MONOGRAPH ON ASCORBIC ACID #116 Vol IV
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VOLUME 4

GRAS MONOGRAPH SERIES

ASCORBIC ACID

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prepared for

THE FOOD AND DRUG ADMINISTRATION
DEPARTMENT OF HEALTH, EDUCATION
AND WELFARE

OCTOBER 7, 1974

prepared by

Tracor Jitco, Inc.

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This publication was prepared under Contract Number FDA 72-100 with the Public Health Service, Food and Drug Administration, Department of Health, Education, and Welfare

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Tracor Jitco, Inc.

Source: Farmakologiya i Toksikologiya 35 (1): 106-107, 1972

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Antagonism Between Ascorbic Acid and N, N, N', N' - Tetraethylthiuram Disulfide in their Effect on Carbohydrate Metabolism

M. A. Evets and M. V. Korablev
(Chair of Pharmacology - Head: Prof. M. V. Korablev-of the Grodno Medical Institute)

Summary

Ascorbic acid prevents an increase in the amount of pyruvic and lactic acids present in the blood and a rise of the enzymic activity of transketolase (KF 2.2.1.1) in the organs and tissues of rats under the effect of N, N, N', N'-tetraethylthiuram disulfide.

N, N, N', N'- tetraethylthiuram disulfide (TETD) causes in patients side reactions, which are cut off (stopped) by using ascorbic acid. In alcoholics, the latter removes asthma, pallor of integuments, a feeling of anxiety, and motor disorders (Niblo and coauthors, 1951; Worner, 1954). The mechanism of the antitoxic action of ascorbic acid has not been established; *in vitro*, it prevents the inhibiting effect of TETD on liver aldehyde dehydrogenase (Graham, 1951).

Our task was to determine the effect of TETD alone and in combination with ascorbic acid on some indices (characteristics) of the carbohydrate metabolism in the organism of rats.

Methods of Investigation

Tests were carried out on 25 rats of both sex, weighing 104-150 g and divided into 3 groups (with 8-9 rats in each group). Animals of the 1st and 2nd group received a single subcutaneous injection of an isotonic sodium chloride solution, while animals in the 3rd group received a single subcutaneous dose of 0.1 g/kg of an ascorbic acid solution. After 10 minutes, a single dose of starch mucilage was introduced into the stomach of rats in the 1st group, while animals of the 2nd and 3rd group received TETD in a dose of 1/5 LD_{50} per kg body weight, in the form of a suspension in starch mucilage introduced into the stomach. After 90 minutes, the animals were decapitated. In the blood, sugar was determined (Georgescu and Painescu, 1963), as well as pyruvic and lactic acids (N. N. Pushkina, 1963), and the enzymic activity of transketolase was determined in organ homogenates (Bruce *et.al.*, 1958). The activity of the enzyme was expressed in micromoles

of sedoheptuloso-7-phosphate formed per 1 g of tissue or as 1 billion erythrocytes per hour at 37°C. Calculation was done according to a standard curve plotted with the aid of sedoheptuloso-7-phosphate.

Results

TETD, without changing the amount of sugar, increases the content of pyruvic and lactic acid in the blood of rats. Under the effect of TETD, the amount of pyruvic acid increased by 86%, and that of lactic acid by 27% over the control data (Table 1).

The compound tested (TETD) also causes a regular rise in the enzymic activity of transketolase (Table 2).

Table 1 - Changes in the amount (in mg%) of sugar, pyruvic and lactic acid in the blood of rats under the effect of TETD alone and in combination with ascorbic acid (average data with confidence range).

Animal group	Sugar	Pyruvic	Lactic Acid
1st	173,5 (109,5 ÷ 237,5)	0,95 (0,88 ÷ 1,02)	22,8 (20,2 ÷ 25,4)
2nd	181,0 (118,3 ÷ 243,7)	1,77 (1,42 ÷ 2,12)	29,0 (26,5 ÷ 28,9)
3rd	188,0 (126,6 ÷ 249,4)	0,96 (0,88 ÷ 1,40)	22,6 (19,4 ÷ 25,8)

Table 2 - Changes in the enzymic activity of transketolase in the organism of rats under the effect of TETD alone and in combination with ascorbic acid.

Animal group	Organ	$\bar{X} \pm S_{\bar{x}}$	P	Animal group	Organ	$\bar{X} \pm S_{\bar{x}}$	P
1st	Brain	29,2 ± 2,73		1st	Spleen	124,74 ± 12,33	
2nd		37,23 ± 1,63	<0,05	2nd		183,70 ± 9,14	<0,002
3rd		27,47 ± 2,02	<0,01	3rd		131,76 ± 6,57	<0,001
1st	Blood erythrocytes	1,23 ± 0,095		1st	Kidneys	108,23 ± 7,18	
2nd		1,77 ± 0,176	0,02	2nd		139,94 ± 11,96	<0,05
3rd		1,30 ± 0,068	<0,05	3rd		104,14 ± 6,79	0,02
1st	Heart	7,77 ± 0,43		1st	Small intestine	127,7 ± 8,77	
2nd		10,84 ± 0,60	0,001	2nd		168,02 ± 6,24	<0,002
3rd		8,14 ± 0,58	<0,001	3rd		121,96 ± 8,54	<0,001
1st	Liver	212,3 ± 12,28		1st	Skeletal muscle	3,76 ± 0,28	
2nd		279,9 ± 15,71	<0,01	2nd		5,45 ± 0,53	<0,02
3rd		221,6 ± 14,83	<0,02	3rd		4,89 ± 0,50	<0,05

As can be seen from Tables 1 and 2, in case of a combined action of ascorbic acid and TETD, the acid prevents an increase in the amount of pyruvic and lactic acid in the blood and also the activation of transketolase in the organs and tissues of animals, caused by TETD.

Discussion of Results

As the data of tests with lactic and pyruvic acid show, TETD contributes to the development of anaerobic glycolysis in the organism of rats. Since TETD inhibits the activity of cytochromoxidase in the organism of these animals (M. V. Korablev and R. P. Simorot, 1965), it can be stated that anaerobic glycolysis is the result (consequence) of tissue hypoxia. We can assume that these changes lead to a compensatory rise in the activity of transketolase.

Ascorbic acid prevents the appearance, in the organism of rats, of glycolysis caused by TETD. Apparently, the action of ascorbic acid is due to its high reducing properties. Not only *in vitro*, but also *in vivo*, ascorbic acid protects and restores the activity of oxido-reductases. As a result, hypoxia does not arise or is removed, and the carbohydrate metabolism and transketolase activity are normalized. There are reasons to believe that the therapeutic and antitoxic action of ascorbic acid is based on the above mechanism, and this assumption is confirmed by clinical data. In alcoholics, ascorbic acid removes those side reactions of TETD which are caused by hypoxia.

Conclusion

Ascorbic acid is a typical antagonist of TETD. It prevents the development, in the rat organism, of anaerobic glycolysis and the rise of the enzymic activity of transketolase, caused by this compound.

Bibliography

1. Korablev, M. V., Simorot, R. P. Farmakol. i toksikol. 1965, No. 2, p. 234 (in Russian).

2. Pushkina, N. N. Biochemical research methods, Moscow, 1963, p. 105 (in Russian).

3. Georgescu, P., Peunescu, E., Biochemical methods of diagnosis and investigation, Bucharest, 1963, p. 118 (in Russian).

4. Bruns, F. H., Dunwald, E., Noltmann, E., Biochem. Z., 1958, Bd 330, S. 497. S. 497

4. Bruns, F. H., Dunwald, E., Noltmann, E., Biochem. Z., 1958, Bd 330, S. 497.

5. Graham, D. W., J. Pharm. (Lond.), 1951, v. 3, p. 160.

6. Niblo, G., Nowinski W., Roark D., Dis. nerv. Syst., 1951, v. 12. p. 340.

7. Worner, H., Dtsch. med. Wschr., 1954, Bd 79, S. 778.

Received for publication
on 19 October, 1970

2396

FARMAKOL. TOKSIKOL. 35(1): 106-107, 1972.

УДК 615.356:577.164.2].015.23:615.246.9:547.496.2

АНТАГОНИЗМ МЕЖДУ АСКОРБИНОВОЙ КИСЛОТОЙ и N, N, N′, N′-ТЕТРАЭТИЛТИУРАМДИСУЛЬФИДОМ ВО ВЛИЯНИИ НА ОБМЕН УГЛЕВОДОВ

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Аскорбиновая кислота препятствует увеличению количества пировиноградной и молочной кислот в крови и повышению ферментативной активности транскетолазы (КФ 2. 2. 1. 1) в органах и тканях крыс под влиянием N, N, N′, N′-тетраэтилтиурамдисульфида (Фармакол. и токсикол., 1972, № 1, с. 106).

N, N, N′, N′-тетраэтилтиурамдисульфид (ТЭТД) вызывает у больных побочные реакции, для купирования которых используют аскорбиновую кислоту. Последняя устраняет у алкоголиков удушье, бледность кожных покровов, головную боль, чувство страха и моторные расстройства (Niblo и соавторы, 1951; Wörner, 1954). Механизм антитоксического действия аскорбиновой кислоты не установлен. In vitro она препятствует ингибирующему влиянию ТЭТД на альдегиддегидрогеназу печени (Graham, 1951).

Мы поставили своей задачей установить влияние одного ТЭТД и его сочетания с аскорбиновой кислотой на некоторые показатели углеводного обмена в организме крыс.

Методы исследования

Опыты ставили на 25 крысах обоего пола весом 104—150 *г*, разбитых на три группы (по 8—9 крыс в каждой). Животным 1-й и 2-й групп однократно подкожно вводили изотонический раствор поваренной соли, а крысам 3-й группы — однократно подкожно раствор аскорбиновой кислоты в дозе 0,1 *г/кг*. По истечении 10 мин. крысам 1-й группы однократно в желудок вводили слизь крахмала, а животным 2-й и 3-й групп — ТЭТД в виде взвеси в слизи крахмала в дозе $^1/_5$ DL_{50} на 1 *кг* веса. Через 90 мин. животных обезглавливали. В крови определяли сахар (Джорджеску и Пэунеску, 1963), пировиноградную и молочную кислоты (Н. Н. Пушкина, 1963), а в гомогенатах органов — ферментативную активность транскетолазы (Bruns и соавторы, 1958). Активность энзима выражали в микромолях образовавшегося седогептулозо-7-фосфата на 1 *г* ткани или 1 млрд. эритроцитов за 1 час при 37°. Расчет проводили по стандартной кривой, построенной с помощью седогептулозо-7-фосфата.

Результаты

ТЭТД, не изменяя количества сахара, повышает содержание пировиноградной и молочной кислот в крови крыс. Под влиянием его количество первой увеличилось на 86%, а второй — на 27% в сравнении с контрольными данными (табл. 1).

Исследуемый препарат закономерно повышает ферментативную активность транскетолазы (табл. 2).

При комбинированном действии аскорбиновой кислоты с ТЭТД кислота, как видно из табл. 1 и 2, препятствует увеличению количества пировиноградной и молочной кислот в крови и активированию транскетолазы в органах и тканях животных, вызываемых ТЭТД.

