillary gland (6), brain cortex (4), brain stem (2) and heart (2); the blood and submaxillary gland reached their maximum content after 90 minutes while the concentrations were declining in the other organs, and after 4.5 hours the concentrations had decreased to very low levels in all organs.

The excretion of nicotine has been reviewed by Larson, Haag and Silvette (1961) and Larson and Silvette (1968). Noether (1923) has reported to have found nicotine in the urine of guinea pigs within 1.5 hours after subcutaneous injection of the drug; excretion reached a peak in 5-7 hours and negligible levels in 18 hours. Ninety per cent of administered C-14 labelled nicotine was found in the urine of guinea pigs 18 hours after injection (Fishman 1963); dogs excreted all of the administered nicotine within 48 hours and chromatographic autoradiography showed at least 10 compounds. Werle and Meyer (1950) as cited by Larson et al. (1961) have reported that the urinary excretion of nicotine by

rats rose to a maximum 3 hours after intraperitoneal injection with 5-10 mg nicotine per kg of body weight. Noether (1923) as cited by Larson et al. (1961) used a leech-muscle bioassay to determine nicotine in the urine: he found that after a 12 hours abstinence of smoking, heavy smokers were not excreting any nicotine, but that nicotine appeared in the urine 1.5-2 hours after smoking and reached a peak after one additional hour. Using chemical methods, other workers have reported that nicotine disappeared from urine within 3-4 days after smoking was discontinued. An increase in the acidity and rate of flow of urine results in a greater and more uniform excretion of nicotine by humans after smoking (Beckett and Triggs 1966); the greatest rate of excretion of nicotine in the urine occurred within 15 minutes after smoking and excretion of unchanged nicotine was virtually complete in 8 hours; smokers excreted about as much cotinine as nicotine.

Whatever the route of administration of nicotine, intravenous injection (1-2 mg), inhalation (0.1-0.5 mg), smoking (0.05-2.3 mg), the amount of unchanged nicotine was greater in the non-smokers (55-70 per cent) than in the smokers (25-50 per cent); thus, habitual smoking apparently induced enzymatic metabolism of nicotine in a manner similar to that reported for the dog, rabbit and rat after chronic exposure to nicotine (Beckett and Triggs 1967).

As reported by Larson and Silvette (1968), other workers have found that autoradiographic chromatography revealed at least 8 metabolites of nicotine in the urine of mice and cats injected with C^{14} labelled nicotine. Papadopoulos and Kinzios (1963) made in vitro studies of the metabolism of nicotine with rabbit liver supernatant and found 8 major metabolites, 5 of which corresponded to cotinine, nornicotine,

demethyl cotinine, pyridylacetic acid, and nicotine-1-oxide. Hansson et al. (1964) have studied the metabolism of C^{14} labelled nicotine with tissue slices from various organs of the mouse; the brain, spleen, stomach, small intestine and adrenal glands did not metabolize nicotine; products of metabolism of nicotine were identified in the lung (cotinine), in the liver (cotinine, Y-(3-Pyridyl)-Y-oxo-N-methylbutyramide, and hydroxycotine) and the kidneys [cotinine and Y-(3-hydidyl-Y-oxo-N-methylbutyramide)]. McKennis et al. (1964) used the results of a variety of mammalian experiments, both in vitro and in vivo to summarize the metabolic alterations to the pyrrolidine ring of nicotine as shown in Figure 4. With regard to this scheme, Morselli et al. (1967) studied the possibility of chemical reduction of cotinine to nicotine by administering (+) -cotinine-2-C^{14} to rats but found no conversion to nicotine and nornicotine. Although the scheme proposed by McKennis et al. (1964) can be supported by isolation of the products of metabolism, the extent, mechanism and biological significance of these reactions remains to be clarified.

Cigarette smoking and diseases

Numerous studies have shown that death rates and the incidence of several diseases are higher among smokers than non-smokers (U.S. Public Health Service, 1964, 1967, 1968, 1969; Doll and Hill 1964; Department of National Health and Welfare, Ottawa (1966). In general, for men between the ages of 35 and 60, about one-third of all deaths of cigarette smokers are excess deaths that would not have occurred as early if the smokers had the same death rates as non-smokers. On the basis of statistical studies, smokers of 20-39 cigarettes between the age of 25 and 45 can expect to die six years earlier than non-smokers; those smoking at 50 years can expect to loose 5 years and those at 65, 3 years of their life.

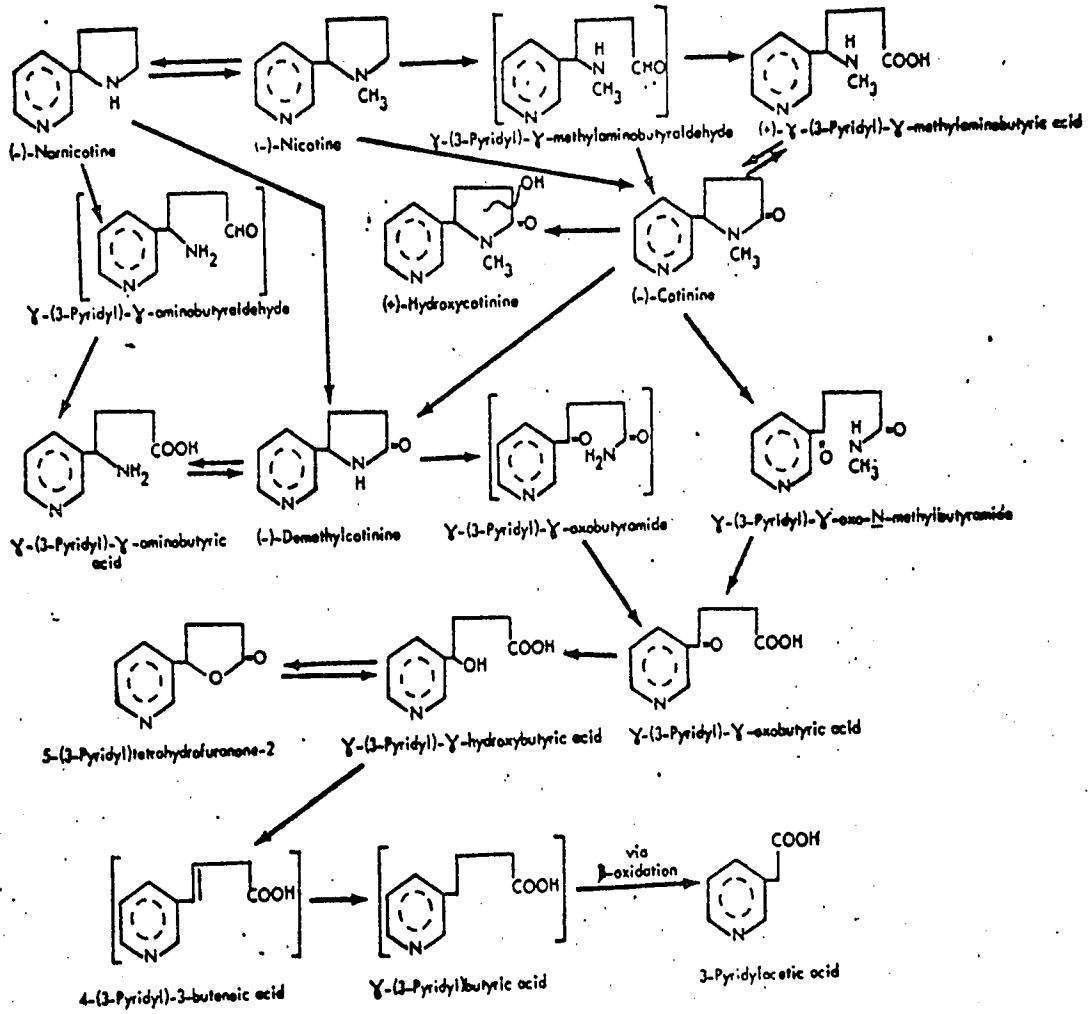


Figure 4. Schematic representation of some routes in the mammalian metabolism of (-) nicotine. (From McKennis et al. 1964, reproduced by permission of copyright owners.)

For the last two decades, coronary heart disease in the United States has been the leading cause of death; during 1967 it was responsible for 1,833,900 deaths. Death rates from coronary heart disease increase with age; these rates per thousand are 2 between 45-54 years, 6 between 55-64, 14 between 65-74, 30 between 75-85 and 70 for 85 years and older. The death rate of male cigarette smokers dying from cornary heart disease is on the average about 70 per cent higher, and in presence of other known risk factors 200 per cent higher than the death rate of male non-smokers. Female cigarette smokers are less seriously affected. Ex-smokers have a lower risk of dying from coronary heart disease than those who continue smoking cigarettes. Cigarette smoking could favor the fatal outcome from coronary heart disease by triggering an oxygen deficit to a critical degree in the myocardium; this could be caused by mechanisms such as (1) myocardial wall tension and velocity of contraction mediated through norepinephrine release; (2) hypoxia due to impaired pulmonary function, and (3) impaired coronary blood flow as a consequence of increased blood viscosity associated with hyperlipemia. Most coronary occlusions are attributed to thromboses; indeed, a pathological exaggeration of normal interaction between the blood elements and the arterial wall is believed to be responsible at least in fact for the development of atherosclerosis (Murphy and Mustard 1966). Several studies on thombus formation as related to cigarette smoking have been reviewed in the 1968 supplement to the U.S. Public Health Service Review (1967). Catecholamine release induced by nicotine can enhance ATP and ADP induced platelet aggregation. In fact, habitual smokers have a greater tendency to platelet aggregation than non-habitual

smokers (Glynn et al. 1966). Murphy and Mustard (1966) believed that the release of epinephrine due to cigarette smoking could have a direct effect in platelet aggregation through acceleration of glycolysis with release of ADP which is one of the basic factors causing platelet aggregation. A study by Murchison and Fye (1966) has shown that a rise in plasma free fatty acids which followed cigarette smoking was associated with platelet adhesiveness and aggregation. Long chain saturated fatty acids (C14-C22) produced extensive thrombosis and death when given intravenously to dogs (Connor et al. 1963), but long chain highly unsaturated fatty acids had no effect (Connor et al. 1965). Kershbaum and Bellet (1968) have summarized results of their studies demonstrating that cigarette smoking in humans resulted in a rapid and consistent increase in free fatty acids; the greater response obtained by cigarette smokers as compared to pipe and cigar smoking was related to the tendency to inhale cigarette smoke; dogs responded to cigarette smoke and nicotine in the same manner as humans. As pointed out by Kershbaum and Bellet (1968) these aberrations in lipid metabolism are recognized to be contributory factors in the development of atherosclerosis. Another aberration in lipid metabolism which is of interest in relation to cigarette smoking is β-lipoprotein levels. Lipoproteins are huge molecules which serve as transport vehicles to carry fats from one place to another; the α-lipoprotein (high density) contains less lipids than the β-lipoprotein (low density). Olsen (1963) has postulated that low density β-lipoproteins were an agent responsible for the development of atherosclerosis. Because corticosteroids might be directly involved in lipoprotein synthesis (Barrett 1966), Kershbaum et al. (1968)

studied the effect of smoking and nicotine on adrenocortical secretions and found a 27 to 77 per cent rise in plasma 11-hydroxycorticosteroid concentrations after heavy cigarette smoking compared to a normal diurnal fall during a non-smoking control period; furthermore, nicotine administered intravenously to anesthetized dogs resulted in a 64 per cent rise in plasma corticosteroids. However, according to Larson and Silvette (1968), there is no evidence indicating that cigarette smoking has a direct effect on β/α lipoprotein fraction.

Lung cancer mortality rate of males has increased from 2 per 10,000 in 1950 to 4 per 10,000 in 1965, while the mortality rate for females increased from 4 per 100,000 to 6 per 100,000. In general death rates from lung cancer increases with age: it becomes about 2 per 1,000 between 55-84 years. Although there is a very strong association between cigarette smoking and lung cancer, this type of cancer contributes to about only 16 per cent (Brownlee 1965) of excess deaths associated with cigarette smoking, while the coronary heart diseases contribute to about 45 per cent. Cancers of other organs (oral cavity, larynx, oesophagus and bladder) are also associated to a greater extent with cigarette smokers than non-smokers, and contribute to about 10 per cent of excess deaths associated with cigarette smoking (Larson and Silvette 1968). Passive inhalation of cigarette smoke has failed to produce lung cancer; voluntary inhalation of cigarette smoke by dogs produced hyperplastic and metaplastic changes in their lungs, but it was found difficult to keep the dog alive long enough to obtain neoplastic transformation (U.S. Public Health Service Review, 1967, and 1968 supplement).

Tobacco smoke condensate (the matter collected in cold traps in conditions simulating human smoking habits) is also called "tar": the amount of tar depends on the type of cigarette and may vary from 4-48 mg per cigarette in U.S.A. (Moore and Bock 1968) or 8-36 mg per cigarette (Department of National Health and Welfare, Canada, 1968). Tobacco smoke condensate is tumorigenic to a variety of animal tissues and species (Wynder and Hoffman 1967): since tobacco smoke condensate is a complete carcinogen (it can induce carcinomas and sarcomas) it must have tumor-initiating as well as tumor-promoting components. The disruption of regularity in cellular life caused by carcinogens is presumably initiated by alterations of chromosomes or more specifically of DNA; tumor promotion could be mediated by mitochondria destruction which would adversely affect cellular respiration (Wilk and Wynder 1967). The initiators consist mainly of polynuclear aromatic hydrocarbons such as dimethyl- a -anthracene, and tumor-promoting agents comprises phenols and phenols derivatives. O-amino-phenols implanted into mouse bladder are carcinogenic. Two of these phenols (3-hydroxyanthranilic acid and 3-hydroxykynurenine) which are normal intermediate metabolites of tryptophan, are about 50 per cent higher in the urine of smokers than in the urine of non-smokers and are accompanied by a decrease in the excretion of N^1-methylnicotinamide (Kerr et al. 1965); the mechanism by which N^1-methylnicotinamide excretion is decreased in proportion to the increase in o-aminophenols was not elucidated. Results reported by Tompsett (1959) indicated that subjects with cancer of the bladder excreted more 3-hydroxyanthranilic acid and o-aminophenols than normal subjects.

Death rates from emphysema of the lungs and chronic bronchitis in U.S. have increased steadily from 4 per 100,000 in 1956, to 14 per 100,000 in 1966; quite a lot of data has been accumulated which clearly relate cigarette smoking to emphysema of the lungs and chronic bronchitis (U.S. Public Health Service review (1967) and 1968 supplement; the mortality risk for cigarette smokers varies between 3-20 times that of non-smokers. Pulmonary fibrosis and emphysema similar to those in human being were found in 5 dogs who smoked daily through a tracheotomy tube for more than 420 days; no such parenchymal changes were found in control dogs (Auerbach et al. 1967).

The 1969 supplement to the U.S. Public Health Service review (1967) reports studies showing that women who smoke during pregnancy have greater risk of spontaneous abortion, stillbirth or deaths of the baby early in infancy; there is also evidence that babies born to smoking mothers are more likely to weigh less than those born to non-smokers.

Solomon et al. (1968) concluded from a 10 year study of about 9,500 patients that cigarette smokers have definitely a higher prevalence of periodontal disease in general (gingivitis, alveolar resorption and tooth loss) than non-smokers.

Cigarette smoking and vitamin C

Some of the various diseases that are associated with cigarette smoking are also associated with lower vitamin C levels. If cigarette smoking caused a significant hypovitaminosis C, this could be an important factor in the development of some of the diseases associated with cigarette smoking. Indeed some reports in the literature indicate that cigarette smoking was associated with lower vitamin C levels.