Обсуждение результатов

Как свидетельствуют данные опытов с молочной и пировиноградной кислотами ТЭТД способствует развитию в организме крыс анаэробного гликолиза. Поскольку ТЭТД тормозит в организме этих животных активность цитохромоксидазы (М. В. Кораблев и Р. П. Симо-

Table 1

Таблица 1

Изменение количества (в *мг*%) сахара, пировиноградной и молочной кислот в крови крыс под влиянием одного ТЭТД и сочетания его с аскорбиновой кислотой (средние данные с доверительными границами)

Animal group Группа животных	Sugar Сахар	Pyruvic acid Пировиноградная кислота	Lactic acid Молочная кислота
1st 1-я	173,5 (109,5÷237,5)	0,95 (0,88÷1,02)	22,8 (20,2÷25,4)
2nd 2-я	181,0 (118,3÷243,7)	1,77 (1,42÷2,12)	29,0 (26,5÷28,9)
3rd 3-я	188,0 (126,6÷249,4)	0,96 (0,88÷1,40)	22,6 (19,4÷25,8)

Animal group Animal group

Таблица 2 Table 2

Изменение ферментативной активности транскетолазы в организме крыс под влиянием одного ТЭТД и сочетания его с аскорбиновой кислотой

Группа животных	Орган Organ	$\bar{X} \pm S_{\bar{x}}$	P	Группа животных	Орган Organ	$\bar{X} \pm S_{\bar{x}}$	P
1st 1-я	Головной мозг	29,2±2,73		1-я	Селезенка	124,74±12,33	
2nd 2-я		37,23±1,63	<0,05	2-я		183,70±9,14	<0,002
3rd 3-я		27,47±2,02	<0,01	3-я		131,76±6,57	<0,001
1st 1-я	Эритроциты крови	1,23±0,095		1-я	Почки	108,23±7,18	
2nd 2-я		1,77±0,176	0,02	2-я		139,94±11,96	<0,05
3rd 3-я		1,30±0,068	<0,05	3-я		104,14±6,79	0,02
1st 1-я	Сердце	7,77±0,43		1-я	Тонкий кишечник	127,7±8,77	
2nd 2-я		10,84±0,60	0,001	2-я		168,02±6,24	<0,002
3rd 3-я		8,14±0,58	<0,001	3-я		121,96±8,54	<0,001
1st 1-я	Печень	212,3±12,28		1-я	Скелетная мышца	3,76±0,28	
2nd 2-я		279,9±15,71	<0,01	2-я		5,45±0,53	<0,02
3rd 3-я		221,6±14,83	<0,02	3-я		4,89±0,50	<0,05

рот, 1965), можно утверждать, что анаэробный гликолиз является следствием тканевой гипоксии. Можно полагать, что эти изменения ведут к компенсаторному повышению активности транскетолазы.

Аскорбиновая кислота препятствует возникновению в организме крыс гликолиза, вызываемого ТЭТД. Действие ее обусловлено, по-видимому, высокими редуцирующими свойствами. Она не только in vitro, но in vivo защищает и восстанавливает активность оксидоредуктаз. В результате этого не возникает или устраняется гипоксия, нормируется обмен углеводов и активность транскетолазы. Есть основания полагать, что указанный механизм лежит в основе лечебно-антитоксического действия аскорбиновой кислоты. Предположение оправдывается клиническими данными. Аскорбиновая кислота устраняет у алкоголиков те побочные реакции ТЭТД, которые вызваны гипоксией.

Заключение

Аскорбиновая кислота является типичным антагонистом ТЭТД. Она препятствует развитию в организме крыс анаэробного гликолиза и повышению ферментативной активности транскетолазы, вызываемых этим препаратом.

ЛИТЕРАТУРА

Кораблев М. В., Симорот Р. П. Фармакол. и токсикол., 1965, № 2, с. 234. — Пушкина Н. Н. Биохимические методы исследования. М., 1963, с. 105. — Джорджеску П., Пэунеску Е. Биохимические методы диагноза и исследования. Бухарест, 1963, с. 118. — Bruns F. H., Dünwald E., Noltmann E., Biochem. Z., 1958, Bd 330, S. 497. — Graham D. W., J. Pharm. (Lond.), 1951, v. 3, p. 160. — Niblo G., Nowinski W., Roark D., Dis. nerv. Syst., 1951, v. 12. p. 340. — Wörner H., Dtsch. med. Wschr., 1954, Bd 79, S. 778.

Поступила 19/X 1970 г.

Gazz. Int. Med. Chir. 69:1268 - 1269, 1964

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NOTES ON THERAPY

ABOUT A CASE OF INGESTION OF IO GRAMS OF VITAMIN C

ALBERT FAZIO

The desire for strange foods, certain cravings which seem caprices in children and also adults, finds a physiological reason in the necessity of a contribution of definite substances indispensable for growth or for filling up the deficit or, yet, for restoring an organic equilibrium wich tends to break.

The classical avitaminoses, scholastic, at least with us, are a rarity today, especially, in a complete symptomatology.

More frequent are the hypovitaminoses with scarce or apparently absent symptomatology, wich shows up only in particular conditions of the organism: overfatigue, breast feeding, convalescence, antibiotic therapy, etc.

Very prevalent, instead, in our population, especially infantile are the so-called non-apparent distrophies, due to still lighter defects, wholly asymptomatic.

In the specific case of vitamin C or ascorbic acid, IOO mg per day are for a youngster, introduced in various manners, for intensifying the action of the adrenocortical hormones for maintaining normal the substance of the bones and of the teeth, for strengthening the power of immunization, for protecting the sanguine crasis and for promoting the absorption and perhaps the utilization of alimentary iron.

With an inadequate contribution, alimentary or not, of factor C, to the very variable individual real requirement, the human organism suc-

ceeds to maintain equally an apparent state of well-being for a time more or less long, due, however, to a precarious condition of equilibrium, such that it could be broken by one of any of the injurious pathogenic agents or not above cited.

Still, it is necessary to vitaminize much food-stuff, so that the daily intake of indispensable vitamins willnot be less than the individual and occasional requirements.

It is above all in children that it is necessary to administer optimal vitamin doses, including also mineral salts and vitamin C. This must be from birth and must last for all the time of development.

In my professional private practice I have the habit of vitaminizing my little patients, with results in weight, in stature, in liveliness and in wellbeing superior to others not treated by the same standard.

One of these, one day, unknown to his parents, swallowed about I0 g of a pharmaceutical preparation containing vitamin C in a confection made very pleasant to the palate.

Held under observation for I8 hours, the little patient did not give any complaint, neither gastric nor acidic, and has maintained unchanged his own good humor and the proper general sensation.

" ViterraPfizer " was used for vitaminizing the patients.

The little patient, four years old, had swallowed in one single turn about 20 pills (for a total of about I0grams) of Vicon Pfizer.

Gazz. Int. Med. Chir. 69: 1268-1269 1964

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SU UN CASO DI INGESTIONE DI 10 G DI VIT. C

ALBERTO FAZIO

Il desiderio di cibi strani, certe voglie che sembrano capricci, nei bambini e persino negli adulti, trovano una ragione fisiologica nella necessità di un apporto di determinate sostanze, indispensabili per l'accrescimento o per colmare un deficit oppure, ancora, per ristabilire un equilibrio organismico che tende alla rottura.

Le avitaminosi classiche, scolastiche, almeno da noi, sono oggi una rarità, soprattutto a sintomatologia completa.

Più frequenti sono le ipovitaminosi con scarsa o apparentemente assente sintomatologia, che si appalesa soltanto in particolari condizioni dell'organismo: sovraffaticamento, allattamento, convalescenza, antibiotico-terapia, etc.

Diffusissime, invece, nella nostra popolazione, specialmente infantile, sono le cosiddette distrofie inapparenti, dovute a carenze ancora più lievi, del tutto asintomatiche.

Nel caso specifico della Vitamina C o Acido ascorbico, occorrono, per un giovinetto, circa 100 mg pro die, introdotti in varia maniera, per intensificare l'azione degli ormoni corticosurrenali, per mantenere normale la sostanza delle ossa e dei denti, per potenziare i poteri immunitari, per proteggere la crasi sanguigna e per favorire l'assorbimento e forse l'utilizzazione del ferro alimentare.

Con un inadeguato apporto, alimentare o non, di fattore C, al variabilissimo fabbisogno reale individuale, l'organismo umano riesce a mantenere egualmente un apparente stato di benessere per un tempo più o meno lungo, dovuto, però, ad una condizione precaria di equilibrio, tale da poter venire rotta da una delle qualsiasi noxae patogene o non sopra riportate.

Pertanto, occorre vitaminizzare molte sostenze alimentari, affinchè l'apporto giornaliero di vitamine indispensabili non risulti inferiore al fabbisogno individuale ed occasionale.

E' soprattutto nei bambini che bisogna somministrare dosi

E' stato usato per vitaminizzare i pazienti il Viterra Pfizer.

Il piccolo paziente, di anni 4, ha ingerito in una sola volta circa 20 compresse (per un totale di circa 10 g) di Vicon Pfizer.

ottimali vitaminiche, comprendenti anche sali minerali e Vitamina C. Ciò deve avvenire sin dalla nascita e deve durare per tutto il periodo dello sviluppo.

Nella mia pratica professionale privata ho l'abitudine di vitaminizzare i miei piccoli pazienti, che risultano così di peso, di statura, di vivacità e di benessere superiori ad altri non trattati alla stessa stregua.

Uno di questi, un giorno, di nascosto ai genitori, ha ingerito circa 10 g di un preparato farmaceutico contenente Vitamina C in confezione tale da renderla gradevolissima al palato.

Tenuto sotto osservazione per 48 ore, il piccolo paziente non ha accusato alcun disturbo, nè gastrico nè acidotico, ed ha mantenuto inalterato il proprio buon umore e la propria cenestesi.

BIBLIOGRAFIA

FAZIO A.: *La fame di Vitamina C.* L'Economia Umana, 3, 1, 1956.
FAZIO A., CATALANI A.: *Sindromi neurologiche e psichiatriche da malnutrizione.* In corso di pubblicazione su « L'Informatore Medico ».
GAMNA C.: Medicina Interna, 1950.
GRAY C. H.: Clinical Chemical Patology, 1955.
MONASTERIO G.: Vitamine. 1949.

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J. Home Econ. 24, 827-832

VITAMIN C IN CANNED CITRUS PRODUCTS

CARL R. FELLERS AND PAUL D. ISHAM

BECAUSE of the increasing consumption of canned citrus products by American people and because physicians and mothers have repeatedly requested information on this point, particularly from the point of view of infant feeding, a vitamin C evaluation seemed desirable and was made at Massachusetts State College, Amherst.

There is little information bearing on canned citrus fruits in the literature. Delf (1) in England in 1925 reported that oranges canned by gradually heating to 100°C. and holding at this temperature for five minutes showed no loss in antiscorbutic property, and only a slight decrease in activity after four years. As canned at the present time, orange juice never exceeds a temperature of 65°C. and great care is taken to prevent oxidation. Apparently, the vitamin C activity of canned orange slices has not been reported. Koch and Koch (2) recently reported that in spite of cold storage or freezing, unstrained orange juice lost practically all of its vitamin C in three months. The method of storage was not stated, but air was probably not excluded. When a sirup was made of the juice, however, by the addition of sugar, citric acid, color, and preservative, there was practically no loss in vitamin C. Priston (3) likewise found orange concentrate to be a good source of vitamin C. The activity gradually decreased upon storage. MacLeod and Booher (4) in 1930 found that canned Florida grapefruit and grapefruit juice in amounts varying from 2.2 to 2.8 grams daily effectively protected young guinea pigs from scurvy and produced normal growth.

Methods and source of canned products. The methods employed in the present study were essentially those of Sherman, LaMer, and Campbell (5). To insure quantitative accuracy, feeding was done by means of a large-bore pipette. The canned slices were pulped by forcing through a coarse strainer.

The amount of fruit or juice fed was judged by weighing the animals before and during feeding process. Young guinea pigs weighing approximately 300 grams were used, the dose being apportioned according to weight. The test was run for 90 days. Autopsies were performed on the animals to determine the Sherman scurvy-score.