Strauss and Scheer (1939) using 2,6-dichloroindophenol for titrating L-ascorbic acid studied the acute effect of smoking 1-3 cigarettes while fasting on the urinary excretion of L-ascorbic acid following a 200 mg dose given to subjects (presumably non-smokers) previously saturated with vitamin C and concluded that a reduction in L-ascorbic acid excretion was obtained; on the contrary Högler (1943) reported that acute tests (smoking 3 cigarettes in succession on an empty stomach) had no effect on blood vitamin C levels. Venulet and his associates in Poland, during the period 1950-1959, published several papers on the effect of smoking on ascorbic acid levels (Venulet 1953, 1954; Larson et al. 1961; and Andrzejenski 1966): an acute test showed that non-smokers who volunteered to smoke about 7 cigarettes per day had a significant decrease in serum ascorbic acid by the third day, and a return to normal levels 5 days after cessation of smoking. Dietrich and Büchner (1960) reported that the average plasma vitamin C of 16 non-smokers decreased by 16 per cent after each person smoked 30 cigarettes during 3 days; 9 of the subjects participating in the experiment complained of the typical acute effects of cigarette smoke: headache, lack of appetite, a feeling of weakness and sickness, heart palpitation, increased salivation, stomach pain and nausea. Because of these side effects, acute smoking tests gave results of a limited value and these results can hardly be extrapolated to chronic smoking.

Harmsen (1941) studied the blood ascorbic acid levels of the personnel of three hospitals and reported that 9 heavy smokers had abnormally low blood ascorbic acid levels, but, as pointed out by Larson et al. (1961), he did not indicate if there were other heavy smokers with normal

levels. Högler (1943) reported that a study of over 100 smokers and non-smokers showed no difference in the vitamin C blood levels of the two groups; this study however was restricted to persons with normal gastrointestinal functions and, since Högler stated that gastrointestinal disturbances were often present in smokers, one can only guess how many smokers were excluded from the study. According to Venulet (1953, 1954), the plasma ascorbic acid levels in a group of 60 medical students varied from 0.6 to 0.9 mg per cent in smokers and from 1 to 1.2 mg in non-smokers. Toporkova (1959) as cited by Larson and Silvetti (1968) reported that smokers excreted less ascorbic acid than non-smokers. In the study by Dietrich and Buchner (1960), the following average values were obtained for ascorbic acid in blood plasma: 0.9 mg per cent (55 smokers) and 1.32 mg per cent (20 non-smokers); the ascorbic acid excreted in the urine during one day was 5.5 mg (smokers) and 7.2 mg (non-smokers). Calder, et al. (1963) published the following results: the average plasma vitamin C content was 0.91 mg per cent for 91 non-smokers, 0.73 mg per cent for 83 smokers of 14 cigarettes or less, and 0.52 mg per cent for 31 smokers of 15 cigarettes or more; in the same order, the quantity of vitamin C in µg per 10^8 leucocytes was 29.1, 24.8 and 21.7. They stated that an approximate assessment of food habits did not indicate that these were causing the differences in vitamin C levels. Taber and Larson (1962) reported that a group of 45 smokers and 45 non-smokers excreted in the urine amounts of ascorbic acid not significantly different after receiving a 500 mg loading dose of L-ascorbic acid. This contrasts with the results obtained by Rupniewska (1965). After intravenous loading

with 500 mg L-ascorbic acid, the mean urinary excretion of ascorbic acid by elderly men was 32.7 mg per cent for 14 smokers and 56.4 mg per cent for 12 non-smokers. Rupniewska (1964) previously found amazingly low mean values for ascorbic acid in the plasma of young soldiers: 0.16 mg per cent for 22 smokers and 0.24 mg per cent for 18 non-smokers, and 4 hours after a saturation test 0.36 mg per cent and 0.45 mg per cent in the same order. In another study published by Durand and his associates (1962), an abnormally high range (4.8 - 8.4 mg per cent) for ascorbic acid in plasma from humans was reported. The average plasma ascorbic acid level of 40 smokers was about half that of 37 non-smokers, and even after loading with 1 g L-ascorbic acid daily for 25 days the plasma ascorbic acid levels of the smokers never reached those of the non-smokers. The most spectacular reports on this subject are those of McCormick (1952), and Goyanna (1955). The first stated, without providing evidence, that he had determined by laboratory and clinical tests that one cigarette neutralized about 25 mg of vitamin C, while the second found from a study of 500 subjects that people smoking more than 20 cigarettes per day had no detectable vitamin C in their urine. A study by Brooke and Grimshaw (1968) was published in the same issue of the same journal in which some of the results included in this thesis were published by Pelletier (1968). Brook and Grimshaw were satisfied that the typical English breakfast supplied a negligible amount of vitamin C and used blood taken from non-fasting subjects between 9 and 10 a.m.; they obtained the following mean vitamin C values in plasma (mg per cent) and leucocytes (mg/10^8 cells): for 32 male non-smokers, (0.62 mg and 24.6 mg),

for 22 male cigarette smokers (0.34 mg and 18.8 mg), for 50 female non-smokers (0.74 mg and 26.0 mg); the plasma vitamin C levels appeared to be inversely related to age and the amount of cigarettes smoked but the leucocyte vitamin C levels did not change with age. Brook and Grimshaw (1968) thought as Calder et al. (1963) that it was unlikely that differences in vitamin C intake accounted for the differences in blood levels between smokers and non-smokers.

Effect of nicotine on vitamin C in vitro

Reif (1943) tested the effect of nicotine on a solution of ascorbic acid in pure distilled water and found that the oxidation of ascorbic acid was minimal even after prolonged reaction time; addition of small amounts of copper in presence of nicotine resulted in a faster oxidation than with copper alone, and the oxidation increased with the alkalinity of the medium; large quantities of nicotine in presence of copper destroyed the vitamin C completely. Bourquin and Musmanno (1953) added an amount of nicotine estimated to be equal to the amount present in the blood of a heavy smoker and reported that in 3 experiments 24.4 to 31.6 per cent of the vitamin C had been lost after 5 to 10 minutes. Goyanna (1955) reported that the macerate of one cigarette oxidized 2 mg of L-ascorbic acid. Calder et al. (1963) reported that tobacco smoke destroyed vitamin C in solution and that an equivalent amount of air or smoke from cigarette paper were without effect. Taber and Larson (1962) had also reported that incubation of aqueous solutions at physiological pH resulted in pronounced destruction of ascorbic acid by nicotine, whereas incubations with added nicotine resulted in little or no destruction of ascorbic acid in either dog or human whole blood. Taber and

Larson (1964) further demonstrated that ascorbic acid destruction was due to pH effects (alkalinity), and that whole blood, due to its buffering activity, protected ascorbic acid from oxidation. Wenzel and Beckloff (1958) found no change in the serum ascorbic acid content of rabbits given 2-8 mg nicotine per kg body weight daily in the drinking water. Maren (1951) reported that treatment of rats with nicotine (2 mg per kg body weight) decreased the ascorbic acid content of adrenals by 20 per cent, but Taber and Larson (1964) found that treatment of rats for 30 days with nicotine (12.5 mg per kg body weight) added to the diet produced no effect on the urinary ascorbic acid excretion or the ascorbic acid content of the whole body.

Rats, mice, rabbits and frogs like most animals synthesize their vitamin C requirement, but guinea pigs like man are not able to synthesize their own vitamin C and must depend on exogenous sources (Chaudhuri and Chatterjee 1969).

Yun and Kim (1938) injected female guinea pigs daily with about 1 mg nicotine per kg body weight for periods ranging from 15 to 190 days and noted an increase in vitamin C (granules in the adrenals) which was proportional to the days of administration. As cited by Frommel et al. (1945), Seisoku (1939) found hyper-vitaminosis C in the adrenals of guinea pigs injected during 130 days with nicotine. Frommel et al. (1945) reported that the acute effect of injecting 20 mg nicotine to guinea pigs resulted in a marked increase in the ascorbic acid content of the adrenals, spleen, and more modest increase in other organs. On the contrary, Taber and Larson (1964) showed that a group of female guinea pigs receiving every second day 15 mg ascorbic acid per kg body

weight orally or subcutaneously and receiving during 12 days 10 mg nicotine per kg body weight in their drinking water excreted urinary ascorbic acid in a quantity not different from a control group receiving no nicotine.

Effect of cigarette smoke and nicotine on the vitamin C status of animals

Venulet and his associates in Poland reported experiments with animals on the effect of cigarette smoke and nicotine on vitamin C (Venulet 1953, 1954; Larson et al. 1961; and Andrzejenski, 1966). Exposure of mice to cigarette smoke for 20 minutes a day resulted in an increased urinary excretion of ascorbic acid during the first day, but a decrease to half the normal excretion in one week and to 1/10 to 1/20 in 4 months; there was a considerable decrease in the ascorbic acid content of the adrenals after exposure to tobacco smoke for 5-10 minutes daily for 20 days. The blood levels of frogs exposed to tobacco smoke first increased due to the mobilization of ascorbic acid and then decreased after two weeks presumably due to increased catabolism: the greatest loss occurred in the adrenals, spleen, myocardium and lung. Dansys and Skupinski (1955) reported that exposure of mice to cigarette paper smoke first caused an increase in the excretion of urinary vitamin C after 3 days followed by a 50 per cent decrease lasting 10 days and a return to normal levels afterwards; nicotine injection into the dorsal lymph sac of the frog during 12 days failed to show a significant effect on the ascorbic acid content of liver, heart, brain, stomach and adrenals. Thus cigarette paper per se or nicotine did not produce the same effect as cigarette smoke. Experiments with rabbits (Lupu et al. 1954) showed that exposure of rabbits to cigarette smoke also resulted in a very

rapid decrease in adrenal ascorbic acid (secretory response) with a return to normal within 24 hours (Wenzel and Beckloff 1958).

Lupu et al. (1954) found that after acute exposure of guinea pigs to cigarette smoke, the adrenals' ascorbic acid content decreased by 40 per cent in one hour, 60 per cent in one day and returned to normal in two days. Evans et al. (1967) reported that a group of 15 male guinea pigs dosed orally with 2 mg L-ascorbic acid per kg body weight and exposed to cigarette smoke for two 10 minutes periods daily during 28 days, had vitamin C levels (22 mg per cent) in the adrenals significantly lower than a control group (31.1 mg per cent) not exposed to cigarette smoke; however, the two groups had comparable levels of vitamin C in the testis and liver.

The scope and approach to the problems in relation to vitamin C and cigarette smoking

Although the studies discussed above indicated that cigarette smokers had a lower vitamin C status than non-smokers, there was a need to confirm this by well controlled studies and to investigate the reasons for these differences.

In most of these studies only L-ascorbic acid was determined and dehydroascorbic acid, which has full vitamin C activity, was not included. It is well known that if necessary precautions are not taken, L-ascorbic acid can be easily oxidized on standing and that this oxidation can be accelerated by the presence of Cu^{++} and nicotine (at alkaline pH). As discussed in Part I, methods that determine L-ascorbic acid on the basis of the decolorization of 2,6-dichloroindophenol dye could introduce a positive error due to reducing substances such as cysteine and glutathione and to

other reducing substances generated in trying to reduce dehydroascorbic acid before titration. Most methods used to determine total vitamin C with 2,4-dinitrophenylhydrazine on the basis of osazone formation are also subject to interference from sugars and diketogulonic acid (the first product of oxidation of dehydroascorbic acid).

Before studying the vitamin C status of cigarette smokers and non-smokers, it was decided to develop a specific practical analytical method (presented in Part I).

The first study, with humans was limited to 5 smokers and 5 non-smokers, and attempted to find evidence for or against the indications of lower vitamin C levels of cigarette smokers and to investigate the possibility of a different metabolism of vitamin C by cigarette smokers; for this purpose blood and plasma vitamin C were measured before and during a depletion period following saturation with vitamin C, and urinary excretion of load doses was also measured.

The second study with humans involved 14 smokers and 14 non-smokers with comparable characteristics and dietary habits. The purpose was not only to confirm the results of the preliminary study indicating lower vitamin C levels in smokers as compared to non-smokers, but also to rule out the possibility that different intakes were causing the difference in levels and to evaluate the magnitude of the effect. For this purpose the habitual vitamin C intakes were correlated with the initial blood levels and with the urinary excretion of load tests; the urinary excretion of vitamin C from normal doses after saturation was also measured in order to determine if smokers utilized the same intakes in the same way as the non-smokers. The value of the load test to evaluate the

vitamin C status of humans was estimated from the relationship of blood levels and per cent urinary excretion of a 1.1 g dose during 7 hours.

A third study was made with a different group of 5 smokers and 5 non-smokers in order to compare the urinary excretion of L-ascorbic acid and D-isoascorbic acid which has only 5 per cent the vitamin C activity of L-ascorbic acid.

Finally the effect of nicctine and cigarette smoke condensate on the vitamin C metabolism of the guinea pig was studied for the purpose of elucidating the mechanism involved in the development of the lower vitamin C status of cigarette smokers. In order to interpret the results obtained, it was necessary to perform experiments to determine the mode of absorption of vitamin C given orally to guinea pigs.

MATERIALS AND METHODS

A. Evaluation of the vitamin C status of cigarette smokers and non-smokers

Experiment 1

Ten healthy male subjects (five smokers and five non-smokers), who were members of the Food and Drug Directorate in Ottawa, participated in this study. The smokers were smoking from 15 to 50 cigarettes per day with an average of about 30 cigarettes (Table I). The non-smokers were only slightly older than the smokers. The weights of the smokers were in general higher, but the effect of this factor could be circumvented by expressing the data per kg body weight. One of the five smokers consumed considerably more alcohol than any of the other subjects and was responsible for the greater average alcohol consumption in his group. The smokers consumed significantly more caffeine mainly in the form of tea or coffee. In the non-smoker group, subject J consumed more caffeine, than his partners and exclusively from "cola" drinks. The previous daily vitamin C intake of the 10 subjects was evaluated from their declared weekly consumption of foods and according to Heinz Nutritional Data (1960). Most of the smokers consumed about 60 mg of vitamin C per day except subject B, who consumed about 136 mg. The average for this group was 76 mg as compared to 87 mg for the non-smokers who showed more variation in their daily intake (43-164 mg).

The plan of the experiment is summarized in Table II. The object of the experiment was to determine if there was any significant difference between smokers and non-smokers with regard to vitamin C:

(a) in the levels of blood, plasma, and 24-hr urine specimens prior to saturation (day 0);

TABLE I

Charácteristics of five smokers and five non-smokers

Subjects	Age	Wt (kg)	Cigarettes/day	Alcohol** /month	Caffeine† /day	Vitamin C (mg)/day per kg body wt
Smokers						
A	26	69.8	25-30 (FT)*	2 (B)	4.5	0.86
B	38	63.4	20-25 (R)	30 (B)	7.5	2.14
C	30	90.6	15-20 (R)	2 (B + A)	5.0	0.74
D	21	63.4	25 (FT)	15 (B» A)	5.0	0.90
E	31	74.8	50 (FT)	3 (A)	7.5	0.82
MEAN	29	72.4	27-30	10	6.0	1.09
Non-smokers						
F	37	70.7	Nil	3 (A)	2.5	1.29
G	29	97.4	"	6 (A)	0.5	0.63
H	39	88.3	"	7 (B + A)	2.5	0.88
I	35	88.3	"	4 (B)	3.0	1.86
J	28	92.9	"	8 (B» A)	4.0	0.46
MEAN	34	87.3	Nil	6	2.5	1.02

* FT = filter tip. R = regular.
** B = refers to 1 pint of beer and A to alcohol equivalent to 1 pint of beer.
† = Caffeine is expressed in terms of cups of tea or coffee.

TABLE II

Vitamin C intake and determinations during experiment

	Days	Intake (g/day) *	Days of determinations	
			Blood & Plasma	24 hr Urine+
Presaturation	0	Nil	0	0
Saturation	1 - 5	2	-	1 - 5
Desaturation	6 - 18	Nil	8,11,18	6,11,18
Resaturation	19 - 23	2	-	19 - 23

* Doses were divided as follows: 250 mg at 4 and 7.30 PM, 550 mg at 10 PM, 250 mg at 7:30, 10 and 12 AM and at 2: 30 PM.

\+ From 4 PM.

(b) in the retention and maximal excretion in subjects while receiving 2 g of vitamin C for 5 days during the saturation period;

(c) in the rate of loss in urine, blood, and plasma during the desaturation period;

(d) and in the retention during resaturation with daily doses of 2 g of vitamin C for 5 days.

During the whole length of the experiment, the subjects were on a non-supervised, restricted diet. All agreed to abstain from alcoholic beverages during the whole period since there are claims that it can interfere with the saturation by vitamin C (Durand et al. 1962). The daily consumption of a medium-sized potato was permitted only during the saturation periods, i.e. days 1-5 and 19-23. During the remainder of the test, including day 0, potatoes were replaced by rice. For the whole period, no fruits or vegetables were permitted. Preserved meats such as bologna, weiners, and vacuum packed meats were not allowed since preliminary tests indicated that they contained either L-ascorbic acid or D-isoascorbic acid. Meats such as liver, kidney, or brain were not permitted. No other restrictions were placed on the subjects. Urine was collected from 4 PM (exclusive) on one day to 4 PM (inclusive) on the next day. The urine was collected in glass bottles containing metaphosphoric acid pellets in sufficient quantities so that, after dilution to 2 liters, its concentration was 4 per cent. Instructions were given to keep the samples cool (about 10°C) at all times. Complete 24-hr samples were kept frozen in polyethylene bottles until assayed (usually within a few days). Blood specimens were collected at 9-9:30 AM with heparin added as an anticoagulant and were immediately cooled and assayed on the

same day by the 2,4-dinitrophenylhydrazine procedure described in Part I-E. Blood and plasma assays were made before and after saturation, but were not carried out during saturation tests since they would have merely reflected the vitamin intake.

Experiment 2

A memorandum for recruiting healthy subject was sent to all personnel of the Food and Drug Directorate in Ottawa; the protocol of the experiment and the required restrictions were explained. Subjects who had taken medicine during the preceding six months were not retained; matching was done with regard to age, weight, sex, dietary and drinking habits. Twenty-eight persons were selected from about 34.

During this experiment, restrictions consisted of avoiding fruits, juices, fruit drinks, instant breakfasts, alcoholic beverates, aspirins and laxatives. Controlled lunches and dinners consisted of T.V. dinners plus bread and cakes. Each person kept a complete record of all foods and drinks consumed during the experiment, the design of which is shown in Table III.

The subjects ate a T.V. dinner on the eve before the blood samples were taken at 8: 30 AM before breakfast. Vitamin C was determined on the day of sampling as described for Experiment 1.

On the next day at 8:30 AM, the subject were given 1.1 g vitamin C and a fairly high dosage of water soluble vitamins. They were told to drink at least one glass of water every two hours. The per cent of the dose excreted in the urine until 4:40 PM was measured. The same dosage was given at 4:30 PM and the urine collected until 8:30 AM the next day. These vitamin doses were continued for 5 days without dietary restrictions

TABLE III

Experimental design

Day	Diet*	Vitamin C (given)	Vitamin C (measured)
(-) 1	1 T.V. (PM)	Dietary	Nil
0	1 T.V. (PM)	Fasting	Blood (mg %)
1	2 T.V.	2 x 1.1 g	% Load excreted
2 to 6	Regular	2 x 1.1 g	(Saturation)
7 to 8	Regular	Dietary	(Desaturation)
9 to 11	2 T.V.	Dietary	(Desaturation)
12	2 T.V.	Fasting	Blood (mg %)
12 to 14	2 T.V.	2 x (57-95 mg)	% Dose excreted
15	2 T.V.	2 x 1.1 g	% Dose excreted
15 - 1	-	-	% Load retained

* No foods or drinks containing vitamin C allowed after T.V. dinners.

in order to saturate the individuals. Restrictions were resumed during the last 3 of the 5 days of desaturation, and until the end of the experiment. After desaturation, blood vitamin C was determined, and the excretion of vitamin C intakes ranging from 58 to 95 mg given twice a day was measured for three days. The two 95 mg intakes included other water soluble vitamins. Finally, two loading doses were given in order to estimate the vitamin C retained from the previous loads.

The average daily vitamin C intakes were based on all the information supplied by the subjects and were evaluated by using food tables from Heinz Nutritional Data (1960), amounts declared on labels of foods and typical determinations of some vegetables such as boiled, mashed, or fried potatoes and potato chips.

Experiment 3

This experiment consisted of comparing the urinary excretion of L-ascorbic acid and D-isoascorbic acid by five smokers and five non-smokers before and after saturation with vitamin C in order to determine the differences in the urinary excretion of these isomers. The design of this experiment was described in Part II-C.

B. Effect of nicotine and cigarette smoke condensate on the vitamin C metabolism of guinea pigs

Experiment 1

The mode of absorption of L-ascorbic acid given orally to guinea pigs was studied as follows. Eight male guinea pigs were given a rabbit ration for two days in order to have a negligible vitamin C intake. At the beginning of the test, the guinea pigs were weighing an average of

350 g. One animal (control) was bled to death under anesthesia, and its blood was kept for analysis. The gastrointestinal tract was removed and the gastrointestinal content was placed into a beaker containing 10 ml of 50 per cent metaphosphoric acid. The interior of the gastrointestinal tract was washed well with distilled water and the washings were added to the same beaker. The content of the beaker was diluted to 250 ml, mixed well, filtered and frozen until analyzed on the next day, after a 1:4 dilution with 2 per cent metaphosphoric acid. The stomach, small intestine, and large intestine were weighed and separately extracted by blending with 25 ml of cold 4 per cent metaphosphoric acid, and diluting to 50 ml with distilled water. The liver, heart, kidneys and adrenals were extracted with cold 4 per cent metaphosphoric acid. The frozen extracts were assayed on the next day as described in Part II-A for the determination of vitamin C and the counting of C^{14}. The other guinea pigs were given orally 0.2 ml of an aqueous solution containing 8 mg L-ascorbic acid and 2.78 μc L-ascorbic acid-1-C^{14} (New England Nuclear Corp.). The other animals were killed one at a time at the following intervals after being given L-ascorbic acid: 15, 45 and 90 min, and 3, 6, 9 and 12 hrs. The analyses of the blood, gastrointestinal tract and content were as described above for the control.

Experiment 2

This preliminary experiment was designed to test the effect of nicotine (one of the main components of tobacco), given orally with the diet for 1 month. The control group (4 guinea pigs) received ground guinea pig pellets to which 1 per cent corn oil was added, and the experimental group (4 guinea pigs) received the same diet which contained 3.88 mg nicotine per 100 g added with corn oil.

Experiment 3

Thirty-six guinea pigs, which weighed 360 g after having been fed for one week with a synthetic diet (described in Part II-A) containing 0.5 per cent vitamin C, were divided into two groups of equivalent weights. Both groups were fed the synthetic diet without vitamin C for 3 weeks. Twice a day (9 AM and 5 PM), the controlled group received subcutaneous injections of 0.9 per cent sodium chloride (0.2 ml), while the experimental group received the same injections containing 0.2 mg nicotine. At the end of the experiment, one double injection was given to 12 animals in each group followed by oral administration of 0.2 ml of an aqueous solution containing 5 mg L-ascorbic acid plus 3.4 μc L-ascorbic acid-1-C^{14}. Each injected guinea pig was immediately placed in a glass metabolic cage as described by MacKenzie et al. (1959) in order to permit collection of $C^{14}O_2$ and urine.

At the top of each cage, one glass tube projecting from a rubber stopper provided an air intake, while another tube was used to draw air from the cage by means of a suction apparatus. The air drawn from the cage was passed through 300 ml of an ethanolanine and ethylene glycol monoethylether 2:1 mixture (Teffay and Alvarez 1961). This efficiently removed and retained CO_2 from the air.

At periods of 1.5, 3, 6, 9, 12 and 24 hours, duplicate 1 ml samples were removed from each flask containing the ethanolanine and ethylene-glycol monoethylether mixture and were added to glass vials containing 15 ml of 2:1 combination of liquid scintillation mixture and ethanol (Hayes and Gould, 1953). The scintillation mixture contained 0.5 per cent 2,5-diphenyloxazole (PPO), and 0.01 per cent 1,4-bis-2-(5-phenyl-

oxazolyl)-benzene (POPOP) dissolved in ethylene glycol monomethyl ether and toluene (1:2) as described by Cuppy et al. (1963).

At the bottom of each cage, a connecting apparatus equipped with a stop cock leading to a flask containing 0.5 g of metaphosphoric acid was used to collect the urine. Twelve hours after L-ascorbic acid was administered, the flask containing the urine was disconnected and replaced by a similar flask for the remainder of the 24 hr period. In each case, the urine was usually diluted to 25 ml and filtered. Duplicate 0.2 ml samples of diluted urine were placed in 15 ml of the scintillation mixture described by Cuppy et al. (1963).

The CO_2 and urine samples were counted in a Nuclear Chicago Liquid Scintillation Counter model number 8401, system 703. The gate was set at 1150 and data at 1050. The per cent efficiency of known amounts (dpm) of toluene-C^{14} plotted against the Channels ratio 'Base to 4.1' over 'Base to 9.9' was used to obtain the efficiency of the samples. Normally, the efficiency was about 50 per cent for counting $C^{14}O_2$ and about 40 per cent for C^{14} from the urine. All radiotracers measurements of the Channel 'Base to 9.9' were made to have an accuracy equivalent to 1 per cent standard deviation.

The C^{14} content of the blood and organs of guinea pigs were determined as described in Part II-A.

Experiment 4

Because of the possibility that the scorbutic state of guinea pigs might have masked the effect of nicotine on the utilization of a dose of L-ascorbic acid, another study was made on the effect of injections of

nicotine and cigarette smoke condensate (supplied by Dr. F.G. Bock, Rosswell Park Memorial Institute, Buffalo, N.Y.) on the utilization of L-ascorbic acid by guinea pigs injected intramuscularly or administered orally with L-ascorbic acid. The guinea pigs used in this study were fed the synthetic diet containing no vitamin C, and received intramuscular injections of 5 mg L-ascorbic acid every second day for a minimum of 8 days before testing the effect of nicotine and cigarette smoke condensate. At the beginning of the test, the guinea pigs weighed an average of about 250 g. Because there were only six glass metabolic cages available for measuring $C^{14}O_2$, the experiment was planned to handle 6 guinea pigs (A-F) at a time after two weeks of the following treatment: A, B and C received daily 2 mg L-ascorbic acid orally while D, E and F received the same dose by intramuscular injection. During one week, the guinea pigs received subcutaneous injections as follows: 0.2 ml alcohol (A and D), 0.2 ml containing 0.66 mg nicotine (B and E), and 0.2 ml containing 11 mg of cigarette smoke condensate equivalent to 0.66 mg nicotine (C and F); during the following week the dose was given twice per day, the first dose in the morning as in the first week, and the second dose 3 hours later. On the last day of treatment, a set of animals (A to F) received 3 μc L-ascorbic acid-1-C^{14} plus amounts of L-ascorbic acid varying from 2 to 8 mg per set. The animals were immediately placed in individual metabolic cages described above for Experiment 3. The $C^{14}O_2$ was measured 1.5, 3, 6, 9 and 12 hrs after administering L-ascorbic acid, and the animals were then killed. Analyses of the urine, blood and organs were performed as described in the previous experiment.

RESULTS

A. Evaluation of the vitamin C status of cigarette smokers and non-smokers

Experiment 1

As shown in Table IV, all the smokers had a low vitamin C level in plasma and blood (0.29 and 0.30 mg per 100 ml, respectively), compared to the non-smokers (0.66 and 0.73 mg per 100 ml). Three days after the saturating doses of vitamin C were discontinued, the plasma and blood vitamin C levels of both smokers and non-smokers had reached about the same values (0.9 mg per 100 ml for plasma, and 1 mg per 100 ml for blood). The vitamin C content of plasma and blood of both groups also decreased to the same extent, 6 and 13 days after the discontinuance of vitamin C. After 13 days, the smokers were above their initial values, while the non-smokers were below theirs. Within about 10 days, there was a 50 per cent decrease in the vitamin C content of blood and plasma of both groups. It was not possible to determine dehydroascorbic acid in whole blood because ascorbic acid was oxidized to dehydroascorbic acid during acid extraction (Kellie and Zilva 1936). Plasma dehydroascorbic acid was determined and yielded mean values of about 0.1 mg per 100 ml in both the smokers and the non-smokers before or after saturation. Because of the lower vitamin C content of the smokers before saturation, the proportion of dehydroascorbic acid was 20 per cent compared to 12 per cent in the non-smokers.

Data of the daily urinary excretion of vitamin C are summarized in Figure 5. Prior to saturation (day 0) and on the second and third day after discontinuing L-ascorbic acid (days 7-8), the smokers excreted only about half the vitamin C excreted by non-smokers. The maximum difference in the excretion of vitamin C occurred during the first day of saturation

TABLE IV

Vitamin C* in blood (Bl) and plasma (Pl)
of 5 smokers and 5 non-smokers

	Smokers	Non-smokers	P
	Presaturation		
Bl	0.30 ± 0.09	0.66 ± 0.21	<0.01
Pl	0.29 ± 0.08	0.72 ± 0.29	<0.05
	Three days after saturation		
Bl	0.92 ± 0.17	0.88 ± 0.08	NS
Pl	1.04 ± 0.15	0.98 ± 0.12	NS
	Six days after saturation		
Bl	0.69 ± 0.16	0.67 ± 0.07	NS
Pl	0.80 ± 0.16	0.84 ± 0.15	NS
	Thirteen days after saturation		
Bl	0.46 ± 0.04	0.44 ± 0.07	NS
Pl	0.46 ± 0.07	0.47 ± 0.10	NS

* Vitamin C and S.D. are in mg per 100 ml.

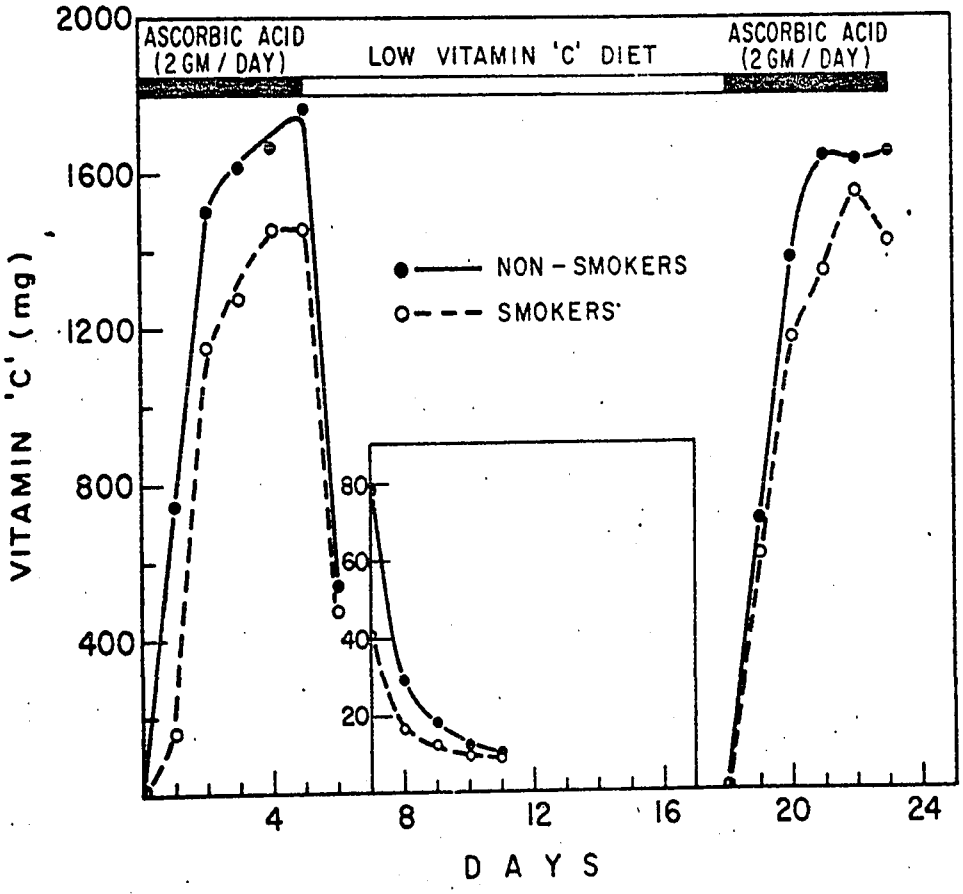


Figure 5. Urinary excretion of vitamin C by smokers and non-smokers.

with 2 g of L-ascorbic acid. As shown in Table V, the smokers excreted only 167 mg, i.e. about 23 per cent of the 740 mg excreted by the non-smokers. Such a difference was not found during the first day of the resaturation period (day 19) when the smokers excreted 610 mg, i.e. about 85 per cent of the 700 mg excreted by the non-smokers. On the second day of the first saturation period, the excretion of vitamin C by the smokers rose to about 75 per cent that of the non-smokers and from there gradually to above 85 per cent. The mean saturation excretion calculated from the last two days of saturation (days 4 and 5) and resaturation (days 21 and 23) was 1,468 mg for the smokers and 1,679 mg for the non-smokers, a difference of 211 mg or 13 per cent. As shown in Figure 6, on days 7 and 8 (the second and third day after discontinuing L-ascorbic acid doses), the percentage excretion by the smokers dropped to about 50 per cent that of the non-smokers. During the next three days (day 9-11), the percentage excretion of the smokers increased linearly to 90 per cent of that of the non-smokers.