The oranges and grapefruit were grown and canned by the Dr P. Phillips Co., Orlando, Florida, and were taken from grocers' stocks in

Buffalo, New York. The fruit was tree-ripened, prepared promptly for canning, heat-exhausted, sealed in plain tin cans, and subjected to a short heat treatment. Free oxygen is never present in cans of fruit after a few days' storage, the oxygen uniting supposedly with the iron and tin

Average gains in weight and survival periods and scurvy scores of guinea pigs receiving canned citrus fruits and juices as sole source of vitamin C

PRODUCT	NUMBER OF ANIMALS	DAILY AMOUNT FED	AVERAGE GAIN IN WEIGHT	AVERAGE SURVIVAL PERIOD	SCURVY SCORE	REMARKS
		grams	*grams*	*days*		
Canned orange juice	3	1	334	90	0	All animals made excellent growth. No trace of scurvy
" " "	3	2	406	90	0	
" " "	2	3	404	90	0	
Canned orange slices	3	1	227	90	2 (3, 4, 0)	Animals in excellent physical condition
" " "	3	2	406	90	0	
" " "	2	3	301	90	0	
Canned grapefruit juice	3	1	326	90	2 (2, 2, 2)	Very slight scurvy lesions on teeth and ribs only. Excellent physical condition
" " "	3	2	370	90	0	
" " "	2	3	292	90	0	
Canned grapefruit slices	3	1	312	90	2 (4, 2, 0)	Very slight scurvy. Animals in excellent physical condition
" " "	3	2	454	90	0	
" " "	2	3	394	90	0	
Fresh orange juice	4	1.5	295	90	0	No scurvy: good physical condition
Fresh grapefruit juice	3	1.5	227	90	0	No definite scurvy lesions; not quite so good as orange
Basal ration only (negative control)	3	0	−112	27	19	Marked scurvy in every case. Extreme emaciation

of the container. The fruits are therefore stored in an essentially reducing medium, for hydrogen gas is slowly evolved during storage as a result of acid-metal interaction. This explanation is given at this place because of its importance in accounting in part for the nature of the results to be given later.

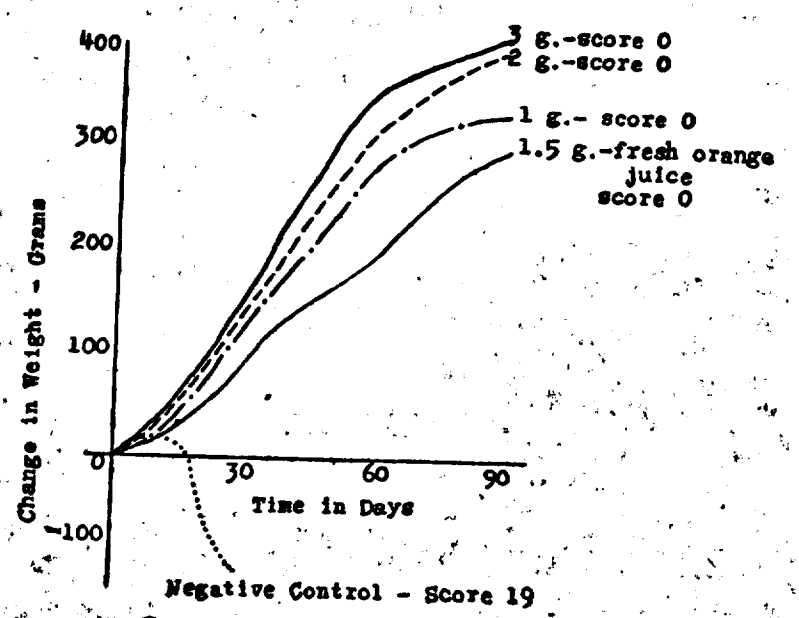

CHART 1. CANNED AND FRESH ORANGE JUICE

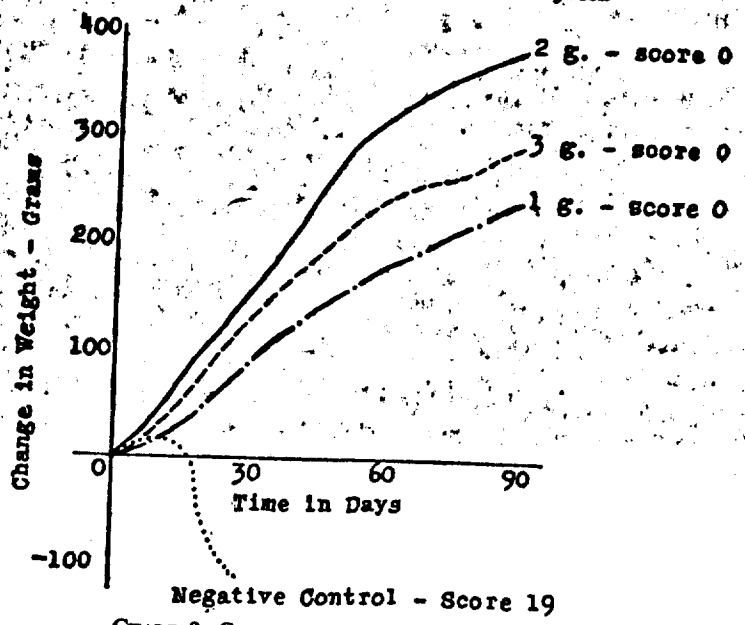

CHART 2. CANNED AND FRESH ORANGE SLICES

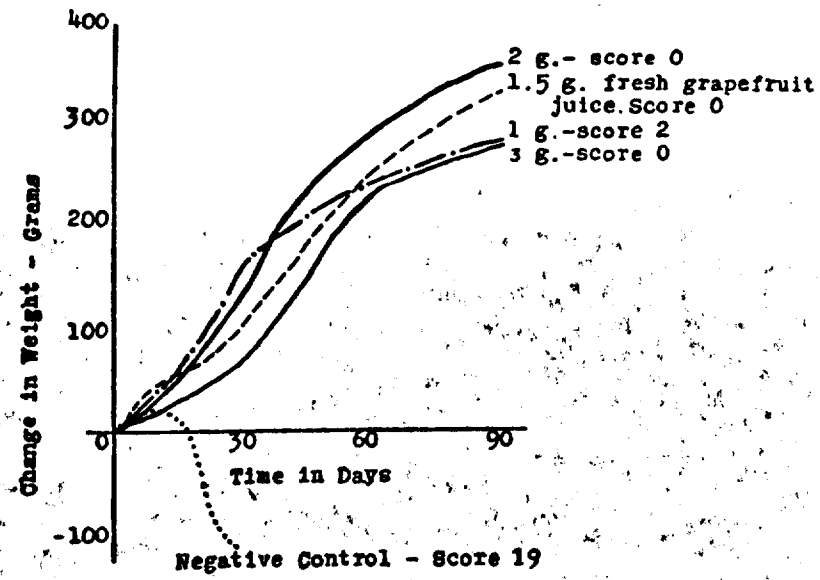

CHART 3. CANNED AND FRESH GRAPEFRUIT JUICE

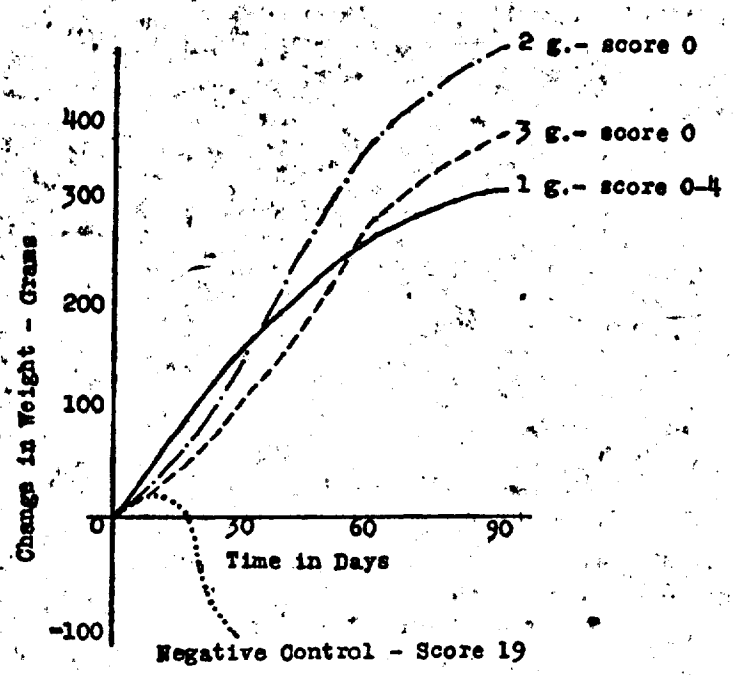

CHART 4. CANNED AND FRESH GRAPEFRUIT SLICES

When examined, the canned citrus products were from seven to ten months old.

Experimental results. The table and charts 1, 2, 3, and 4 present the experimental data.

One gram per day (6 days a week) of canned orange juice fully protected against scurvy in a 90-day test period. Canned orange slices were slightly less potent, but since a 1-gram dose gave only the slightest indications of scurvy (scores of 3, 4, and 0) and 2 grams fully protected, it is safe to assume that the protective dose of canned orange slices was only slightly more than 1 gram. Freshly expressed Florida orange juice fully protected from scurvy when fed at levels of 1.5 grams daily.

One gram of canned grapefruit juice or canned grapefruit slices did not quite afford protection over a 90-day test period. However, since two grams gave ample protection and excellent growth, the minimum protective dose may be estimated at slightly in excess of 1 gram, possibly 1.2 grams. It is apparent that grapefruit is only very slightly inferior to orange juice in its antiscorbutic properties.

Freshly expressed grapefruit juice afforded protection from scurvy when fed at the 1.5-gram daily level. Growth and physical condition were slightly inferior to those of animals fed on fresh orange juice. These juices were expressed from Florida citrus fruits bought in the public market at Amherst during August and September, that is, the fruit had undergone from 6 to 9 months' storage and still retained a high vitamin C potency. These results are in accord with those reported by Bracewell and Zilva (6), who found the time of picking and storage period had but little effect upon the vitamin C content of Honduras or South African citrus fruits. They found no correlation between the soluble solids, acid content, or pH of the juice and its antiscorbutic potency.

The negative controls, fed only the vitamin C-free basal ration, lived less than 30 days, showed marked emaciation, swollen joints, lameness, and inability to eat. At autopsy the characteristic scurvy lesions were found, namely, swellings and hemorrhages of the joints and rib junctions; hemorrhages in the intestines, bladder, and muscles; and marked disintegration and decomposition of the jaw and teeth.

Summary. 1. Canned orange juice was fully protective when fed to guinea pigs in daily doses of 1 gram. Canned orange slices, canned grapefruit slices, and canned grapefruit juice were slightly less effective than canned orange juice. Slightly more than 1 gram daily of these products afforded protection from scurvy and gave excellent growth curves in all animals.

2. The pipette method of feeding proved very satisfactory and is more exact than other technics used in feeding guinea pigs. All the animals took the food greedily.

3. Commercially canned orange juice, orange slices, grapefruit juice, or grapefruit slices after nine months' storage contained fully as much vitamin C as fresh citrus fruits. Canned citrus products are excellent sources of vitamin C and may be used in place of the fresh fruits. The hermetically sealed tin can prevents destructive oxidations of vitamin C in stored citrus fruits.

REFERENCES

1. Delf, E. M. *Biochem. J.* 19, 141–152 (1925).
2. Koch, E. M., and Koch, F. C. *J. Ind. Eng. Chem.* 24, 351–352 (1932).
3. Priston, J. L. *J. Roy. Naval Med. Service* 12, 1–20 (1926).
4. MacLeod, G., and Booher, L. J. Home Econ. 22, 588–593 (1930).
5. Sherman, H. C., La Mer, V. K., and Campbell, H. L. *J. Am. Chem. Soc.* 44, 165–172 (1922).
6. Bracewell, M. F., and Zilva, S. S. *Biochem. J.* 25, 1081–1089 (1931).