The relative retention was estimated in a manner similar to that described by Lowry et al. (1946) by subtracting the daily excretion of the first 3 days of saturation and resaturation from the mean saturation excretion, and the results are given in Table VI. During the first period, the smokers retained 1,794 mg and the non-smokers retained 1,164 mg, thus giving a difference of 630 mg. In the second period, after being 13 days on a restricted diet containing practically no vitamin C, the smokers retained about the same amount as the non-smokers (1,264 mg vs. 1,312 mg), but 530 mg less than in the first period, while the non-smokers had retained 148 mg more.

TABLE V

Daily urinary-excretion of vitamin C* on an intake of 2 g of L-ascorbic acid

Intake	Day	Smokers	Non-smokers	Difference	P
		Initial saturation period			
2 Gm	1	167 ± 124	740 ± 259	573	<0.01
"	2	1151 ± 105	1514 ± 287	363	<0.05
"	3	1276 ± 115	1617 ± 146	341	<0.01
"	4	1454 ± 124	1667 ± 210	213	NS
"	5	1455 ± 98	1771 ± 132	316	<0.01
Nil	6	467 ± 70	535 ± 217	68	NS
		Resaturation after 13 days on deficient diet			
2 Gm	19	610 ± 178	700 ± 61	90	NS
"	20	1171 ± 237	1383 ± 144	212	NS
"	21	1342 ± 269	1640 ± 200	298	NS
"	22	1525 ± 156	1627 ± 165	102	NS
"	23	1417 ± 302	1649 ± 125	232	NS
	Mean-S+	1463 ± 141	1679 ± 131	246	<0.05

* Vitamin C and S.D. are expressed in mg per day.

+ Mean-S = days $\frac{4 + 5 + 22 + 23}{4}$.

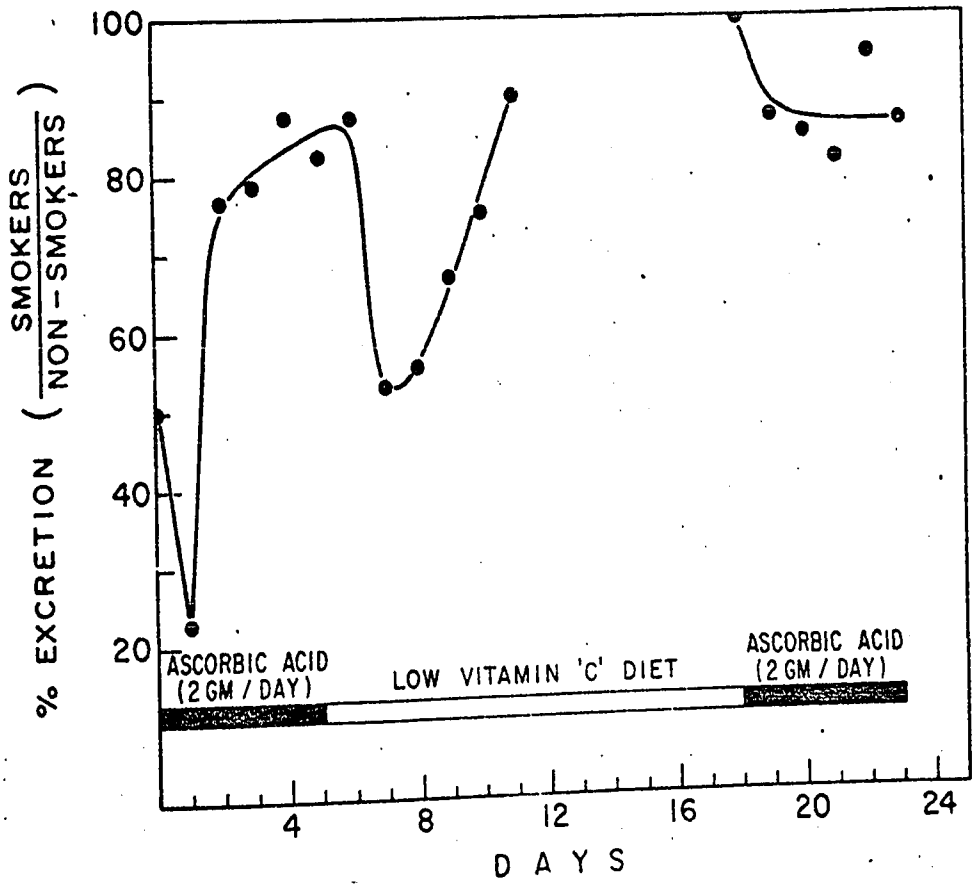


Figure 6. Percentage urinary excretion of vitamin C by smokers in comparison to non-smokers.

TABLE VI

Relative retention* of vitamin C+ by smokers and non-smokers during saturation (A) and resaturation (B) periods of 5 days

	Smokers	Non-smokers	Difference	P
A	1794 ± 463	1164 ± 614	630	NS
B	1264 ± 324	1312 ± 280	(-) 48	NS
B - A	530 ± 542	148 ± 365	678	<0.05

* Relative Retention = (3 x mean saturation excretion per day) - excretion on the first 3 days on 2 g of vitamin C per day; where the mean saturation excretion is derived from the last 2 days on 2 g of vitamin C.

\+ Vitamin C and S.D. are expressed in mg.

Values for absolute retention given in Table VII are more exact than those for relative retention because they include continued vitamin C excretion of the saturating doses during the 4 days after discontinuing these doses. The vitamin C excreted during the first 9 days of the first saturation period corrected for blanks (day 0), was subtracted from the total possible excretion (also corrected for blanks), during 5 days by subjects already saturated by a continuous intake of 2 g per day. The mean absolute retention in mg of vitamin C per kg body weight was 18.45 for smokers and 5.2 for the non-smokers. Subject J (non-smoker) had a high retention of 19.46 mg, which was in accord with his low vitamin C levels in blood and plasma. The retention by the other non-smokers varied from 0.46 to 3.85 mg, while the range in all the smokers was from 8.91 to 27.7 mg. Subject B (smoker) who had the highest vitamin C intake of the subjects in his group, retained less vitamin C than they did, but he retained much more than four of the five non-smokers. Since absolute retention per se comprises both intake and smoking effects, the intake was dissociated by multiplying the absolute retention by the individual intake and dividing by the mean intake of all subjects. This corrected retention, although somewhat arbitrary, represents the smoking effect more accurately. The mean values of corrected retention (17.36 mg per kg body weight for the smokers and 3.25 for the non-smokers) were statistically different ($P < 0.25$).

While on a restricted diet, the daily excretion of dehydroascorbic acid in both groups varied from about 2 to 4 mg (10-30 per cent of the total vitamin C content), but while receiving vitamin C doses, it varied from 20 to 95 mg (1.5-6 per cent of the total vitamin C). The sample

TABLE VII

Absolute retention* of vitamin C (mg/kg body wt) in smokers and non-smokers

Subject	Absolute Retention	Absolute retention + X Intake factor
	Smokers	
A	19.28	15.64
B	10.67	21.54
C	8.91	6.22
D	27.70	23.52
E	25.69	19.87
Mean	18.45	17.36
S.D.	8.52	6.87
	Non-smokers	
F	1.74	2.12
G	3.85	2.29
H	0.50	0.42
I	0.46	0.81
J	19.46	8.44
Mean	5.20	2.82
S.D.	3.62	3.25
P	$<.10$	$<.025$

* Absolute retention = $\frac{\text{(excretion days 1 to 9) - (5X mean saturation excretion)}}{\text{Kg body wt}}$

+ Intake factor = (Intake individual subject)/(Mean intake of subjects A-J)

blanks of all subjects when read against standard blanks were giving absorbancies too low to yield any significant amount of diketogulonic acid.

Experiment 2

Characteristics of the two groups are summarized in Table VIII. There was no significant difference with regard to age, weight, consumption of tea, coffee, alcohol and vitamin C. The higher "colas" intake of the smokers' group was due to two individuals taking about 8 "cokes" per day.

As shown in Table IX, before the load tests, vitamin C blood levels of the smokers were significantly lower than those of the non-smokers by about 30 per cent. The smokers excreted significantly less of the first vitamin C load than the non-smokers, indicating a greater retention. The second load produced no difference. After saturation and desaturation, vitamin C blood levels were higher and about the same for both groups. The amounts of vitamin C excreted from the load doses were also higher than on the first day and there was no significant difference between groups. According to the difference in excretion between the 8:30 AM loads at the beginning and end of the experiment, the smokers retained significantly more vitamin C. They also retained more from the 4:30 PM load, but the difference was not statistically significant because there were several individual variations. By that time, the restrictions and T.V. dinners were a burden. One smoker resigned on the last day of the experiment and his non-smoker partner was excluded so that the values for the excretion on the last day for the retention were calculated for 13 subjects per group.

TABLE VIII

Characteristics of 14 smokers and 14 non-smokers

	Smokers		Non-smokers	
	Mean ± S.E.	Range	Mean ± S.E.	Range
Age (years)	33 ± 3	18 - 50	35 ± 3	18 - 49
Wt. (lbs.)	158 ± 7	104 - 197	158 ± 6	130 - 190
Cigarettes/day	31 ± 4	8 - 68	-	-
Years (cig.)	12 ± 3	3 - 34	-	-
Tea & Coffee*/day	3.0 ± 0.7	1 - 8	2.5 ± 0.5	1 - 6.5
Colas*/day	0.7 ± 0.7	0 - 8	0.4 ± 0.2	0 - 2
Alcohol+/month	15.3 ± 6.5	0 - 75	13.0 ± 5.3	0 - 60
Vit. C (mg/day)	93 ± 13	30 - 177	89 ± 11	35 - 182

* Cups.

+ Average drinks.

TABLE IX

Vitamin C in blood and excretion of 1.1 g doses (± S.E.)

	Day	Smokers	Non-smokers	P
Blood (mg %)	0*	0.42 ± 0.06	0.60 ± 0.05	<0.05
Excreted (%)	1+	10.0 ± 2.5	20.1 ± 3.5	<0.05
Excreted (%)	1**	24.4 ± 3.6	26.5 ± 4.4	NS
Blood (mg %)	12*	0.88 ± 0.05	0.81 ± 0.04	NS
Excreted (%)	15+	25.6 ± 2.5	27.6 ± 2.5	NS
Excreted (%)	15**	41.7 + 4.4	35.4 ± 3.4	NS
Retained (%)	15+ - 1	14.9 ± 3.6	5.9 ± 2.1	<0.05
Retained (%)	15* - 1	14.5 ± 4.1	6.9 ± 4.3	NS

* Fasting; +8:30 AM dose; **4:30 PM dose.

In order to ascertain that the initial lower vitamin C levels of the smokers were not due to the difference in the intakes, regression straight lines fitting of the relationship between intakes, initial blood levels and excretion of the first load were done. Figure 7 shows the best straight line fits, from the least square point of view, of the intakes as related to blood level. The slopes are obviously different. The correlation coefficient was significant for the non-smokers but not for the smokers.

The women (3 smokers, 3 non-smokers) did not appear to have a vitamin C pattern different from the men. There was no apparent relationship between the blood levels and the number of cigarettes smoked or the number of years of smoking. Consuming large quantities of cokes showed no effect on the blood and intake relationship. Three volunteers who had quit cigarettes 3-6 months earlier had blood and intake relationships in line with the other non-smokers.

As shown in Figure 8, the relationship between vitamin C intake and per cent excretion of the first load test showed about the same pattern for the difference between the two groups and the same correlation coefficient as was obtained for blood levels.

Figure 9 shows a good relationship between blood levels of all the subjects and excretion of the first load test. The correlation coefficient 0.73 was highly significant. A regression straight line analysis showed no difference between groups.

The urinary excretion of thiamine and riboflavin loads (15 and 10 mg respectively) indicated no difference between groups. Although the smokers excreted less pyridoxic acid from 5 mg of vitamin B_6, the differences were not statistically significant.

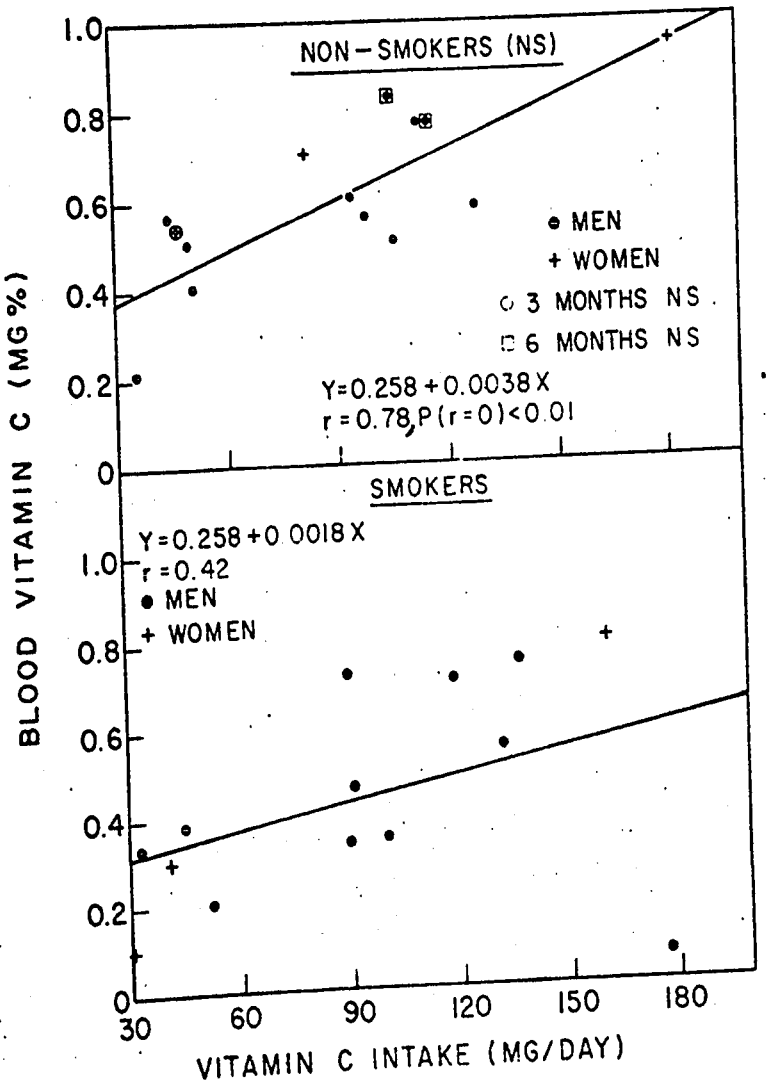


Figure 7. The relationship between blood vitamin C (mg %) and intake of vitamin C (mg per day) of smokers and non-smokers.

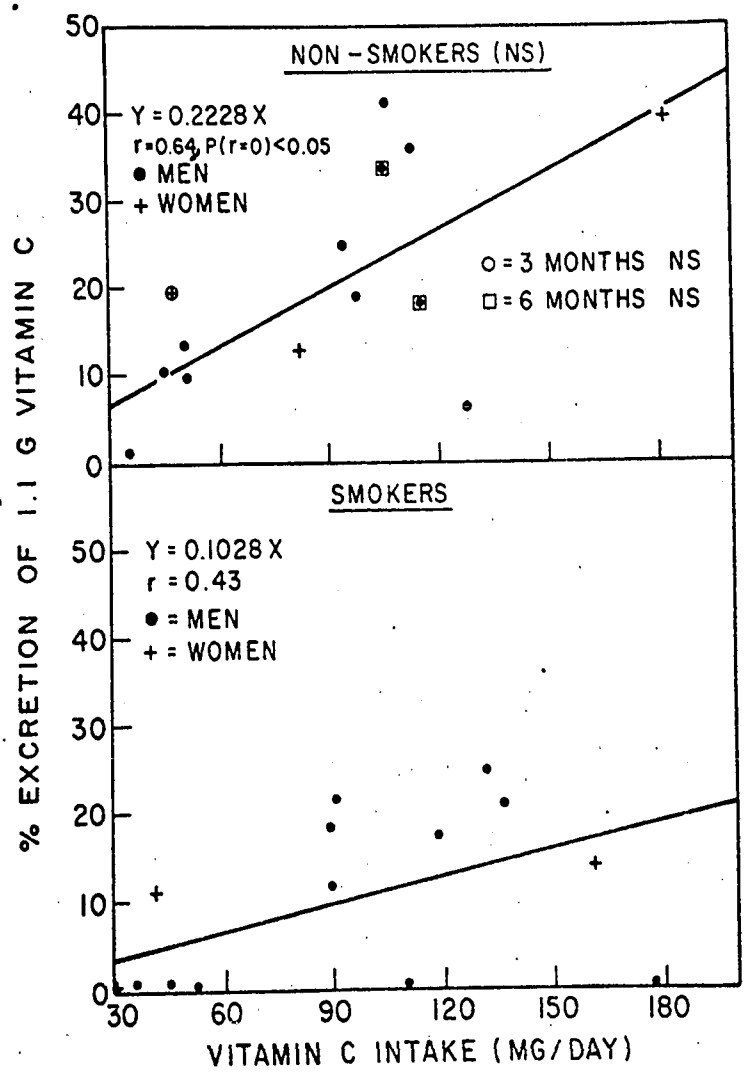


Figure 8. The relationship between per cent excretion of 1.1g vitamin C and intake of vitamin C (mg per day) of smokers and non-smokers.

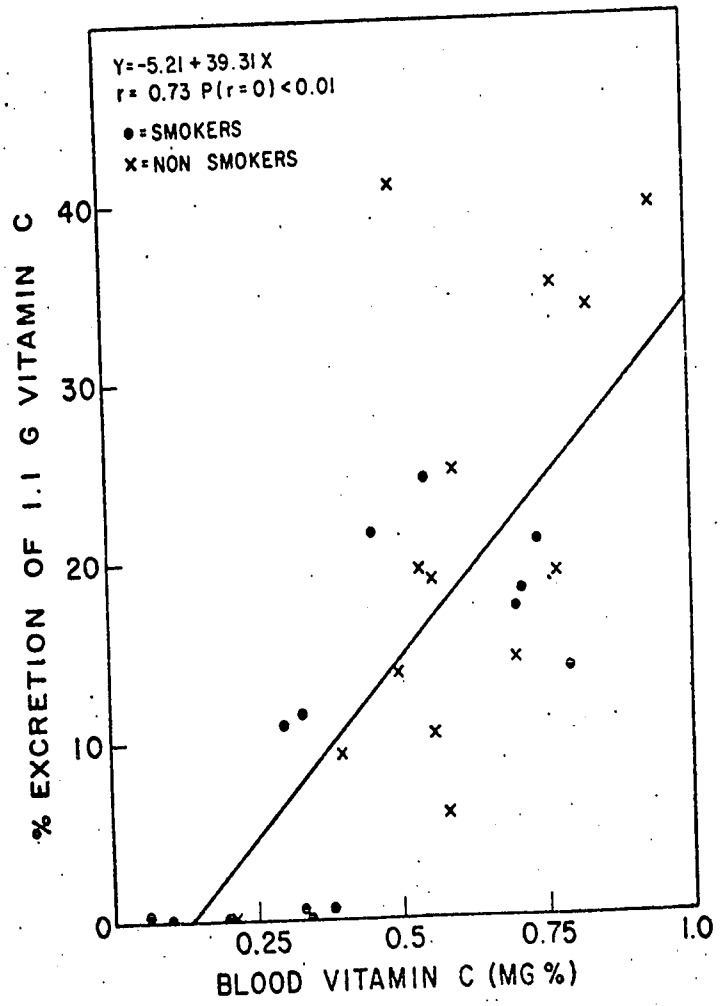


Figure 9. The relationship between per cent excretion of 1.1g vitamin C and blood vitamin C (mg %).

Table X shows the percentage excretion of 58 to 95 mg doses of vitamin C given twice a day after the desaturation period. The excretion of the first dose was low for both groups but from all the other doses the smokers excreted about 40 per cent less vitamin C than the non-smokers.

Experiment 3

The results of this experiment were reported in Part II-C. It was shown that cigarette smokers excreted less L-ascorbic acid and D-isoascorbic acid than non-smokers after receiving 75 mg doses of these compounds.

B. Effect of nicotine and cigarette smoke condensate on the vitamin C metabolism of guinea pigs

Experiment 1

As shown in Figure 10, about 80 per cent of L-ascorbic acid given orally to a guinea pig was recovered in the gastrointestinal content 15 minutes after being administered; at the same time, about 60 per cent of C^{14} from administered L-ascorbic acid-1-C^{14} was found in the gastrointestinal content. Forty-five minutes after oral administration only about 33 per cent of the L-ascorbic acid dose was recovered in the gastrointestinal content while the percentage of C^{14} was twice that amount. The percentage of L-ascorbic acid decreased to less than 4 per cent after $1\frac{1}{2}$ hrs, but the percentage of C^{14} did not decrease below 65 per cent until 3 hours after administration of L-ascorbic acid-1-C^{14}. Six hours after administration, the percentage of C^{14} had decreased to about 7 per cent.

TABLE X

Per cent excretion (± S.E.) of vitamin C intake after saturation

Intake	Smokers	Non-smokers	P
58 mg*	11.4 ± 1.6	14.4 ± 2.8	NS
61 mg+	17.9 ± 2.8	27.8 ± 3.4	<0.05
95 mg*	12.3 ± 2.1	25.6 ± 3.4	<0.005
95 mg+	25.6 ± 4.2	39.4 ± 2.7	<0.01
62 mg*	28.3 ± 4.3	43.1 ± 5.1	<0.05
61 mg+	38.2 ± 5.2	66.0 ± 7.6	<0.005

* 8:30 AM + 4:30 PM

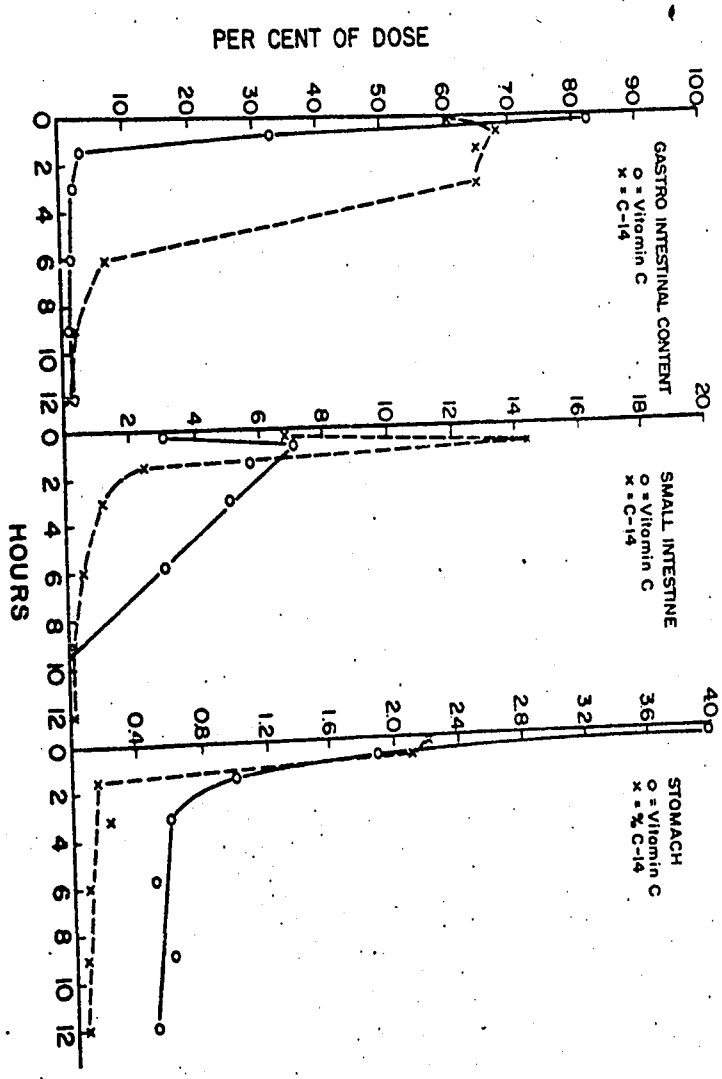


Figure 10. Per cent recovery of L-ascorbic acid and C^{14} after oral administration of 8 mg L-ascorbic acid and 2.78 μc L-ascorbic acid to guinea pigs.

In the tissues of the small intestine the maximal uptake of L-ascorbic acid (7 per cent) and of C^{14} (14 per cent) occurred 45 minutes after oral administration. Thereafter, the loss of C^{14} was more rapid than that of L-ascorbic acid.

In the tissues of the stomach the uptake of L-ascorbic acid (about 4 per cent) and C^{14} (about 2.2 per cent) was maximal 15 minutes after oral administration. Both L-ascorbic acid and C^{14} decreased rapidly thereafter.

As shown in Figure 11, about 2.6 per cent of the C^{14} (from the L-ascorbic acid-1-C^{14}) was found in the tissues of the large intestine after 45 minutes, and this amount decreased constantly to 0.4 per cent after 6 hours.

The uptake of C^{14} by the liver rose gradually to about 1 per cent of the dose after 6 hours. In the blood, assuming a total volume of 25 ml, C^{14} rose gradually to 0.4 per cent of the dose after $1\frac{1}{2}$ hours and then decreased to about 0.04 per cent after 12 hours. The plot of C^{14} in the kidneys was similar to that of the blood, but rose only to about 0.2 per cent of the dose. The maximal uptake of C^{14} by the heart, spleen, or adrenals was about 0.02 per cent of the dose and occurred after 3 hours.

Experiment 2

As shown in Table XI, results of this preliminary experiment indicated that the vitamin C levels of blood and several organs were lower in a group of 4 guinea pigs receiving nicotine in their diet than in a control group receiving no nicotine.

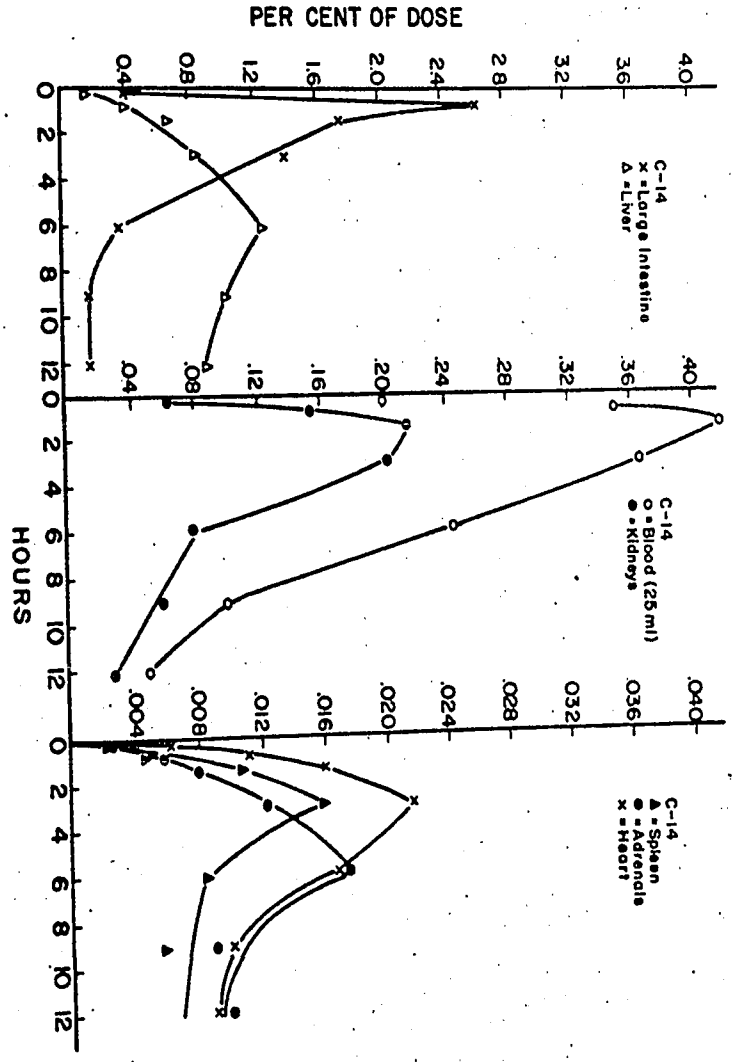


Figure 11. Per cent recovery of C^{14} after oral administration of 8 mg L-ascorbic acid plus 2.78 μc L-ascorbic acid-1-C^{14} to guinea pigs.

TABLE XI

Effect of nicotine* treatment for 28 days on vitamin C in organs†

Organs	With nicotine	Control
Liver	3.7 ± 1.0	5.6 ± 0.6
Brain	10.7 ± 1.7	12.9 ± 1.7
Spleen	15.2 ± 2.6	19.3 ± 2.3
Adrenal	15.3 ± 9.2	30.2 ± 5.6
Heart	1.0 ± 0.1	1.9 ± 0.1
Kidney	1.6 ± 0.1	3.2 ± 0.3
Blood	0.21± 0.006	0.26 ± 0.03

* 3.88 mg dissolved in corn oil per 100 g of ground guinea pigs pellets.

† Mean values for 4 guinea pigs and S.E. are in mg %.

Experiment 3

Guinea pigs, which had received daily subcutaneous injections of 0.4 mg nicotine while being fed a diet deficient in vitamin C during 3 weeks, lost weight in a manner similar to the control guinea pigs receiving no nicotine. The organs' weights as shown in Table XII were also similar in both groups, except the spleen which was larger in the nicotine-treated animals. The percentage of C^{14} remaining in the organs of guinea pigs one day after oral administration of 5 mg L-ascorbic acid containing 3.4 μc L-ascorbic acid-1-C^{14}, was very small and was not significantly different for both groups. On the other hand, as shown in Table XIII, from 70 to 80 per cent of the C^{14} was expired as $C^{14}O_2$ during 24 hours, but during that period no significant difference between the two groups could be observed. About 10 per cent of the C^{14} from L-ascorbic acid-1-C^{14} was found in the urine of guinea pigs in twenty-four hours, but there was again no significant difference between the two groups.

Experiment 4

It is interesting to note in Table XIV that a two week treatment of guinea pigs, with 2 mg L-ascorbic acid given orally, resulted in vitamin C levels similar to those obtained by intramuscular injection of the vitamin. There was, however, a higher incorporation of C^{14} in the spleen, adrenals, brain and heart of guinea pigs twelve hours after intramuscular injection of L-ascorbic acid-1-C^{14} than after oral administration.