Food Protection Committee. 1972
Comprehensive GRAS Survey
National Academy of Sciences, National Research Council,
Wadhington, D.C.

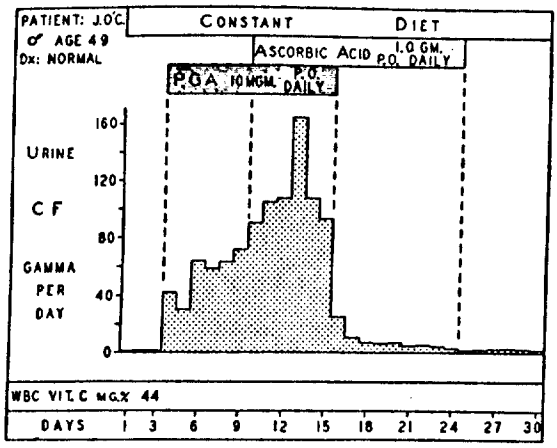

FIG. 3. URINARY EXCRETION OF CF FOLLOWING ORAL PGA IN A NON-SCORBUTIC MALE BEFORE AND AFTER ASCORBIC ACID THERAPY

during the first three days of study. Urinary CF ranged from 7.9 to 16.1 gamma daily when 10 mg. PGA were given orally daily for six days. During the next six days of study, 1.0 gm. of ascorbic acid was given intramuscularly daily to this patient in addition to PGA. Urinary CF was 12.6 gamma on the first day of ascorbic acid therapy, 15.2 gamma on the second day, and ranged from 61.8 to 129.3 gamma daily for four additional days. Ascorbic acid was then continued, but PGA omitted for six days. Urinary CF returned promptly to initial levels. For the next six days, PGA was again given in addition to ascorbic acid. The readministration of PGA was accompanied on the first day of this medication by the urinary excretion of 77.9 gamma of CF, 69.7 gamma on the second day, and 72.5 to 106.0 gamma daily for four additional days of this study period. Finally, ascorbic acid was discontinued, but PGA continued for five days during which time urinary CF excretion averaged 49.3 gamma daily.

J. O'C., Figure 3, a non-scorbutic control patient, excreted approximately 0.9 gamma of urinary CF daily during the first three days of study. When 10 mg. of PGA were given orally, urinary CF excretion rose promptly to 41.8 gamma on the first day and averaged 54.3 gamma daily during the six days of PGA administration. One gram of ascorbic acid was then given orally daily for six days in addition to the PGA. Urinary CF excretion was immediately further increased, and averaged 116.1 gamma daily for this period. Then for nine days, ascorbic acid was continued alone. The urinary CF excretion gradually decreased to 3.0 gamma daily. The urinary CF excretion during a final period of study which was comparable to the initial study period was again very low, ranging from 1.9 to 2.9 gamma daily.

DISCUSSION

A small amount of CF was excreted in the urine by the two patients with scurvy before PGA administration and was slightly increased by the oral administration of PGA before ascorbic acid therapy. However, maximal urinary CF excretion occurred in these patients only when both PGA and ascorbic acid were given. The relatively small increases in urinary CF excretion which were observed in the scorbutic patients when PGA alone was given during the scorbutic state or during the first several days of combined ascorbic acid and PGA therapy, which has also been noted by others (16), is in marked contrast to the immediate and large increases in urinary CF excretion which occurred in these same patients given PGA following adequate therapy with ascorbic acid. A prompt and great increase in urinary CF occurs when PGA is given orally to normal subjects and this increase is further augmented by the administration of ascorbic acid (14, 16). Similar results were obtained in the present study of a non-scorbutic subject during six days of PGA administered orally alone, and then six days of combined PGA and ascorbic acid. Thus the findings in the patients with scurvy given PGA are also markedly different from those observed in the non-scorbutic individual. However, following adequate therapy with ascorbic acid, these patients responded to PGA administration in a manner similar to that observed in the non-scorbutic individual. The failure to observe high urinary CF values in the three subjects studied during those periods of dietary supplementation with ascorbic acid alone indicates that the maximal increases in urine CF excretion observed when both PGA and ascorbic acid were given cannot be attributed to an effect of ascorbic acid upon the constituents of the diets provided.

Large amounts of PGA are known to stimulate the growth of *L. citrovorum* (1). Since the CF

(Quart. Jour. Stud. Alcohol 14(1):19-21.1953.

Effect of Intraperitoneal Administration of Alcohol on the Adrenal Levels of Cholesterol and Ascorbic Acid in Rats and Guinea Pigs[1]

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IN A PREVIOUS PUBLICATION (1) we reported on the marked reduction in the levels of cholesterol and ascorbic acid in the adrenal glands of rats and guinea pigs following intoxication by the oral administration of alcohol. Various doses were prepared from pure commercial 95 per cent ethyl alcohol (1 ml. equivalent to 0.76 g.) and administered as a solution of 50 per cent by volume in water. Smith (2, 3) has reported a similar effect from the oral administration of alcohol but the concentrations used were not stated in the published work.

Since the concentration of alcohol which we used is somewhat irritating to the gastrointestinal tract and tends to cause pyloric constriction with marked distension of the stomach, it was felt advisable to repeat some of the work with alcohol solutions known to be nonirritating to mucous membranes. Because it was not feasible to give the required dose of these more dilute solutions orally it was decided to use the intraperitoneal route. Accordingly we prepared solutions of 10 to 15 per cent by volume of the same commercial alcohol, diluted either with 5 per cent glucose or with 0.85 per cent sodium chloride, and administered them at the desired dose levels. Control animals received an equivalent amount of the diluting fluid only. All animals were fasted overnight and the injections made the next morning.

Adult albino rats of both sexes were used, although only one sex

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Received for publication: August 20, 1952.

was permitted in any one experiment. They were given 3 g. of alcohol per kg. of body weight as a 10 per cent by volume solution. The diluent was 5 per cent glucose in part of the experiments and 0.85 per cent saline in the others. The animals were sacrificed by rapid decapitation 4 to 5 hours after the intraperitoneal injections. In our previous work it was found that a definite reduction of both adrenal constituents had resulted by this time following oral administration of alcohol. The adrenals were removed at once, carefully dissected from surrounding tissue and weighed to the nearest 0.1 mg. One adrenal was used for ascorbic acid determination by the method of Roe and Keuther (4) and the other for cholesterol by the procedure of Outhouse and Forbes (5). The experimental results are shown in Figure 1. The values for the two diluents are given separately.

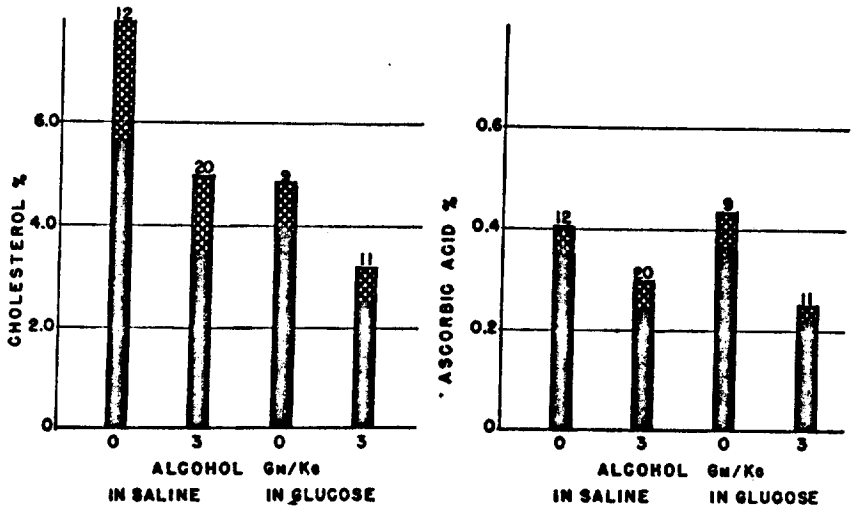

FIGURE 1.—*Changes in Cholesterol and Adrenal Ascorbic Acid Levels Following Intraperitoneal Injection of Alcohol in Fasted Rats.* The solid bars represent mean values expressed as per cent of wet adrenal tissue. The shaded areas indicate the positive half of the standard deviation. The numerals above the bars show the number of animals used; those below the bars, the amount of alcohol injected in grams per kilogram of body weight. Control animals received diluent only.

It will be seen that the cholesterol content of the adrenals of the saline controls was definitely higher than was the case with the glucose controls. Whether the difference between these groups of animals was due to an effect of the diluent upon the adrenal cholesterol cannot be stated at the present time. However, since the

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alcohol-treated and the control animals were always run at the same time and on animals comparable in age and sex, variation between the groups was controlled as far as the immediate purpose of the investigation is concerned. At autopsy, none of the animals showed any visible evidence of irritation. It will thus be noted that those animals which received alcohol showed an average reduction in adrenal cholesterol of 40 and 36 per cent, and in ascorbic acid of 32 and 40 per cent, over the corresponding controls. Thus the same general reduction was obtained as was previously reported (1) from the oral administration of alcohol.

Repetition of these experiments with male guinea pigs showed a reduction in adrenal cholesterol and ascorbic acid similar to that obtained in the rat. These animals were sacrificed approximately 24 hours after receiving the alcohol, since our earlier work with orally administered alcohol had shown that a marked reduction in both adrenal substances could be demonstrated at that time.

It appears evident, therefore, that the adrenal changes previously noted after alcohol administration must have been due primarily to the alcohol. One experiment was carried out with 10 guinea pigs in which a 25 per cent by volume solution of alcohol was given orally. Although this solution caused no visible sign of irritation, the reduction in the levels of adrenal cholesterol and ascorbic acid was as great as that obtained from the more concentrated but irritating solution.

Summary

Intraperitoneal injections of nonirritating alcohol solutions have been shown to cause the same general reduction in adrenal cholesterol and ascorbic acid concentration as was previously reported from the oral administration of a 50 per cent by volume solution of 95 per cent alcohol.

REFERENCES

1. Forbes, J. C. and Duncan, G. M. The effect of acute alcohol intoxication on the adrenal glands of rats and guinea pigs. Quart. J. Stud. Alc. **12:** 355–359, 1951.
2. Smith, J. J. The endocrine basis and hormonal therapy of alcoholism. N. Y. St. J. Med. **50:** 1704–1706, 1711–1715, 1950.
3. Smith, J. J. Effect of alcohol on the adrenal ascorbic acid and cholesterol of the rat. J. clin. Endocrin. **11:** 792, 1951.
4. Roe, J. H. and Keuther, C. A. 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, 1943.
5. Outhouse, E. L. and Forbes, J. C. A micromethod for determination of tissue lipids. J. Lab. clin. Med. **25:** 1157–1163,1940.

Science (Washington) 169(3949): 989-991 1970

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Cadmium Toxicity Decreased by Dietary Ascorbic Acid Supplements

Abstract. *Feeding the environmental toxicant cadmium to young Japanese quail for 4 weeks produced growth retardation, severe anemia, low concentrations of iron in the liver, and high concentrations of cadmium in the liver. Dietary ascorbic acid supplements almost completely prevented the anemia and improved the growth rate but did not markedly alter concentrations of iron or cadmium in the liver.*

The hazards of industrial exposure to high concentrations of cadmium have been recognized for many years. More recently, attention has focused on cadmium intake from food, water, air, and cigarettes as a source of risk to the general population, primarily in the production of cardiovascular and respiratory diseases (*1*).

We have investigated the effect of a wide range of dietary components upon the toxic effects of cadmium in the young Japanese quail (*Coturnix coturnix japonica*). This bird has an exceptionally rapid growth rate and is very sensitive to dietary deficits and to toxic materials. We observed that dietary ascorbic acid supplements produce a marked protective effect on the anemia and a lesser protective effect on the depressed growth rate caused by cadmium.

Day-old birds of both sexes were fed an adequate purified soybean protein diet containing a total of 75 mg of zinc per kilogram and approximately 100 mg of iron per kilogram (*2*) for a period of 4 weeks. The basal diet was either fed alone or supplemented with cadmium, ascorbic acid, or cadmium plus ascorbic acid at the concentrations indicated in Table 1. Birds received

Table 1. Protective effects of ascorbic acid against cadmium toxicity in young Japanese quail. Mean values ± standard error at 4 weeks of age.

Dietary cadmium (mg/kg)	Dietary ascorbic acid (% by weight)	Mortality*	Body weight (g)	Hematocrit value (% by volume)	Trace elements in liver (μg/g fresh weight)			
					Zinc	Iron	Copper	Cadmium
				Experiment 1				
0	0	0/8	80 ± 1.6	40 ± 1.2	26 ± 1.4	78 ± 7.8	5.7 ± 0.26	<0.1
75	0	3/9	57 ± 3.1	17 ± 1.0	21 ± 1.1	35 ± 2.9	5.0 ± 0.27	20 ± 2.5
75	1.00	1/7	78 ± 2.2	35 ± 1.4	25 ± 1.7	45 ± 5.8	5.6 ± 0.31	21 ± 0.9
				Experiment 2				
0	0	0/8	77 ± 1.4	40 ± 1.0	21 ± 1.4	89 ± 8.0	4.7 ± 0.20	<0.1
0	1.00	0/8	78 ± 2.4	41 ± 1.2	20 ± 0.5	123 ± 20.9	5.0 ± 0.14	<0.1
75	0	1/6	66 ± 2.5	21 ± 1.0	20 ± 0.9	30 ± 2.1	4.6 ± 0.30	17 ± 2.0
75	0.05	1/7	62 ± 4.3	29 ± 1.4	21 ± 1.0	32 ± 1.6	5.5 ± 0.27	23 ± 1.4
75	0.10	0/7	65 ± 1.8	33 ± 0.5	24 ± 1.5	36 ± 1.5	5.5 ± 0.25	21 ± 1.6
75	0.50	1/7	69 ± 5.0	34 ± 0.7	27 ± 1.2	36 ± 4.4	5.6 ± 0.63	22 ± 2.0
75	1.00	0/8	72 ± 1.6	35 ± 0.8	25 ± 1.9	43 ± 7.4	6.1 ± 0.53	20 ± 1.0

* Number of deaths during period from 1 to 4 weeks of age divided by the total number of birds at 1 week.

diets and demineralized drinking water freely. Rigid procedures were followed to obviate environmental contamination with trace elements (*2*). Eight or nine birds were assigned to each group. Some deaths during the first week were unrelated to the experimental treatment; therefore all deaths during this period were excluded from the mortality values. At 4 weeks of age, birds were weighed, microhematocrits were determined for blood collected from the wing vein, and livers were removed, weighed, wet-ashed, and assayed for zinc, iron, copper, and cadmium by atomic absorption spectrophotometry (*3*).

The data in Table 1 show that anemia was the most marked sign of cadmium toxicity. For every concentration of dietary ascorbic acid supplement used, there was a significant improvement in the hematocrit reading over that of birds receiving cadmium alone. In another experiment (data not shown) identical in design to that of experiment 1, the hematocrit values were very similar to those in experiment 1 and the hemoglobin concentrations, as determined by the cyanmethemoglobin method, were 11.7, 2.7, and 9.2 g of hemoglobin per 100 ml of blood, respectively.

The concentrations of zinc and copper in the liver were little affected by supplemental administration of either cadmium or ascorbic acid. In both experiments the birds receiving 1 percent (by weight) dietary ascorbic acid with cadmium had significantly higher ($P < .05$) concentrations of zinc in the liver than birds receiving cadmium alone. The physiological importance of this effect is questionable because of the variation between the two control groups not receiving supplements. The concentration of iron in the liver was markedly decreased by the administration of cadmium. Birds fed ascorbic acid in combination with cadmium showed a small increase in the concentration of iron in the liver over that in birds fed cadmium alone. The difference was statistically significant ($P < .01$) when data for identical treatments from experiment 2 were combined with those from experiment 1 and compared by Student's *t*-test. High concentrations of cadmium accumulated in the livers of all birds receiving cadmium, irrespective of ascorbic acid supplements.

The depressed growth rate of birds fed cadmium was improved by the administration of ascorbic acid. Some deaths in experiment 1 were produced by cadmium but not in experiment 2. Mortality rates and the severity of the anemia have been somewhat variable during the 2-year period spanned by these and the previously reported experiments (*2*). It seems likely that part of this variation may be related to variability in the soybean protein of inorganic components other than zinc, iron, and cadmium. The concentrations of zinc, iron, and cadmium were monitored throughout.

We reported earlier that the anemia produced by cadmium was accompanied by elevated concentrations of transferrin and low concentrations of iron in blood plasma (*2*). These results and the present data on iron concentrations in the liver support the conclusion that a primary effect of cadmium under these conditions was the production of an iron-deficiency anemia. The ascorbic acid probably improved the utilization of iron, but not sufficiently to correct the anemia completely or to permit storage of iron in the liver. Copper concentrations in the liver were normal throughout; it thus appears that neither cadmium nor ascorbic acid influenced copper metabolism. Hill and Starcher reported that ascorbic acid supplements decreased copper absorption from the intestine and exacerbated symptoms of copper deficiency in chicks fed a diet low in copper (*4*). A concentration of 8 to 9 mg of total copper per kilogram, as present in our diet, was probably in excess of the requirement. It is not known whether the growth depression caused by cadmium was related to antagonism of zinc, iron, or some other factor. The zinc content of the diet was approximately three times the required level (*5*).

On the basis of the amounts of cadmium in the liver, the protective effect of ascorbic acid does not appear to involve prevention of cadmium absorption. It has been shown in in vitro perfusion studies that ascorbic acid depresses the uptake of zinc and cadmium by the isolated small intestine of the rat but that ascorbic acid enhances the subsequent transport (*6*). It is probable that ascorbic acid did not accelerate the loss of cadmium, although Shcherbakova showed that the combination of ascorbic acid and cysteine does accelerate the loss of cadmium from rabbits (*7*).

These data do not preclude the possibility that cadmium interferes with the normal synthesis of ascorbic acid by this bird. The lowest amount of ascorbic acid fed, which appears to be about the minimum protective level, is approximately two and one half times the vitamin C requirement of the guinea pig (*8*).

In this study a very high intake of cadmium, superimposed on an adequate diet, was chosen for the experimental advantage of providing marked effects in a short period of time. On the basis of these results and the well

known cadmium-zinc antagonism, it is possible that dietary zinc, iron, and ascorbic acid are important factors in modifying the adverse effects of cadmium in human beings.

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References and Notes

1. H. A. Schroeder, *J. Chronic Dis.* **18**, 647 (1965); ———, A. P. Nason, I. H. Tipton, J. J. Balassa, *ibid.* **20**, 179 (1967); R. E. Carrol, *J. Amer. Med. Ass.* **198**, 267 (1966); M. Nandi, H. Jick, D. Slone, S. Shapiro, G. P. Lewis, *Lancet* **1969-II**, 1329 (1969); G. P. Lewis, H. Lyle, S. Miller, *ibid.*, p. 1330.
2. R. M. Jacobs, M. R. S. Fox, M. H. Aldridge, *J. Nutr.* **99**, 119 (1969).
3. Trace element analyses were carried out with an atomic absorption spectrophotometer (Perkin-Elmer model 303).
4. C. H. Hill and B. Starcher, *J. Nutr.* **85**, 271 (1965).
5. M. R. S. Fox and R. M. Jacobs, *Fed. Proc.* **26**, 524 (1967).
6. B. M. Sahagian, I. Harding-Barlow, H. M. Perry, Jr., *J. Nutr.* **93**, 291 (1967).
7. I. V. Shcherbakova, *Sb. Nauch. Tr. Ryazan. Med. Inst.* **15**, 190 (1962).
8. Committee on Animal Nutrition, *Nutrient Requirements of Laboratory Animals* [*Nat. Acad. Sci. Nat. Res. Counc. Publ.* **990** (1962), p. 19].
9. We thank J. J. Gantt, Mrs. M. S. Smith, and Mrs. L. M. Mines for excellent technical assistance.

27 April 1970

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J Am Med Assoc 162, 1224-6 (1956)

BLOOD ASCORBIC ACID LEVEL IN BIOFLAVONOID AND ASCORBIC ACID THERAPY OF COMMON COLD

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and
Henry L. Heyl, M.D., Hanover, N. H.

• *The incidence and course of common colds were followed in 89 medical students and nurses who volunteered in a study of the efficacy of naringin, a bioflavonoid extracted from grapefruit peel and used in remedies for colds. One group of 22 subjects received 333 mg. of naringin and 65 mg. of ascorbic acid three times daily for three months; a second group received naringin only, another ascorbic acid only, and the fourth a placebo. These substances were administered in capsules as nearly alike as possible. Symptoms of colds were systematically recorded, and the levels of ascorbic acid in the blood were determined periodically. The average level of ascorbic acid in 22 men who were to receive ascorbic acid by mouth was initially 0.96 mg. per 100 cc. of blood; after 12 weeks of taking only ascorbic acid the average for this group was 1.41 mg. per 100 cc. of blood. In all groups and at all stages, the blood ascorbic acid levels for males were significantly lower than those for females. There was no evidence that the naringin affected the ascorbic acid level of the blood, prevented colds, or cured them.*

During the past year treatment with the bioflavonoids has been widely publicized as a means of preventing or modifying the common cold. One of the reasons given for the effect has been that they potentiated ascorbic acid (vitamin C) in alleviating cold symptoms. Since there was no convincing proof available, either in support or in denial, we undertook a pilot study to explore the validity of these statements. Within the limits of this study, the bioflavonoid tested showed effect neither on the incidence or cure of colds nor on the ascorbic acid content of the blood. In the course of the study, interesting data were developed regarding the relationship of blood ascorbic acid levels to the incidence and cure of colds. These are reported in detail.

Method

The study was made on 89 volunteer medical students and student nurses for the three-month period from February to May, 1956. Four groups were established and given capsules (three times daily,

From the Dartmouth Medical School and the Hitchcock Foundation.

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orally) made up as follows: group 1, bland placebo; group 2, naringin (bioflavonoid), 333 mg., and ascorbic acid, 65 mg.; group 3, ascorbic acid, 65 mg.; and group 4, naringin (bioflavonoid), 333 mg. (Naringin is an extract of grapefruit peel.) Every effort was made to disguise the contents of the capsules. All looked and tasted alike. Even the doctors conducting the study did not know the key to the code numbers used. Groups were assigned in rotation. Thus the first volunteer in line received bland placebo, the second naringin (bioflavonoid) and ascorbic acid, and the fifth bland placebo, as the rotation started over again.

TABLE 1.—*Blood Ascorbic Acid Level Determinations in Group 1: Patients Given Placebo*

Patient No.	Sex	Level, Mg./100 Cc. Initial Wk.	6th Wk.	12th Wk.	During Cold	Wk. of Cold	Results of Treatment
1	M	1.02	0.60	1.50	0.80	5th	No change
5	M	1.04	1.32	1.60	1.36	5th	Improved
9	M	1.12	1.08	1.52			
13	M	0.72	1.08	1.80	1.24	7th	No change
					1.84	4th	Cured
17	M	1.12	1.12	1.10	1.32	4th	Improved
21	M	0.80	0.76	1.30			
25	M	1.00	1.28	0.88			
29	M	0.40	0.44	0.40			
33	M	1.12	1.16	0.44			
37	F	1.60	1.40	1.28			
41	M	1.16	1.52	1.44			
45	M	1.08	1.12	0.96			
49	M	1.08	1.28	1.10			
53	F	1.60	1.80	...			
57	F	1.20	1.20	...			
61	M	0.76	0.88	1.12			
65	F	1.60	1.16	1.08			
69	F	1.12	1.32	...			
73	F	1.44	1.40	1.40			
77	M	0.44	0.28	0.50	0.40	5th	Worse
81	F	0.52	0.70	1.54	0.80	6th	No change
85	F	1.48	1.64	1.12			
89	M	1.36	...	...			