Neither nicotine nor cigarette smoke condensate administered subcutaneously had an effect on the total vitamin C content of guinea pigs' organs. Table XV shows the effect of nicotine and cigarette smoke

TABLE XII

Effect of nicotine treatment* on L-ascorbic acid-1-C^{14}** (expressed as per cent of dose ± S.E.) in the organs of guinea pigs depleted of vitamin C for 3 weeks

Organ	Control		Nicotine	
	wt.(g)	% dose x 10^2	wt.(g)	% dose x 10^2
Liver	11.4 ± 0.5	11.6 ± 0.8	12.4 ± 0.6	14.6 ± 1.0
Kidneys	3.3 ± 0.1	6.4 ± 1.5	3.3 ± 0.1	5.1 ± 0.9
Spleen	0.6 ± 0.1	1.6 ± 0.2	1.0+ ± 0.1	2.5 ± 0.4
Adrenals	0.3 ± 0.1	1.7 ± 0.1	0.3 ± 0.1	2.1 ± 0.2
Brain	3.3 ± 0.1	2.1 ± 0.2	3.3 ± 0.1	2.2 ± 0.2
Heart	1.1 ± 0.1	0.8 ± 0.1	1.1 ± 0.1	0.9 ± 0.1

* 0.2 ml (1 mg nicotine per ml 0.96 per cent NaCl) given daily intraperitoneally to 12 guinea pigs at 9 AM and 5 PM for 3 weeks: 12 guinea pigs (controls) received 0.9 per cent NaCl only.

** 5 mg L-ascorbic acid containing 3.4 µc L-ascorbic acid-1-C^{14} in 0.4 ml H_2O given orally.

\+ Significantly different from control ($P < 0.05$).

TABLE XIII

Effect of nicotine treatment* on C^{14} excreted as CO_2, or in urine (expressed as per cent of dose ± S.E.) after oral administration of 5 mg L-ascorbic acid (3.4 μc L-ascorbic acid-1-C^{14}) to guinea pigs previously depleted for 3 weeks.

Material	Hours	Control	Nicotine
CO_2	1½	1.7 ± 0.4	1.7 ± 0.3
"	3	10.2 ± 2.3	7.6 ± 1.1
"	6	29.2 ± 4.4	33.3 ± 4.4
"	9	47.8 ± 5.1	57.8 ± 4.3
"	12	57.7 ± 4.7	67.3 ± 3.9
"	24	80.0 ± 2.7	70.2 ± 5.1
Urine	12	7.9 ± 1.2	5.9 ± 0.6
"	24	11.7 ± 2.0	9.2 ± 1.0

* 0.2 ml (1 mg nicotine per ml 0.9 per cent NaCl) given daily intraperitoneally to 12 guinea pigs at 9 AM and 5 PM for 3 weeks: 12 guinea pigs (controls) received 0.9 per cent NaCl only.

TABLE XIV

Vitamin C in the organs of guinea pigs after two weeks treatment with 2 mg L-ascorbic acid orally (o) or intramuscularly (i) and 12 hours after administration of C^{14} labelled vitamin C*

Organ		Weight (g)	Vitamin C (mg %)	% dose x 10^2 (per organ)
Liver	o	12.8 ± 0.6	9.6 ± 0.9	39.0 ± 8.2
	i	11.7 ± 0.9	9.6 ± 1.0	44.8 ± 3.2
Kidneys	o	3.3 ± 0.1	3.8 ± 0.3	11.6 ± 2.3
	i	3.3 ± 0.1	4.3 ± 0.2	13.3 ± 0.7
Spleen	o	1.1 ± 0.1	15.0 ± 1.7	4.0 ± 0.6
	i	0.9 ± 0.2	18.8 ± 1.8	9.5 ± 0.6
Adrenals	o	0.28 ± 0.01	25.4 ± 3.1	3.9 ± 0.7
	i	0.27 ± 0.01	29.8 ± 4.0	10.4 ± 0.8
Brain	o	3.5 ± 0.1	11.3 ± 0.4	3.7 ± 0.4
	i	3.6 ± 0.1	12.1 ± 0.3	6.4 ± 0.4
Heart	o	1.1 ± 0.1	2.3 ± 0.1	1.5 ± 0.2
	i	1.2 ± 0.1	3.3 ± 0.2	3.6 ± 0.2

* All guinea pigs received 3 µc L-ascorbic acid-1-C^{14}; in each group, 3 animals received 2 mg L-ascorbic acid, 2 received 4 mg and 5 received 8 mg.

TABLE XV

Effect of subcutaneous injections of nicotine and cigarette smoke condensate on C^{14} (expressed as per cent of controls content* ± S.E.) in the organs, blood and urine of guinea pigs 12 hours after administration of 2, 4 and 8 mg of C^{14} labelled (3 μc) vitamin C to guinea pigs.

	Nicotine		Cigarette Condensate	
Specimen	C, oral (10)+	C, i.m. (8)+	C, oral (11)+	C, i.m. (8)+
Liver	61** ± 10	142 ± 22	94 ± 18	112 ± 10
Kidneys	70** ± 11	115 ± 8	89 ± 15	116 ± 11
Spleen	74 ± 12	158 ± 34	96 ± 17	125 ± 14
Adrenals	84 ± 14	160 ± 55	109 ± 25	108 ± 18
Brain	80** ± 8	111 ± 12	120 ± 27	103 ± 13
Heart	80** ± 8	87 ± 9	98 ± 13	114 ± 7
Blood	64** ± 12	114 ± 19	88 ± 16	144 ± 45
Urine	66** ± 10	87 ± 9	100 ± 15	83 ± 10

* Percentage calculated for per cent of dose retained per 100 g.

\+ Values between parenthesis indicate number of guinea pigs; these include animals who received 2 mg labelled vitamin C (3 in each group), 4 mg (2 in each nicotine group and 1 in each cigarette condensate group), other received 8 mg.

** Significantly different from control group ($P < 0.05$).

condensate on the incorporation of C^{14} in guinea pigs' organs 12 hours after administration of L-ascorbic acid-1-C^{14}. The results were expressed as the percentage incorporated or excreted in comparison to control animals. This way of expressing the results was selected because the limited number of metabolic cages available permitted only one animal of each group to be studied each day. The guinea pigs that received L-ascorbic acid orally had a depressed incorporation of C^{14} (from L-ascorbic acid-1-C^{14}) when they were injected subcutaneously with nicotine, but no such effect was found after injections of cigarette smoke condensate. On the other hand, when guinea pigs were given L-ascorbic acid intramuscularly, there was usually an increased incorporation of C^{14} (from L-ascorbic acid-1-C^{14}), but the increase was not statistically significant.

It can be seen in Table XVI that guinea pigs previously given 2 mg L-ascorbic acid daily, expired a considerable proportion of $C^{14}O_2$ from the breakdown of L-ascorbic acid-1-C^{14} given orally. The rate at which $C^{14}O_2$ was expired most rapidly occurred in the period 3 to 6 hours after oral administration. More than half of the C^{14} from L-ascorbic acid-1-C^{14} was expired 12 hours after oral administration. The pattern of $C^{14}O_2$ expired by these guinea pigs was comparable to that obtained with depleted guinea pigs in the previous experiment. Intramuscular administration of L-ascorbic acid-1-C^{14} produced about 20 to 25 per cent the amount of $C^{14}O_2$ expired after oral administration. Nicotine caused a marked increase in the amount of $C^{14}O_2$ expired, particularly for measurements taken 3 and 6 hours after oral administration of L-ascorbic acid-1-C^{14}, and cigarette smoke condensate caused a less marked and statistically non-significant increase in the amount of

TABLE XVI

Effect of subcutaneous injections of nicotine and cigarette smoke condensate on CO_2-C^{14} (expressed as per cent of controls content ± S.E.) expired after administration of 3 µc L-ascorbic acid-1-C^{14}

Hours	% dose Control	Nicotine	Cigarette † condensate
	C, oral	(10)†	(11)†
1.5	0.8 ± 0.2	153 ± 26	117 ± 18
3.0	7.2 ± 1.2	269 ± 74*	152 ± 28
6.0	28.7 ± 3.6	200 ± 43*	152 ± 39
9.0	43.9 ± 4.1	158 ± 27	133 ± 27
12.0	51.5 ± 4.5	145 ± 21	130 ± 19
	C, i.m.	(9)†	(8) †
1.5	0.5 ± 0.1	92 ± 7	108 ± 20
3.0	1.9 ± 0.1	94 ± 9	99 ± 10
6.0	5.0 ± 0.4	96 ± 9	108 ± 10
9.0	7.7 ± 0.6	102 ± 8	112 ± 12
12.0	10.1 ± 0.7	104 ± 9	112 ± 11

† Values between parenthesis indicate number of guinea pigs: these include 3 animals receiving 2 mg vitamin C in each group, 2 animals dosed with 4 mg vitamin C, 2 in each nicotine group and 1 in each cigarette condensate group; other received 8 mg.

* Significantly different from control group ($P < 0.05$).

$C^{14}O_2$ expired. Neither nicotine nor cigarette smoke condensate affected the amount of $C^{14}O_2$ expired after intramuscular administration of L-ascorbic acid-1-C^{14}.

DISCUSSION

A. Evaluation of the vitamin C status of cigarette smokers and non-smokers

The results obtained for vitamin C in blood and/or plasma in two different studies demonstrate a significant difference between the mean levels of smokers (about 0.3 to 0.4 mg) and non-smokers (about 0.6 to 0.7 mg). Similar results had been previously reported but the lack of proper control or the use of rather non-specific methods of analysis in several of these studies rendered most of these results rather inconclusive. For example, Harmsen (1941), had studied the blood ascorbic acid levels of the personnel of three hospitals in Germany during World War II and reported that 9 heavy smokers had abnormally low blood ascorbic acid levels but failed to indicate if there were other heavy smokers with normal levels. Durand et al. (1962), using a colorimetric 2-6, dichloroindophenol determination, found that in general the plasma of the smokers had about half the ascorbic content of the non-smokers. Unfortunately, the normal range reported (4.8 - 8.4 mg per cent) is much above the normal value of 1 mg which has been reported by several authors (Clemetson and Andersen, 1967; Stewart et al., 1953; Baker, 1967; and Pearson, 1967). It seems unlikely that this difference was due to interfering reducing substances but, if this was the case, the results would indicate a lower level of reducing substances in the smokers; it seems more likely that the higher normal values are due to either miscalculations or improper standardization. In studies on 60 medical students by Venulet (1953), blood ascorbic acid levels of smokers were in the range of 0.6 and 0.9 mg, while those of non-smokers were in the range of 1.0 and 1.2 mg. Similarly, Dietrich and Buchner

(1960) reported the following average values for ascorbic acid in blood plasma; 0.9 mg (55 smokers) and 1.32 mg (20 non-smokers). Calder et al. (1963), from the Beecham Group in England, obtained vitamin C values of 0.52 mg for 31 smokers of 15 cigarettes or more a day, and 0.91 mg for 91 non-smokers; similar results were published by Brook and Grimshaw (1968) from the Beecham Group also. The various workers cited above have found blood and plasma vitamin C levels higher than those in the present studies, but a similar difference between smokers and non-smokers. In view of the specificity of the analytical method used in the present study, and the care which was taken to prevent loss of vitamin C in the blood samples, and to avoid the interference due to the recent dietary intake, it can be concluded that cigarette smokers have blood vitamin C levels lower than those of non-smokers.

It could be argued that blood vitamin C levels do not necessarily reflect the vitamin C which is incorporated into the various organs of the body. The vitamin content of white blood cells (W.B.C.) may be a good index of body stores but, as already described, such a determination is difficult (Griffiths, 1968). The retention of vitamin C from large doses of L-ascorbic acid would be expected to vary inversely with the initial levels of vitamin C in the organs, and the urinary excretion of a test dose should be proportional to the levels in the organs. The present studies did show a good correlation between urinary excretion of vitamin C after a test dose and the blood vitamin C levels (Figure 9). The cigarette smokers were found to excrete significantly less of a test dose than non-smokers before but not after a saturation period with L-ascorbic acid.

Relative retention values, estimated in a manner similar to that of Lowry et al. (1946), are useful in showing a difference between groups; this was determined in the first study and it was found that smokers required 630 mg more than non-smokers to become saturated. In the same study, the absolute retentions from the saturating doses were inversely proportional to the basic blood and plasma levels and showed a significant difference between the two groups when the effect of previous vitamin C intake was taken into account. In the second study, the smokers retained significantly more L-ascorbic acid from the first load test than the non-smokers. The greater retention of vitamin C by the smokers is in accord with results reported by Rupniewska (1965) in studies with 26 old men from a State Home for Pensioners. They found a decreased excretion of vitamin C in the urine of 14 cigarette smokers (about 60 per cent the amount excreted by 12 non-smokers) during 4 hours following an intravenous injection of 500 mg of vitamin C. It is difficult to explain the results of Taber and Larson (1962) who reported that a group of 45 smokers and 45 non-smokers excreted in the urine amounts of ascorbic acid not significantly different after receiving a 500 mg loading dose of L-ascorbic acid.

It can be concluded that cigarette smokers in general not only have lower blood vitamin C levels but also have lower body stores of the vitamin. The cause of this could be due to a different utilization of vitamin C or to a different intake.

Calder et al. (1963) and Brook and Grimshaw (1968) had stated that an approximate assessment of the food habits of the volunteers participating in their studies did not indicate that differences in intake of

vitamin C between smokers and non-smokers were causing the differences in blood vitamin C levels. In the first study reported above, the initial difference in vitamin C levels between smokers and non-smokers could not be attributed to a difference in the intakes, since these were comparable. In the second study also, both groups had comparable vitamin C intakes. Furthermore, as shown in Figure 7, the slopes relating vitamin C intakes and blood levels were obviously different for the smokers and the non-smokers. According to these slopes, the smokers would have needed about twice as much vitamin C as non-smokers in order to maintain comparable levels. The correlation coefficient was significant for the non-smokers, but not for the smokers. This indicates that a fair prediction of the blood levels of non-smokers from their intakes would be possible, but predictions for the smokers would be misleading. The relationship of the vitamin C intakes with the percentage excreted after a load test gave a pattern similar to that obtained with blood levels. This further indicated that the load test was useful in evaluating the vitamin C status.

On the basis of the above discussion, the lower vitamin C levels found in the cigarette smokers were not due to lower vitamin C intakes. In order to develop lower vitamin C levels, the smokers would need to either utilize more vitamin C or to have less vitamin C available for utilization than the non-smokers although receiving the same intakes. A significant difference in the utilization of vitamin C by the organs and tissues of the smokers appear unlikely because, as shown in Table IV, when 5 smokers and 5 non-smokers were placed on a deficient diet for 13 days, the blood and plasma levels of both groups decreased at the same

rate and there was no significant difference in the subsequent retention of vitamin C from load tests.

The most likely explanation for the lower blood vitamin C levels of the smokers would be that less vitamin C is effectively available to the smokers receiving intakes comparable to those of non-smokers. This hypothesis is directly supported by urinary excretion data. In the first study, the 5 smokers showed a tendency to excrete less of L-ascorbic acid doses, particularly on the second and third day after the saturating doses were discontinued. In the second study, after the saturation and desaturation period, the smokers excreted about 60 per cent of the vitamin C excreted by the non-smokers following doses ranging from 58 to 95 mg. In the third study, after saturation with loading doses of L-ascorbic acid, the smokers again excreted less vitamin C than non-smokers following 75 mg doses of L-ascorbic acid; they also excreted less D-isoascorbic acid than non-smokers following 75 mg doses of D-isoascorbic acid. Since it is recognized that D-isoascorbic acid is poorly retained by vital organs, the lower excretion of D-isoascorbic acid by the smokers suggests that the smokers have less D-isoascorbic acid available to the body than the non-smokers when receiving the same dose of this isomer.

The lower availability of ascorbic acid to smokers than to non-smokers could be explained by a difference in the absorption process. More ascorbic acid could be oxidized to dehydroascorbic acid or further degraded to diketogulonic acid in the gastrointestinal tract of the smokers, due to the secretion of some oxidative enzyme (such as ceruloplasmin involved in the oxidation of serotonin which is known to be released by nicotine), or because of a different bacterial flora (although this is unlikely). Perhaps

following absorption, there is a difference in the ascorbic acid/dehydroascorbic ratio reaching the liver which favours the degradation of vitamin C in the liver. However, according to results of the first study, the urinary excretion of the dehydroascorbic acid by the cigarette smokers was not significantly different from that of non-smokers, and neither group excreted a significant amount of diketogulonic acid, which is an oxidation product of dehydroascorbic acid.

B. Effect of nicotine and cigarette smoke condensate on the vitamin C metabolism of guinea pigs

Experiment 1

The mode of absorption of L-ascorbic acid by guinea pigs has been clarified by the results represented in Figures 10-11. Fifteen minutes after oral administration of L-ascorbic acid, about 80 per cent of the dose was still present in the gastrointestinal content. At that time the uptake of L-ascorbic acid by the stomach was maximal while the uptake by the small intestine was maximal about 45 minutes after administration. The small intestine was the major site for the uptake of L-ascorbic acid. The uptake of L-ascorbic acid by the large intestine was also maximal after 45 minutes but was less important that in the small intestine. Looking at the amount of L-ascorbic acid and C^{14} in the gastrointestinal content, it is evident that there was massive destruction of L-ascorbic acid in the period extending from 15 minutes to one and a half hours after administration, but the product of degradation remained unabsorbed for up to three hours. This massive destruction of L-ascorbic acid is certainly, in addition to the short half-life of L-ascorbic acid in guinea pig organs, a major cause for the higher requirement of vitamin C by guinea pigs in

comparison to humans. It is practically impossible to determine directly the uptake of non-labelled L-ascorbic acid by the blood and by the organs of guinea pigs. However, the measurement of C^{14} in the body, after administration of L-ascorbic acid-1-C^{14}, is generally considered to represent L-ascorbic acid-1-C^{14}; this seems acceptable in view of the fact that, $C^{14}O_2$, the main product of oxidation of L-ascorbic acid-1-C^{14}, is rapidly expired, and that oxalic acid, the other breakdown product, is rapidly excreted in the urine. The level of C^{14} in the blood became maximal one and a half hours after the administration of L-ascorbic acid-1-C^{14}, remained nearly the same for three hours, and then started to decrease gradually. The uptake of C^{14} by the kidneys reflected that of the blood, but that of other organs except the liver and adrenals was maximal after three hours. The uptake of C^{14} by the liver and the adrenals was maximal after six hours only, and occurred as the C^{14} content of the blood started to decrease. In summary, the L-ascorbic acid ingested by guinea pigs was absorbed to a relatively small extent in the stomach during about 15-30 minutes. Hughes and Lewis (1965) had concluded that the bulk of vitamin C absorption occurred in the stomach, but they were measuring only the appearance of vitamin C in the blood. The results of the present study showed that appearance of vitamin C in the blood was maximal only one and a half hours after ingestion. The small intestine had the greatest absorption activity with the maximum occurring somewhat less than one hour after ingestion; at that time, however, there had already been an extensive destruction of the vitamin C in the gastrointestinal content and this destruction progressed until about one and a half hours after ingestion. This contrasts with the results of Farmer et al. (1940) who

had found that about half of the vitamin C administered by stomach tube was still present in the gastrointestinal tract 6 hours after administration; it seems possible that the way of administration could have caused this difference.

Experiment 2

The value of this preliminary experiment was very limited since it involved only 4 guinea pigs per group. The results indicated that nicotine given orally depressed the vitamin C levels of organs. However, it was felt that oral administration of nicotine was too different from the absorption of nicotine by the lungs as in cigarette smoking and that ingested nicotine might be metabolized differently and have an effect different from that of nicotine absorbed by the lungs.

Experiment 3

To make guinea pigs breathe nicotine in a manner similar to cigarette smoking in humans would have required specially designed apparatus and would have been too time-consuming. Because the absorption of nicotine after subcutaneous injections had been reported to be rapid and efficient (Travell, 1960), this mode of administration was selected. Nicotine apparently had no significant effect on the metabolism or turnover of vitamin C because the nicotine-treated guinea pigs, when fed a vitamin C-deficient diet, developed the symptoms of scurvy and lost weight at the same rate as guinea pigs injected with saline. The incorporation of L-ascorbic acid-1-C^{14} given orally to depleted guinea pigs, and the amount of $C^{14}O_2$ expired or C^{14} excreted in the urine were not significantly affected by the injection of nicotine. These results were not considered conclusive because scurvy might have masked the effect of nicotine.

Experiment 4

The study of the metabolism of L-ascorbic acid by guinea pigs revealed unexpected results. A dosage of 2 mg L-ascorbic acid produced about the same vitamin C levels, whether given orally or by injection. However, L-ascorbic acid-1-C^{14} yielded about 5 times more $C^{14}O_2$ when given orally than when injected intramuscularly. In fact, the guinea pigs that received L-ascorbic acid-1-C^{14} intramuscularly incorporated more C^{14} after 12 hours than the guinea pigs that received it orally (Table XIV). This suggests that, after the intramuscular injection of L-ascorbic acid-1-C^{14}, the initial incorporation in tissue was greater. This difference was not detectable when non-labelled ascorbic acid was used.

Neither cigarette smoke condensate nor nicotine affected the tissues of non-labelled vitamin C level when L-ascorbic acid was given orally, but nicotine decreased the incorporation of C^{14} from L-ascorbic acid-1-C^{14} while the cigarette smoke condensate did not. According to analyses, the cigarette smoke condensate injected supplied the same amount of nicotine as the injections of pure nicotine. The difference in the effect may have been due to the less efficient absorption of nicotine from the smoke condensate which produced deposits of matter under the skin. When L-ascorbic acid-1-C^{14} was given intramuscularly, nicotine did not decrease the incorporation of C^{14} but rather increased it (Table XV), and was without effect on the amount of $C^{14}O_2$ expired. However, nicotine and smoke condensate increased the amount of $C^{14}O_2$ expired when L-ascorbic acid-1-C^{14} was given orally. This increase was significant in the 3 to 6 hour period following the oral administration of L-ascorbic acid-1-C^{14} to nicotine-treated animals (Table XVI). Undoubtedly, the breakdown of

L-ascorbic acid-1-C^{14} to $C^{14}O_2$ is subsequent to the breakdown of L-ascorbic acid to an unidentified compound in the gastrointestinal tract as found for Experiment 1 in Part III-B. As mentioned earlier, the question whether L-ascorbic acid-1-C^{14} can yield significant $C^{14}O_2$ in humans remains unsettled. If L-ascorbic acid-1-C^{14} did break down significantly to $C^{14}O_2$ in humans, this could be the route by which cigarette smokers make a less efficient utilization of their vitamin C intake. If humans do not break down L-ascorbic acid-1-C^{14} into a significant amount of $C^{14}O_2$, it would still appear very likely that a significant proportion of their L-ascorbic acid intake of cigarette smokers could be degraded in the intestinal tract or by the liver, as might occur if a greater proportion of dehydroascorbic acid reached that organ.

REFERENCES

Andrzejerewiki, S.W. 1966. Studies on the toxicity of tobacco smoke. Acta Medica Polona 7: 407-408.

Auerbach, O., C.E. Hammond, D. Kirman and L. Garfinkel. 1967. Emphysema produced in dogs by cigarette smoking. J. Am. Med. Ass. 199: 241-246.

Barrett, A.M. 1966. The role of plasma free fatty acids in the elevation of plasma cholesterol and phospholipids produced by adrenaline. J. Endocrinol. 36: 301-316.

Battesti, J.P. and A. Thuillier. 1968. Carences en acide folique: causes et diagnostic biologique. La Presse Médicale 76: 2209-2210.

Beaton, G.H., D.M. Hellebust, W. Paul and A.M. Wright. 1960. The effect of scurvy on thyroid activity in the guinea pig. J. Nutr. 60: 321-328.

Beckett, A.H. and E.J. Triggs. 1966. Determination of nicotine and its metabolite cotine, in urine by gas chromatography. Nature 211: 1415-1417.

Beckett, A.H. and E.J. Triggs. 1967. Enzyme induction in man caused by smoking. Nature 216: 587.

Bodanski, O., F. Wroblewski and B. Markard. 1951. Concentrations of ascorbic acid in plasma and white cells of patients with cancerous and non-cancerous diseases. Cancer Res. 11: 238.

Bourquin, A. and E. Musmanno. 1953. Preliminary report on the effect of ascorbic acid content of whole blood. Am. J. Digestive Dis. 20: 75-77.

Briggs, M.H. 1962. Malnutrition and mental disease. Brit. Med. J. April 14: (1): 1078.

Brook, M. and J.J. Grimshaw. 1968. Vitamin C concentration of plasma and leukocytes as related to smoking habit, age and sex of humans. Am. J. Clin. Nutr. 21: 1244-1258.

Brownlee, K.A. 1965. A review of "Smoking and Health". J. Amer. Statis. Ass. 60: 722-739.

Calder, J.H., R.C. Curtis and H. Fore. 1963. Comparison of vitamin C in plasma and leucocytes of smokers and non-smokers. Lancet 1: 556.

Chaudhuri, C.R. and I.B. Chatterjee. 1969. L-ascorbic acid synthesis in birds, phylogenic trend. Science 164: 435-436.

Cheraskin, E., W.M. Ringsdorf, Jr. 1968. A lingual vitamin C test: VII. Relationship of nonfasting serum cholesterol and vitamin C state. Int. J. for Vit. Res. 38: 415-420.

Cheraskin, E., W.M. Ringsdorf, D.W. Aspray, D. Michael and D. Preskitt. 1968A. A lingual vitamin C test: IX. Relationship to gingival state. Int. J. for Vit. Res. 38: 424-432.

Cheraskin, E., W.M. Ringsdorf, D.W. Aspray, D. Michael and D. Preskitt. 1968B. A lingual vitamin C test: X. Relation to tooth mobility. Int. J. for Vit. Res. 38: 433-437.

Clemetson, C.A.B. and L. Andersen. 1966. Plant polyphenols as anti-oxidants for ascorbic acid. Ann. N.Y. Acad. Sci. 136: 339-378.

Comroe, Jr. J.H. 1960. The pharmacological actions of nicotine. Ann. N.Y. Acad. Sci. 98: 48-51.

Comroe, Jr. J.H. 1964. The physiological effects of smoking. Physiology for physicians 2: 1-6.

Connor, W.E., J.C. Hoak and E.D. Warner. 1963. Massivethrombosis produced by fatty acid infusion. J. Clin. Invest. 42: 860-866.

Connor, W.E., J.C. Hoak and E.D. Warner. 1965. The effect of fatty acids on blood coagulation and thrombosis. Thromb. Diath. Haemorrag., Suppl. 17: 89-102.

Conrad, M.E. and S.G. Shade. 1968. Ascorbic acid chelates in iron absorption: a role for hydrochloric acid in bile. Gastroenterology 55: 35-45.

Crandon, J.H., C.C. Lund and D.B. Dull. 1940. Experimental human scurvy. The New England J. of Med. 223: 353-369.

Cuppy, D. and L. Crevasse. 1963. An assembly for $C^{14}O_2$ collection in metabolic studies for liquid-scintillation counting. Anal. Biochem. 5: 462-463.

Dansys, S. and S. Skupinski. 1955. The influence of nicotine and cigarette paper smoke on the level of vitamin C in frog organs and mouse urine. Przegl. Lek. No. 10: 314-315.

Department of National Health and Welfare, Ottawa. 1966. A Canadian study of smoking and health.

Department of National Health and Welfare, Ottawa. 1968. News Release number 1968-104.

Dietrich, G. and M. Büchner. 1960. A contribution to the vitamin C metabolism of smokers. Deutsch Gesundh. 55: 2494-2495.

Doll, R. and A.B. Hill. 1964. Mortalities in relation to smoking: ten years' observation of British doctors. Brit. Med. J. 1: 1399-1410 and 1460-1467.

Durand, C.H., M. Audinot and S. Frajdenrajch. 1968. Hypovitaminose C latente et tabac. Concours Médical 84: 4801-4806.

Eposito, R. and R. Valentini. 1968. Vitamin C and gastrointestinal disorders. Brit. Med. J. No. 7568, 118.

Evans, J.P., R.E. Hughes and P.R. Jones. 1967. Some effect of cigarette smoke on guinea pigs. Proc. Nutr. Soc. 26: XXXV.

Farmer, C.J., A.F. Abt and H. Chinn. 1940. The absorption of vitamin C (L-ascorbic acid) from the intestinal tract, in health and disease. Quarterly Bull. of Northwestern Univ. Med. School 14: 114-119.

Fishman, S.S. 1963. Studies on the excretion and distribution of radioactive nicotine and some evidence on the urinary metabolites. Arch. Int. Pharmacodyn. 145: 123-136.

Franchimont, P. and P.J. Delwaide. 1966. Action vasculaire de la sérotonine chez l'homme. Revue Française d'études chimiques et biologiques 11: 867-885.

Frommel, E., J. Piquet, C.L. Cuénod and M. Loutfi. 1945. La nicotine l'yohimbine et le tartrate d'ergotamine étudiés dans le cadre de l'hypovitaminose C médicamenteuse. Helv. Physiol. Acta 3: 613-615.

Gabuzda, Jr. G.J., G.B. Phillips, R.F. Schelling and C.S. Davidson. 1952. Metabolism of pteroylglutamic acid and the citrovorum factor in patients with scurvy. 31: 756-761.

Glynn, M.F., J.F. Mustard, M.R. Buchanan and E.A. Murphy. 1966. Cigarette smoking and platelet aggregation. The Can. Med. Ass. J. 95: 549-553.

Goldberg, A. 1963. The anemia of scurvy. Quat. J. Med. 32: 51-64.

Goyanna, C. 1955. Tabac et vitamine C. Brasil Medico No. 14-18: 173-177.

Greenberg, S.M., J.E. Tucker, A.E. Henung and J.K. Mathues. 1957. Iron absorption and metabolism. J. Nutr. 63: 19-31.

Griffith, L.I. 1968. Biochemical finding of the Farborough survey. Pages 34-39. In: Vitamins in the Elderly, John Wright and Sons, Ltd. Bristol.

Hansson, E., P.C. Hoffman and C.G. Schmiterlow. 1964. Metabolism of Nicotine in mouse tissue slices. Acta Physiol. Scand. 61: 380-392.

Harmsen, H. 1941. Vitamin C blood levels in a hospital personnel, seasonal differences and influence of nutrition, working conditions and misuse of nicotine. Dtsch. Med. Wschr. 29: 790-795.

Hayes, F.N. and R.G. Gould. 1953. Liquid scintillation counting of tritium-labeled water and organic compounds. Science 117: 480-482.

Heinz Nutritional Data. 1964. H.J. Heinz Co: Pittsburgh, Pennsylvania.

Hitier, Y. 1968. Evolution de l'activité de la deoxyribonucléase acide ou DNASE II au cours de la carence en acide ascorbique chez le cobaye. Ann. Nutr. Alim. 22: 83-88.

Hitier, Y. and T. Terroine. 1968. Contribution de l'acide ascorbique au contrôle de la déoxyribonucléase neutre ou DNASE I. Ann. Nutr. Alim. 22: 89-93.

Hoffer, A. and H. Osmond. 1963. Scurvy and schizophrenia. Diseases of the nervous system 24: 273-285.

Hogler, F. 1943. Nicotine induced damage. Wien. Med. Wochschr. 93: 309-362.

Hsu, J.M. 1969. Effect of scurvy on deiodination of ^{131}I-labeled thyroxine in guinea pigs. J. Nutr. 97: 505-511.

Hughes, R.E. and S.C. Lewis. 1965. The absorption of vitamin C in the stomach of small animals. Proc. Nutrition Soc. 24: XXXV.

Jeffay, H. and J. Alvarez. 1961. Liquid scintillation counting of Carbon-14. Use of ethanolamine-ethylene monomethylether-toluene. Anal. Chem. 33: 612-615.