TABLE 2.—*Blood Ascorbic Acid Level Determinations in Group 2: Patients Given Bioflavonoid and Ascorbic Acid*

Patient No.	Sex	Level, Mg./100 Cc. Initial Wk.	6th Wk.	12th Wk.	During Cold	Wk. of Cold	Results of Treatment
2	M	1.12	1.48	2.00			
6	M	1.08	1.12	1.48	1.08	2nd	Cured
10	M	0.80	1.16	1.28	0.96	2nd	Cured
					1.16	6th	Worse
14	M	0.96	1.36	1.30			
18	M	1.12	1.60	1.38	1.12	1st	Improved
					1.20	4th	Improved
22	M	0.56	1.52	1.10			
26	M	0.72	1.28	1.20			
30	M	0.72	1.52	...			
34	M	0.96	1.60	1.30			
38	F	1.48	2.00	1.72			
42	M	1.12	1.48	1.30	1.60	4th	Cured
46	M	0.92	1.08	1.04			
50	M	1.08	1.44	1.50			
54	F	1.40	1.84	...			
58	F	1.76	1.84	1.28			
62	F	1.84	1.64	...			
66	F	1.40	1.56	1.72			
70	F	1.88	1.76	1.56			
74	F	1.48	1.44	1.84			
78	M	1.32	1.40	1.84			
82	M	0.96	1.48	...			
86	F	1.28	1.92	1.68			

Each subject received a regional physical examination and had blood taken for a determination of ascorbic acid level the day he started the study. All the volunteers were started on the regimen within a single week. Thereafter they continued taking their particular capsule for the three months of the study. At the 6-week and 12-week points in the study, determinations of blood ascorbic acid level were made on all 89 participants. The subject was instructed to report cold symptoms (such as coryza, headache, malaise, fever, or sore throat) the day of onset. At this time the physical examination and determination of blood ascorbic acid level were repeated. He was asked to take no specific cold remedies, not even aspirin or nasal sprays, during the course of the infection. He continued taking the same dosage of the capsule assigned to his group. No antibiotics were used. During the infection the same physician made a record (daily when possible) of the cold's progress or regression. On the fifth day the cold was evaluated as cured, improved, unchanged, or worse.

Two variables were accepted as calculated risks to the accuracy of the study: diet and individual reliability. Although the majority of the subjects ate in the same hospital cafeteria, precise control of their diet was felt to be beyond the scope of this essentially pilot study. We recognized, of course, the varying degrees of reliability with which 89 persons would take 3 capsules a day for three months; we believe, however, that close supervision and the medical orientation of the participants kept this factor of error at a minimum.

TABLE 3.—*Blood Ascorbic Acid Level Determinations in Group 3: Patients Given Ascorbic Acid*

Patient No.	Sex	Level, Mg./100 Cc. Initial Wk.	6th Wk.	12th Wk.	During Cold	Wk. of Cold	Results of Treatment
3	M	1.08	1.32	1.50	1.40	3rd	Improved
7	M	1.28	1.52	2.00	1.92	3rd	Cured
11	M	1.12	1.36	2.00	1.32	3rd	Cured
15	M	0.96	1.68	1.24	1.00	3rd	Improved
19	M	1.12	1.08	0.80	1.08	3rd	Cured
23	M	1.08	1.52	1.10			
27	M	0.72	1.28	1.40			
31	M	1.08	1.16	1.70			
35	M	0.72	0.96	...			
39	F	0.96	...	...			
43	M	1.12	1.48	1.20			
47	M	0.76	1.44	1.50			
51	F	1.16	1.28	...			
55	F	2.04	2.08	1.32			
59	F	1.32	1.28	1.20	1.68	1st	Cured
63	F	1.40	1.52	1.84	1.52	4th	Cured
67	F	1.28	1.72	...			
71	F	1.28	1.40	1.52			
75	F	1.12	1.56	2.72			
79	M	0.88	1.36	1.08	1.52	4th	Improved
83	M	0.96	...	...			
87	M	0.52	...	...			

Tables 1, 2, 3, and 4 give the detailed blood ascorbic acid levels accumulated during this study. They are included in their complete form, partly because they are the basis for the conclusions of this pilot study and partly because comparable records of blood ascorbic acid levels in normal young adults are relatively rare. (Ascorbic acid blood levels were determined according to the technique of Roe and Kuether [1] under the supervision of John P. Davison, Ph.D., assistant professor of the physiological sciences in the Dartmouth Medical School.)

Results

Table 5 summarizes the effectiveness of bioflavonoid with and without ascorbic acid. When used alone, it produced the same results as a placebo. When used with ascorbic acid, the results were comparable to

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nose with ascorbic acid alone. Table 6 indicates that the prolonged administration of bioflavonoid had no effect on the blood ascorbic acid level. Females consistently showed a higher blood ascorbic acid level than males (table 6—statistically significant at the 0.01 level). Most of those receiving ascorbic acid showed an increased blood ascorbic acid level. Those receiving ascorbic acid developed just as many colds as those not receiving it (table 5). In this small series, those receiving ascorbic acid showed more rapid improvement in their colds than those not receiving it (tables 1 to 5 inclusive—statistically significant at the 0.05 level).

Comment

Ascorbic acid deficiencies have been noted not only in classic scurvy but in other infectious and metabolic disorders, such as rheumatoid arthritis, rheumatic fever, tuberculosis, and certain virus infections. The importance of ascorbic acid in maintaining capillary integrity has long been recognized. We will discuss neither the related background nor the literature.

Table 4.—*Blood Ascorbic Acid Level Determinations in Group 4: Patients Given Bioflavonoid*

Patient No.	Sex	Level, Mg. /100 Cc. Initial Wk.	6th Wk.	12th Wk.	During Cold	Wk. of Cold	Results of Treatment
4	M	1.20	1.28	1.70	1.28	5th	Cured
8	M	1.08	0.88	1.20			
12	M	0.72	1.20	1.20			
16	M	0.96	0.72	1.00	1.06	5th	Improved
					0.96	1st	Worse
20	M	1.08	1.08	1.00			
24	M	1.00	1.20	0.96			
28	M	1.12	1.08	0.80			
32	M	1.28	1.40	1.00			
36	M	0.88	1.08	1.20	1.00	3rd	Worse
40	M	1.40	0.88	1.36			
44	M	1.08	0.96	1.52	1.08	1st	Improved
48	M	1.28	0.76	1.20			
52	F	1.08	1.20	1.28	1.12	7th	Improved
56	F	1.75	1.64	...			
60	F	1.56	1.48	1.32	1.08	3rd	Improved
					1.20	5th	Worse
64	F	1.36	1.40	1.44			
68	F	1.80	1.84	1.60			
72	F	1.48	...	...			
76	F	1.72	1.84	...			
80	M	0.64	0.96	0.60			
84	F	1.24	...	...			
88	F	1.36	1.48	1.50			

Recently certain naturally occurring substances have been found to have a related function in maintaining the integrity of the intercellular cement substance. The materials as a group have been classified as flavone glycosides and include such substances as hesperidin, rutin, and naringin. We are particularly concerned with those substances derived from citrus fruit peels, grouped together under the title of bioflavonoids. The significant literature on this subject was summarized at a meeting of the New York Academy of Sciences in 1955, which is reported in the annals of that organization.[2] Various articles in this monograph suggest a relationship between ascorbic acid and the bioflavonoids, with particular reference to their effects on capillary fragility. The monograph also reports the clinical use of the bioflavonoids and ascorbic acid in the treatment of disorders related to decreased capillary permeability. These include poliomyelitis, rheumatic fever, and bleeding related to pregnancy. The hypothesis that the bioflavonoids may prevent or modify colds is apparently based on this same property.

With this possibility in view, a number of drug compounds appeared on the market during the winter of 1955-1956 featuring a bioflavonoid content. Most of these compounds also contained such old cold treatment stand-bys as aspirin, caffeine, and ascorbic acid. The limited statements regarding this treatment of colds reported the use of the whole compound, without reference to specific studies based on the effect of the bioflavonoid alone.

We are reporting a pilot study on the effectiveness of one bioflavonoid per se. It is, of course, not definitive. But, in the absence of any comparable evaluation of a material whose commercial distribution is already widespread, we feel that even this limited study may be helpful to the medical profession. The approximately 300 determinations of blood ascorbic acid level were a by-product of the bioflavonoid study. The series is far too small to warrant conclusive interpretations, but the data raise several points of interest that may serve as points of departure in more definitive studies.

Table 5.—*Incidence and Cure of Colds with Bioflavonoid and Ascorbic Acid*

Group	No. in Group	No. Colds in Group	Colds Cured or Improved in 5 Days
Placebo	23	7	3
Bioflavonoid plus ascorbic acid	22	6	5
Ascorbic acid	22	8	8
Bioflavonoid	22	8	4

Table 6.—*Average Blood Ascorbic Acid Level in Males and Females*

	Level, Mg./100 Cc. Placebo		Bioflavonoid Only		Ascorbic Acid Only		Bioflavonoid and Ascorbic Acid	
	M	F	M	F	M	F	M	F
Initial	0.95	1.32	1.06	1.48	0.96	1.32	0.96	1.56
6th wk.	0.99	1.33	1.04	1.55	1.35	1.55	1.39	1.76
12th wk.	1.12	1.28	1.12	1.43	1.41	1.72	1.39	1.63

Summary

We are reporting a controlled pilot study of the clinical effect of bioflavonoids and ascorbic acid (vitamin C) on the incidence and cure of the common cold, correlated with serial determinations of blood ascorbic acid level on the 89 medical students and student nurses comprising the test group. The administration of a bioflavonoid had effect neither on the incidence or cure of colds nor on the ascorbic acid level of the blood.

This study was supported by grants from the Nepera Chemical Co., Inc., Yonkers, N. Y., and the Harriet M. Spaulding Charitable Trust.

Statistical analyses were done by J. Laurie Snell, assistant professor of mathematics, Dartmouth College.

All drug preparations were provided by the Nepera Chemical Co., Inc.

References

1. Roe, J. H., and Kuether, C. A.: Determination of Ascorbic Acid in Whole Blood and Urine Through 2, 4-Dinitrophenylhydrazine Derivative of Dehydroascorbic Acid, J. Biol. Chem. 147: 399-407 (Feb.) 1943.

2. Bioflavonoids and the Capillary, edited by R. W. Miner, Ann. New York Acad. Sc. (special issue) 61: July 8, 1955.

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Proc. Amer. Assoc. Cancer Res. 3(2): 112, 1960

85. THE ANTICARCINOGENIC EFFECT OF ASCORBIC ACID AND OF ASCOPHYLLUM NODOSUM ON URETHAN-INDUCED PULMONARY ADENOMAS IN STRAIN A MICE. Frederic A. French* and B. L. Freedlander. (Research Labs., Mount Zion Hosp. and Medical Center, San Francisco, Calif.)

The thesis that dietary factors can mitigate carcinogenesis has been under study in this laboratory for some time. The authors' earlier work (Proc. Am. Assoc. Cancer Research, 3:21, 1959), wherein it was shown that high levels of nicotinamide were anticarcinogenic, has been extended. Female strain A mice, 4 months of age, were divided into experimental and control groups. Experimental Group 1 (43 mice) received 3 per cent Norwegian Kelp meal (Ascophyllum nodosum) in the diet. Experimental Group 2 (42 mice) received 0.5 per cent ascorbic acid (neutralized to pH 5) in their drinking water. Eighty-one control mice were used. Two weeks later all mice were given urethan intraperitoneally (1 mg/gm mouse). Eleven weeks later the mice were sacrificed. Pulmonary adenomas were counted visually. The average number of tumors per mouse was: controls, 7.77 ± 0.39; ascorbic acid-treated, 5.17 ± 0.48; kelp-treated, 5.23 ± 0.40. The observed differences and P values were: controls vs. ascorbic acid-treated group, 2.60, $P < 0.00005$; controls vs. kelp-treated group, 2.54, $P < 0.00005$. All mice remained healthy throughout the experiment. Weight gains were normal, and no abnormalities were revealed on autopsy. Replicate and additional experiments are in progress and will be reported.

Amer. J. Clin. Nutr. 18:256-260 1966

Interrelationships Between Estrogenic Activity, Serum Iron and Ascorbic Acid Levels During the Menstrual Cycle

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PLASMA iron alternations during the menstrual cycle have been studied previously and reported to decrease during menstruation.[1,2] The plasma total iron-binding capacity, in addition to the plasma iron level, provides a more accurate biochemical index of iron deficiency. However, serial changes in this parameter (plasma total iron-binding capacity) during menstruation have not been reported. Since menstruation has been shown to result in an appreciable loss of body iron,[3] alterations in the iron-binding capacity of serum or plasma among menstruating women would be anticipated. Plasma iron levels have been shown also to vary with different levels of dietary ascorbic acid[4] and exogenous estrogens.[5]

These relationships stimulated our simultaneous study of each of these indices in women with normal menstrual cycles. The levels of blood ascorbic acid and plasma iron, plasma total iron-binding capacity and biological assay of estrogenic activity were determined serially through two complete menstrual cycles of six healthy young women. The results have been examined for statistical significance and possible interrelationships.

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This work was supported in part by a grant-in-aid from E. Dalton Co.

MATERIAL AND METHODS

Six young women in their early twenties who were employed as nurses and laboratory technicians volunteered for the study. These women, judged healthy on the basis of physical examination, were menstruating normally. The initial phase of the study was devoted to familiarizing the women with the purposes of the investigation. Each subject was instructed in the procedure to be used for obtaining her own vaginal smears and told to abstain from those foods containing large amounts of ascorbic acid and iron on the day prior to blood sampling.

The estrogen levels of the subjects were estimated by the cornification index of their vaginal smears.[6,7] Smears were taken each morning, preserved in a 1:1 ether ethanol mixture and brought to the laboratory the same day or on the Monday following the weekend. Smears were not obtained during actual menses.

Heparinized blood samples (2.0 ml.) were drawn every third day in each subject when she was not menstruating, and daily during menstruation. Aliquots of each blood sample were utilized for hemoglobin, hematocrit and ascorbic acid determinations. Blood ascorbic acid (ascorbic, dehydroascorbic and diketogulonic acids) was measured by the microprocedure of Lowry, Bessey and Burch.[8] The remaining portion was centrifuged (3,000 r.p.m. for 15 minutes) to obtain the plasma. The plasma iron and total iron-binding capacity were determined by a modification of the method of Beale.[9] All measurements were performed in duplicate. In order to avoid recording changes in plasma iron due to diurnal variation,[10] all blood samples were obtained, without exception, between the hours of 9:00 A.M. and 10:00 A.M.

The parameters studied were measured in the subjects through two complete menstrual cycles

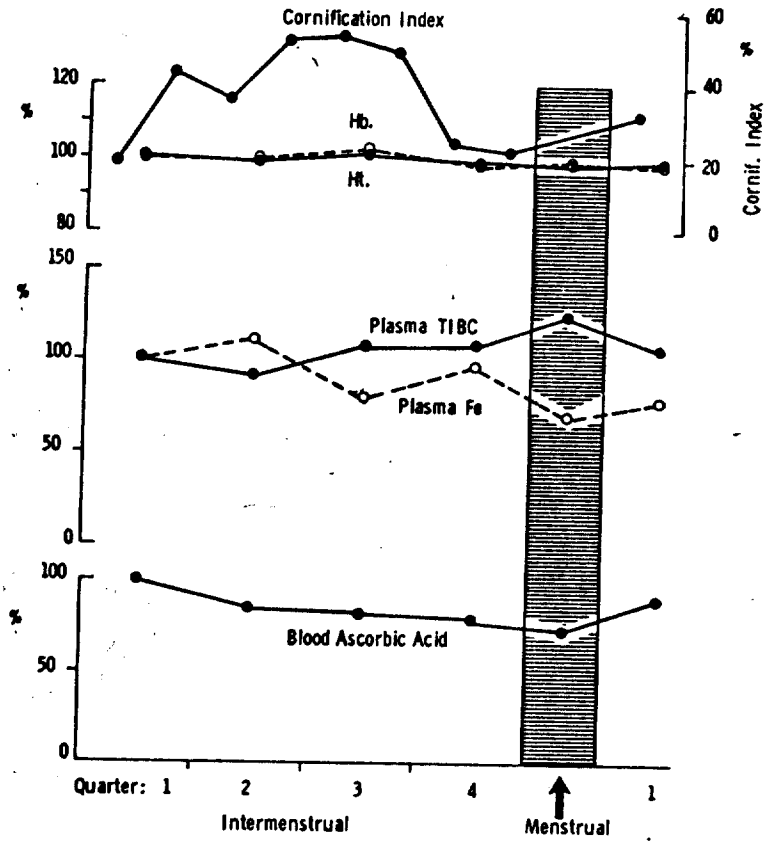

Fig. 1. Graph of mean changes which occurred during one menstrual cycle.

(i.e., three menstrual periods). The initial cycle was utilized to familiarize the technicians and subjects with the methods and procedures. The second cycle was studied by graphing this cycle in each subject, and then dividing the graph into the intermenstrual and menstrual phases according to menstrual flow. A planimeter was used to integrate the total change in each of the parameters measured during both phases in each subject, and this change was differentiated with respect to the number of phase days to obtain an accurate daily level. These values were analyzed statistically to determine the group mean for each parameter and the student's t test was used to determine the significance of the difference between the mean levels in the two phases of the menstrual cycle.

Secondarily, all measurements obtained during the second menstrual cycle were analyzed by the product-moment correlation for the different parameters to determine if a metabolic relationship existed between serial changes in estrogen activity, hemoglobin, hematocrit, plasma iron and blood ascorbic acid levels and plasma total iron-binding capacity.

RESULTS

Figure 1 is a graph of the group per cent changes in the parameters measured. As expected, the peak of the cornification index curve occurred midway between the limits of the intermenstrual phase whereas the minimum level occurred immediately prior to the onset of menstruation. The hemoglobin and hematocrit levels paralleled one another throughout the cycle with a slight rise in each at the time of ovulation. The plasma iron and blood ascorbic acid levels progressively decreased from the intermenstrual phase to much lower levels during menstruation. There was a simultaneous increase in mean plasma total iron-binding capacity which was inversely proportional to iron and ascorbic acid changes.

The results of statistical analysis and the student's t test of the intermenstrual and menstrual mean phase levels are given in Table

TABLE I
Blood and Plasma Levels in Intermenstrual and Menstrual Phases

Data	Intermenstrual Phase	Menstrual Phase	Changes
Blood ascorbic acid (mg. %)	0.347	0.338	−0.009
	1.088	0.935	−0.153
	0.535	0.441	−0.094
	0.779	0.608	−0.171
	0.482	0.417	−0.065
	0.864	0.808	−0.056
Mean ± S.D.	0.682 ± 0.276	0.591 ± 0.237	−0.091 ± 0.06*
Plasma total iron-binding capacity (ug. %)	393.5	388.2	−5.3
	302.3	431.7	+129.4
	483.5	435.3	−48.2
	380.0	531.7	+151.7
	394.7	491.7	+97.0
	430.5	394.0	−36.5
Mean ± S.D.	397.4 ± 59.8	445.4 ± 56.2	+48.0 ± 88.4
Plasma iron (ug. %)	157.9	98.5	−59.4
	141.2	105.0	−36.2
	133.5	117.6	−15.9
	151.2	86.8	−64.4
	123.8	114.1	−9.7
	109.8	33.8	−76.0
Mean ± S.D.	136.2 ± 17.8	92.6 ± 30.9	−43.6 ± 27.2*
Hemoglobin (%)	14.44	14.47	−0.03
	12.10	12.07	−0.03
	12.44	12.00	−0.44
	13.50	13.88	+0.38
	13.01	12.97	−0.04
	12.01	12.11	+0.10
Mean ± S.D.	12.92 ± 0.94	12.92 ± 1.05	−0.00 ± 0.27
Hematocrit (%)	43.7	43.8	+0.1
	38.7	37.1	−1.6
	38.6	37.1	−1.5
	42.8	42.5	−0.3
	39.0	37.9	−1.1
	36.7	36.8	+0.1
Mean ± S.D.	39.9 ± 2.7	39.2 ± 3.1	−0.7 ± 0.8

* Significant at P = <0.02.

I. Mean blood ascorbic acid and plasma iron levels during the menses were lower by 0.09 ± 0.06 mg. per cent and 43.6 ± 27.2 μg. per cent, respectively (P <0.02). Hemoglobin and hematocrit levels varied little throughout the menstrual cycle and were not significantly altered during menstruation. Plasma total iron-bindary capacity levels fluctuated during the cycle so that half values obtained showed an increase and half showed a decrease during menstruation. The mean change was 48.0 ± 60.1 μg. per cent increase in plasma total iron-binding capacity during menstruation; however, this change was not statistically significant because of the large variations in the levels measured.

The results of the product-moment correlation studies are listed in Table II. The progressive changes in blood ascorbic acid levels throughout one complete menstrual cycle were inversely related to plasma total iron-binding capacity levels (P <0.01), but not to the other parameters. Both the hematocrit and hemoglobin levels varied inversely with plasma iron levels (P <0.01). The hematocrit and hemoglobin levels were correlated with a lesser

degree of confidence ($P < 0.05$). The measured changes in plasma iron levels were inversely correlated with estrogen activity ($P < 0.05$). Other correlations between the measured parameters were not significant.

TABLE II
Correlations Between Blood and Plasma Levels in One Menstrual Cycle

Parameters	r value	P
Blood ascorbic acid vs. plasma total iron-binding capacity	−0.704	<0.01
Blood ascorbic acid vs. plasma iron	−0.051	N.S.
Blood ascorbic acid vs. hematocrit	+0.450	N.S.
Blood ascorbic acid vs. hemoglobin	−0.274	N.S.
Blood ascorbic acid vs. estrogen	−0.165	N.S.
Plasma total iron-binding capacity vs. plasma iron	+0.085	N.S.
Plasma total iron-binding capacity vs. hematocrit	−0.234	N.S.
Plasma total iron-binding capacity vs. hemoglobin	+0.575	N.S.
Plasma total iron-binding capacity vs. estrogen	−0.194	N.S.
Plasma iron vs. hematocrit	−0.844	<0.01
Plasma iron vs. hemoglobin	−0.704	<0.01
Plasma iron vs. estrogen	−0.250	<0.05
Hematocrit vs. hemoglobin	+0.637	<0.05

NOTE: N.S. = not significant.