Kellie, A.E. and S.S. Zilva. 1936. The alleged presence of dehydro-ascorbic acid in blood. Biochem. J. 30: 361-368.

Kendler, J. and M. Perek. 1968. Effect of dietary ascorbic acid on vitamin A deficiency in chicks.

Kerr, W.K., M. Barkin, P.E. Levers, S.K.C. Woo and Z. Menczyk. 1965. The effect of cigarette smoking on bladder carcinogens in man. Can. Med. Ass. J. 93: 1-7.

Kershbaum, A. and S. Bellet. 1968. Cigarette, cigar, and pipe smoking. Some differences in biochemical effects. Geriatrics 23: 126-134.

Kershbaum, A., O.J. Pappajohn, S. Bellet, M. Hirabayaski and H. Shafiha. 1968. Effect of smoking and nicotine on adrenocortical secretion. J. Am. Med. Ass. 203: 275-278.

King, C.G. 1967. Present knowledge of ascorbic acid (vitamin C). Pages 76-79. In: Present Knowledge of Nutrition. Nutrition Foundation, Inc. Ninety-nine Park Avenue, New York. N.Y. 10016.

Kirshner, N. 1966. Summary of discussion and commentary. Pharmacological Rev. 18: 83-84.

Krause, R.F. 1964. Laboratory aids in the diagnosis of malnutrition. Page 593. In: Modern Nutrition in Health and Disease. Edited by Wohl and Goodhart, Lea and Febiger, 1964.

LaDu, B.N. and V.G. Zannoni. 1961. The role of ascorbic acid in tyrosine metabolism. Ann. N.Y. Acad. Sci. 161: 175-191.

Larson, P.S., H.B. Haag and H. Silvette. 1961. Tobacco. Experimental and clinical studies. Williams and Wilkins.

Larson, P.S. and H. Silvette. 1968. Tobacco. Experimental clinical studies. Supplement 1. Williams and Wilkins.

Levin. E.Y., E. Levenberg and S. Kaufman. 1960. The enzymatic conversion of 3,4-dihydroxyphenyl-ethylamine to norepinephrine. J. Biol. Chem. 235: 2080-2086.

Lowry, O.H. 1952. Biochemical evidence of nutrition status. Physiol. Rev. 32: 431-448.

Lowry, O.H., O.A. Bessey, M.J. Brock and J.A. Lopez. 1946. The interrelationship of dietary serum, white blood cell, and total body ascorbic acid. J. Biol. Chem. 166: 111-119.

Lucchesi, B.R., C.R. Schuster and G.S. Emley. 1967. The role of nicotine as a determinant of cigarette smoking frequency in man with observations of certain cardiovascular effects associated with the tobacco alkaloid. Clin. Pharmacol. Therapeutics 8: 789-796.

Lupu, N.G., C. Velican and C. Mihaescu. 1954. Contributii experimentale privind rolul siste-mului nervos in reglarea raspunsului secretor al corticosupranenalei la actiunea funului de tutun. Med. Sc. Sect. Rumanian Acad., Session Jan. 22-24, pages 141-168.

Mackenzie, C.G., J.P. Choulder, E.B. Keller, J.R. Rachele, N. Gross and V.J. du Vigneaud. 1949. Oxidation and distribution of the methyl group administered as a methionine. J. Biol. Chem. 180: 99-111.

Maren, T.H. 1951. Pharmacology of nicotine: antipyretic, renal and adrenocorticotrophic effects. Proc. Soc. Exp. Biol., N.Y. 77: 521-523.

Mazur, A. 1961. Role of ascorbic acid in the incorporation of plasma protein into ferritin. Ann. N.Y. Acad. Sci. 92: 223-229.

McCormick, W.J. 1952. Ascorbic acid as a chemotherapeutic agent. Arch. Pediat. 69: 151-155.

McCurdy, P.R. and R.J. Dean. 1968. Some therapeutic implications of ferrous-sulfate ascorbic acid mixtures. Am. J. Clin. Nutr. 21: 284-288.

McKennis, Jr. H., S.L. Schwartz and E.R. Bowman. 1964. Alternate routes in the metabolic degradation of the pyrrolidine ring of nicotine. J. Biol. Chem. 239: 3990-3996.

Moore, C.E. and F.G. Bock. 1968. "Tar" and nicotine levels of American cigarettes. National Cancer Inst. Monograph No. 28: 89-94.

Morselli, P.L., H.H. Ong, E.R. Bowman and H. McKennis, Jr. 1967. Metabolism of (±)-cotinine-2-C^{14} in the rat. J. Medicinal Chem. 10: 1033-1036.

Murchison, L.E. and T. Fype. 1966. Effects of cigarette smoking on serum-lipids, blood glucose and platelet adhesiveness. Lancet 2(7456), 182-184.

Murphy, E.A. and J.F. Mustard. 1966. Tobacco and thrombosis. Am. J. Public Health, 56: 1061-1073.

Nagatsu, T., J.B. Van der Schoot, M. Levitt and S. Udenfriend. 1968. Factors influencing dopamine β-hydroxylase activity and epinephrine levels in guinea pig adrenal gland. J. Biochem. 64: 39-43.

Noether, P. 1923. Quantitative studiem uber das schicksal des nikotins im organismus nack tabakrauchen. Arch. Exp. Path. Berl. 98: 370-377.

Olsen, R.E. 1963. Atherosclerosis. In: Cecil-Loeb Textbook of Medicine edited by Beeson and McDermott, 11th ed., pages 704-710.

Papadopoulos, N.N. and J.A. Kintzios. 1963. Formation of metabolites from nicotine by a rabbit liver preparation. J. Pharmacol. Exp. Ther. 140: 269-277.

Patterson, J.C. 1941. Some factors in the causation of intimal haemorrhages and in the precipitation of coronary thrombosis. Can. Med. Ass. J. 44: 114-120.

Patterson, M.S. and R.C. Greene. 1965. Measurement of low energy beta-emitters in aqueous solution by liquid scintillation counting emulsions. Anal. Chem. 37: 854-857.

Pauling, L. 1968. Orthomolecular psychiatry. Science 265-271.

Pearson, W.N. 1967. Blood and urinary vitamin levels as potential indices of body stores. Am. J. Clin. Nutr. 20: 514-525.

Pelletier, O. 1968. Smoking and vitamin C levels in humans. Am. J. Clin. Nutr. 21: 1259-1267.

Reid, M.E. 1948. Gastrointestinal tract of guinea pig and elimination of ascorbic acid given intraperitoneally. Proc. Soc. Exp. Biol. Med. 68: 403-406.

Reif, G. 1943. Study on the effect of nicotine and caffeine on ascorbic acid. Biochem. Z. 315: 310-319.

Rupniewska, Z.M. 1964. L-ascorbic acid in smokers and non-smokers. Polski Tygod. Lekar. 19: 1259-1263.

Rupniewska, Z.M. 1965. Smoking and ascorbic acid level in the organism. Polski Tygod. Lekar. 20: 1069-1071.

Russel, R.I., J.M. Williamson, A. Goldberg and E. Wares. 1968. The Lancet. No. 7568, 603.

Schlegel, O.J. and G.E. Pipkin. 1968. A report in Rodale's Health Bulletin 6 (No. 28) Page 2.

Sears, M.L. 1969. Vitamin C as a requirement for the storage of norepinephrine by the iris. Biochem. Pharmacol. 18: 253-256.

Sokoloff, B., H. Michiteru, C. Saelhop, B. McConnell and T. Imai. 1967. Effect of ascorbic acid on certain blood fat metabolism factors in animals and man. J. Nutr. 91: 107-118.

Solomon, H.A., R.L. Priore and I.D.J. Bross. 1968. Cigarette smoking and periodontal diseases. J. Am. Dental Ass. 77: 1081-1084.

Stevenson, N.R. and M.K. Brush. 1969. Existence and characteristics of Na - dependent active transport of ascorbic acid in guinea pigs. Am. J. Clin. Nutr. 22: 318-326.

Stewart, J.S. and C.C. Booth. 1964. Ascorbic acid absorption in malabsorption. Clin. Sci. 27: 15-22.

Strauss, L.H. and P. Scheer. 1939. The effects of nicotine on the metabolism of vitamin C. Int. Z. Vitaminforschg. 9: 39-48.

Taber, R.I. and P.S. Larson. 1962. Interactions between smoking, nicotine and ascorbic acid. Pharmacologist 4(2): 177.

Taber, R.I. and P.S. Larson. 1964. In vivo and in vitro studies on the question of a biologic reglationship between nicotine and ascorbic acid. Arch. Int. Pharmacodyn. 151: 243-259.

Tappel, A.L. 1968. Will antioxidant nutrients slow aging process. Geriatrics 23: 97.

Thoa, N.B., R.J. Wurtman and J. Axelrod. 1966. A deficient binding mechanism for norepinephrine in hearts of scorbutic guinea pigs. Proc. Soc. Exp. Biol. Med. 121: 267-271.

Tompsett, S.L. 1959. The determination in urine of some metabolites of tryptophan-kynurenine, anthranilic acid and 3-hydroxyanthranilic acid and reference to the presence of o-aminophenol in urine. Clin. Chim. Acta 4: 411-419.

Toporkova, I.B. 1959. Vitamin C balance in connection with chronic exposure of tobacco industrial workers to tobacco dust. Tr. Leningr. Sanitarnogig Med. Inst. 47: 243-253.

Tosbert, P., H.J. Bremer and A. Spliedt. 1966. Zur tyrosinstoffwechselstörung frühgeborener: die vitamin C abhangigkeit des tyrosinspiegels im blut fruhgeborener. Helvetica Pediatrica Acta 21: 439-445.

Travell, J. 1960. Cardiovascular effects of nicotine and smoking. Part 1. The absorption and fate of nicotine. Absorption of nicotine from various sites. Ann. N.Y. Acad. Sci. 90: 10-30.

U.S. Public Health Service. 1964. Smoking and Health. Report of the Advisory Committee to Surgeon General of the Public Health Service (Washington). Public Health Service Publication, No. 1103. U.S. Department of Health, Education and Welfare.

U.S. Public Health Service. The Health Consequences of Smoking. A Public Health Service review: 1967. Public Health Service Publication No. 1696. 1968 Supplement and 1969 Supplement. U.S. Department of Health, Education and Welfare.

VanderKamp, H. 1966. A biochemical abnormality in schizophrenia involving ascorbic acid. Int. J. Neuropsych. 2: 204-206.

Venulet, F. 1953. Tobacco smoke and ascorbic acid. Endokrinology 30: 345-351.

Venulet, F. 1954. Harmful effects of smoking caused by loss of ascorbic acid. Med. Klin. 24: 959-962.

Warwick, K.M. and H.J. Eysenck. 1963. The effect of smoking on critical flicker fusion (CFF) threshold. Life Sciences 2: 219-225.

Weisman, A. 1967. Blocking serotonin biosynthesis. Bioscience 17: 792-795.

Wenzel, D.G. and G.L. Beckloff. 1958. The effect of nicotine on experimental hypercholesterolemia in the rabbit. J. Am. Pharm. Ass., Sc. Ed. 47: 338-343.

Werle, E. and A. Meyer. 1950. Uber den Abbau von Tabakalkaloiden durch tierische Gewebe. Biochem. Zschi. 321: 221-235.

Willis, G.C. 1953. An experimental study of the internal ground substance in atherosclerosis. Can. Med. Ass. J. 69: 17-22.

Willis, G.C. and S. Fishman. 1955. Ascorbic acid content of human arterial tissue. Can. Med. Ass. J. 72: 500-503.

Wilk, M. and E.L. Wynder. 1966. The action of polycyclic aromatic hydrocarbons on isolated mouse liver mitochondria. Z. Nuturforsch. 216: 161-166.

Wynder, E.L. and D. Hoffman. 1967. Tobacco and tobacco smoke, studies in experimental carcinogenesis. Academic Press, New York.

Yamamoto, I., R. Inoki and K. Iwatsuto. 1968. Penetration of Nicotine-C-14 into several rat tissues in vivo and in vitro. Toxicology and Applied Pharmacology 12: 560-567.

Yun, I.S. and S.S. Kim. 1938. The influence of continuous subcutaneous injection of nicotine on the adrenal gland and the anterior lobe of hypophysis. Tr. Soc. Path. Japan 28: 426-428.

Youmans, J.B. and E.W. Patton. 1942. The laboratory diagnosis of nutritional deficiencies. Clinics 1: 303-345.

Yudkin, J. 1969. Sucrose and heart disease. Nutrition Today 4: 16-20.

Zaitsev, V.F., L.A. Kyasnikov and M.E. Sheikman. 1964. Effect of ascorbic acid on distribution of cholesterol-4-C^{14} in tissues of animals with experimental atherosclerosis. Kardiologiya 4: 30-32.

GENERAL CONCLUSIONS

A rapid, accurate and specific analytical method was needed in order to obtain conclusive data on vitamin C nutrition and metabolism. The new method, presented in this thesis, can measure L-ascorbic acid and dehydro-L-ascorbic acid separately or together (total vitamin C), and is not affected by substances causing interference in other published methods.

By modifying a chromatographic procedure for separating L-ascorbic acid, and D-isoascorbic acid, it was possible to ascertain that the D-isoascorbic acid specimen used contained no L-ascorbic acid and that the effects obtained were due only to D-isoascorbic acid.

The different rates of osazones formation of L-ascorbic acid and D-isoascorbic acid were used to quantitatively determine and differentiate these two isomers. This provided essential methodology for the study of the antiscorbutic activity and metabolism of D-isoascorbic acid.

It was demonstrated that D-isoascorbic acid definitely has antiscorbutic activity. The organs of guinea pigs retain this isomer but not as well as L-ascorbic acid. This difference, however, can be compensated by feeding daily 20 times more of the isomer. D-isoascorbic acid also accelerated the loss of L-ascorbic acid from guinea pigs. There appeared to be some retention of D-iscascorbic acid by humans, specially when they had a lower vitamin C status.

It was found that cigarette smokers receiving a vitamin C intake similar to that of non-smokers retained less of the vitamin in their organism but utilized the vitamin C retained at the same rate as non-smokers.

A very important aspect of the vitamin C nutrition of guinea pigs has been clarified: there is an intensive destruction of the vitamin in the gastro-intestinal tract.

Although it is difficult to extrapolate the results obtained with guinea pigs to man, it seemspossible that nicotine is the cause of the lower vitamin C status of cigarette smokers. In guinea pigs, nicotine caused less incorporation of C^{14} and an increase in $C^{14}O_2$ after oral ingestion of L-ascorbic acid-1-C^{14}.

CLAIMS TO ORIGINAL FINDINGS

1. A highly specific, original and relatively simple method for the determination of L-ascorbic acid and dehydroascorbic acid separately or together (total vitamin C).
2. The chromatographic separation of one part of L-ascorbic acid in presence of 100 parts D-isoascorbic acid.
3. The first quantitative method for differentiating L-ascorbic acid and D-isoascorbic acid.
4. The proof that D-isoascorbic acid can be incorporated into guinea pigs organs.
5. The demonstration of a more rapid loss of L-ascorbic acid from the organs of guinea pigs fed D-isoascorbic acid than from normal guinea pig organs.
6. A conclusive proof that D-isoascorbic acid has complete antiscorbutic activity for guinea pigs.
7. The demonstration that humans having lower vitamin C status can utilize some D-isoascorbic acid.
8. Conclusive proof that, (a) cigarette smokers have lower vitamin C status than non-smokers, and that (b) this lower status is not due to a poorer vitamin C intake but to a less efficient utilization of normal intakes.
9. The demonstration that: (a) there is massive destruction of vitamin C in the gastro-intestinal tract of guinea pigs, and that (b) the small intestine is the major site for the absorption of the vitamin.
10. Evidence that nicotine injections (a) do not affect the utilization of the vitamin C stores of guinea pigs, but (b) cause a decreased incorporation of C^{14} into organs and an increased production of $C^{14}O_2$ arising from L-ascorbic acid-1-C^{14} given orally.