COMMENTS

The results of this study revealed that the 32 per cent decrease in plasma iron levels found during menstruation was in close agreement with the 25 per cent decrease in serum iron levels reported by Powell[1] and the 30 per cent decrease reported by Hemmeler.[2] Our measured changes were verified by both the agreement with results of previous studies performed by Powell and Hemmeler and the level of confidence obtained in our data. Inasmuch as plasma iron levels decreased progressively and not abruptly during menstruation, it appears that the plasma iron changes may not have been directly due to menstrual blood loss, although this was indicated by the results of statistical analysis comparing mean values. A much higher level of correlation was found between plasma iron levels and hematocrit and hemoglobin levels, suggesting a closer causal relationship between the changes in these parameters than in menstrual blood loss. This relationship is in agreement with a basic fact of iron metabolism, that hematocrit and hemoglobin levels vary with the diurnal variation in serum iron levels.[11] Evidence that these observed changes may be due in part to cyclic variations can be based on the inverse correlation found between estrogen activity (as measured by the cornification index) and plasma iron levels during the intermenstrual, but not the menstrual, phase. This supports the findings of Palmer that the injection of estrogens into the female rabbit, a nonmenstruating mammal, rapidly depressed plasma iron levels.[5] There are two plausible explanations for the rather low level of probability found in this correlation. We measured estrogen activity by visual microscopic count of cornified vaginal epithelial cells smeared on a glass slide. Probably, the values for estrogen activity reported herein are the least accurate of all the values obtained for the parameters we studied. In addition, there is no method, at present, that allows for a meaningful estimation of the number of metabolic pathways interrelating *in vivo* estrogenic activity and plasma iron.

Plasma total iron-binding capacity levels, which increased progressively during the intermenstrual phase and attained a maximum peak during menstruation, were not changed significantly by menstruation. However, the very significant inverse correlation between total iron-binding capacity levels and blood ascorbic acid levels suggests a close metabolic relationship. Plasma total iron-binding capacity is the expression of the total amount of iron transferrin present in plasma and, according to Mitchell et al.,[12] exists in two forms: circulating transferrin in serum and sequestered transferrin at cellular sites. These two forms exist in equilibrium. When the need for iron turnover at cellular sites is increased, the sequestered transferrin is increased at the expense of the circulating transferrins. The *in vivo* study of Moore demonstrated a promoting effect of ascorbic acid on iron assimilation at the cellular level.[4] This was confirmed *in vitro* recently by Mazur, who used rat liver tissue.[13] In these experiments the increased iron turnover at cellular sites was accompanied

by an increase in sequestered transferrin associated with a decrease in circulating transferrin. The results of our clinical study suggest a metabolic relationship between ascorbic acid and serum iron transfer which is in complete agreement with results reported previously.

As iron is metabolized at the cellular level or lost during menstruation, the over-all rate of iron movement appears to be reflected in the gradually increased capacity of plasma to combine with plasma iron, and the blood levels of ascorbic acid may exert a controlling influence on plasma total iron-binding capacity.

An abrupt decrease in urinary ascorbic acid levels has been reported to occur at the time of ovulation.[14] A careful scrutiny of the individual graphs of the six women studied did not reveal an abrupt decrease in blood ascorbic acid levels coincident with ovulation. However, had blood values been measured daily, a definitive result would have been obtained. The lack of statistical evidence to verify the proposed relationship between estrogen activity and blood ascorbic acid metabolism tends to support the hypothesis that fluctuations in blood ascorbic acid levels are due to nutritional and menstrual variations in iron metabolism.

SUMMARY

Plasma iron and total iron-binding capacity, hematocrit, hemoglobin and blood ascorbic acid levels were measured on alternate days throughout two complete menstrual cycles in six healthy young women. Estrogen activity was measured daily by the vaginal cornification index. Several statistically significant changes in these measured parameters were observed. The mean plasma iron levels were depressed 32 per cent during menstruation from premenstrual control levels. A paired sample analysis indicated that changes in plasma iron levels were correlated to cyclic changes in the estrogen activity and to both the hematocrit and hemoglobin changes, although the changes in hematocrit and hemoglobin, *per se*, were insignificant.

The blood ascorbic acid levels were 13 per cent lower during menstruation and correlated statistically with changes in plasma total iron-binding capacity throughout the menstrual cycles. Mean plasma total iron-binding capacity was increased approximately 12 per cent during menstruation.

ACKNOWLEDGMENT

We are grateful to Mrs. Sue Kre and William Harris for their technical assistance.

REFERENCES

1. Powell, J. F. Serum-iron in health and disease. *Quart. J. Med.*, 13: 19, 1944.
2. Hemmeler, G. Serum iron and iron therapy. *Schweiz. med. Wchnschr.*, 69: 316, 1939.
3. Millis, J. Iron losses in healthy women during consecutive menstrual cycles. *M. J. Australia*, 2: 874, 1951.
4. Moore, C. V. The importance of nutritional factors in the pathogenesis of iron-deficiency anemia. *Am. J. Clin. Nutrition*, 3: 3, 1955.
5. Palmer, H. The influence of sex hormones on plasma iron. *Scandinav. J. Clin. & Lab. Invest.*, 4: 81, 1952.
6. Papanicolaou, G. The general survey of the vaginal smear and its use in research and diagnosis. *Am. J. Obst. & Gynec.*, 51: 316, 1946.
7. Arronet, G. H. and Turnbull, L. Colpocytogram and cornification index. *Obst. & Gynec. Surv.*, 13: 539, 1958.
8. Lowry, O. H., Bessey, O. A. and Burch, H. B. Effects of prolonged high dosage with ascorbic acid. *Proc. Soc. Exper. Biol. & Med.*, 80: 361, 1952.
9. Beale, R. N. Improved rapid methods for determination of iron content and binding capacity of serum. *J. Clin. Path.*, 15: 156, 1962.
10. Bothwell, T. H. and Finch, C. A. Iron Metabolism. Boston, 1962. Little, Brown and Co.
11. Ramsay, W. N. M. In: Advances in Clinical Chemistry, vol. 1, p. 20. Edited by Sobotka, H. and Stewart, C. P. New York, 1958. Academic Press, Inc.
12. Mitchell, J., Halden, E. R., Jones, F., Bryan, S., Stirman, J. A. and Muirhead, E. E. Lowering of transferrin during iron absorption in iron deficiency. *J. Lab. & Clin. Med.*, 56: 555, 1960.
13. Mazur, A. Role of ascorbic acid in the incorporation of plasma iron into ferritin. *Ann. New York. Acad. Sc.*, 92: 223, 1961.
14. Nakajima, T. Vitamin C Spiegel and Ovulation. *Zentralbl. Gynack.*, 81: 337, 1959.

Furia, T.E. (Editor). 1972
Handbook of Food Additives, 2nd Edition
Chemical Rubber Company, Cleveland, Ohio
pp. 91; 101-102; 106-107

Clin. Invest. 31, 756-61 (1952)

METABOLISM OF PTEROYLGLUTAMIC ACID AND THE CITROVORUM FACTOR IN PATIENTS WITH SCURVY [1, 2]

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(Submitted for publication March 26, 1952; accepted June 13, 1952)

The citrovorum factor (CF) is closely related chemically and metabolically to pteroylglutamic acid (PGA) (1–5). CF replaces PGA in the nutrition of certain microorganisms (3, 6) and in some species of animals (6, 7); and also reverses aminopterin activity (3, 7–11). A relationship of CF to PGA is further indicated by the increased urinary excretion of CF which occurs when PGA is fed (12–16). The hematopoietic effect of CF given to patients with pernicious anemia (17–20), or with other nutritional anemias (21, 22), or to monkeys with megaloblastic anemia induced by dietary means (23) also attests to the metabolic activity of this factor.

The synthesis of CF from PGA by rat liver slices is augmented by ascorbic acid (24). This indicates that ascorbic acid may play a role in the conversion of PGA to CF. In order to investigate the role of ascorbic acid in the metabolic relationship of PGA to CF in man, the urinary excretion of CF was determined following PGA administered orally to two patients with scurvy and to a non-scorbutic subject before, during and after ascorbic acid therapy. The results of these studies are presented and indicate that one role of ascorbic acid in man is to facilitate the conversion of PGA to CF.

[1] The expenses of this investigation were defrayed in part by grants from Sharp and Dohme, Inc., West Point, Pennsylvania, and from Merck and Co., Inc., Rahway, New Jersey, to Harvard University.

[2] A preliminary report of this investigation appeared in the Proceedings of the Forty-third Annual Meeting of the American Society for Clinical Investigation held in Atlantic City, New Jersey, April 30, 1951 (J. Clin. Invest., 1951, 30, 639).

[3] Welch Fellow in Internal Medicine of the National Research Council.

PATIENT MATERIAL AND METHODS

Two men with scurvy and one non-scorbutic male comprise the subjects of this study. The two scorbutics, T. C. age 66, and F. O'D. age 65, were bachelors who lived alone in single rooms, and gave histories of ingesting inadequate quantities of food and of prolonged dietary ascorbic acid deficiency. Clinical evidences of scurvy included multiple perifollicular hemorrhages most prominent on the legs and forearms, ecchymoses especially of the posterior thighs, and hyperkeratotic folliculosis with crinkled, brittle hairs, most apparent on the abdominal wall and thighs. Neither patient showed hypertrophic or bleeding gums. Ascorbic acid was not present in the buffy coat of peripheral venous blood samples obtained from each of these patients. Neither patient had significant anemia. The hemoglobin concentrations were 13.7 gm. and 12.8 gm. per 100 ml., and the red blood cell counts 4.19 million and 4.20 million per cu. mm., respectively. Other blood studies including white blood cell counts, differential count, platelet count, prothrombin time, bleeding time, and coagulation time were within normal limits. A tourniquet test done on a forearm of each of these patients was markedly positive. All of the abnormalities noted were promptly and completely reversed following the administration of ascorbic acid.

The non-scorbutic subject, J. O'C. age 49, was a chronic alcoholic and an epileptic. He had been in the hospital for two months during which time an adequate diet was provided prior to the initiation of the studies described here. At this time physical examination and the usual routine laboratory studies, including tests of liver function, revealed no abnormalities. The peripheral venous buffy coat ascorbic acid content was 44 mg. per cent.

The patients with scurvy were examined shortly after hospital entry and immediately transferred to the Thorndike Metabolic Ward. They did not receive food or medication prior to the institution of the dietary regimen and the studies described. During the entire periods of study these patients were kept at rest in bed and were offered diets consisting of boiled milk and rice, crackers, sugar, coffee, and water. These items of food supplied minimal, if any, quantities of ascorbic acid. The non-scorbutic subject was ambulatory and was maintained on a constant well-balanced dietary regimen pro-

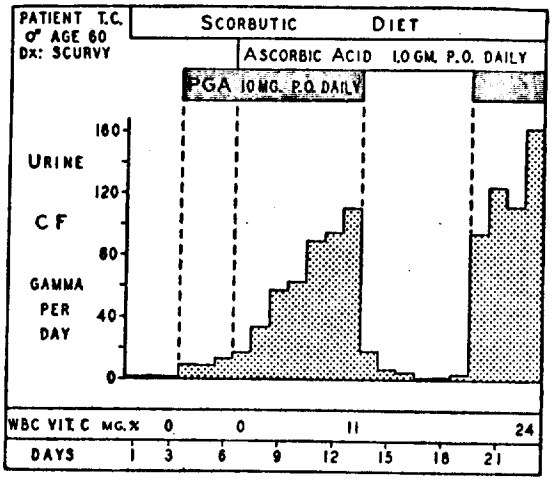

FIG. 1. URINARY EXCRETION OF CF FOLLOWING ORAL PGA IN A PATIENT WITH SCURVY BEFORE AND AFTER ASCORBIC ACID THERAPY

viding 75 gm. of protein and 2,800 calories daily during this study.

The three subjects were observed during initial control periods of three days. Each was then given 10 mg. of PGA[4] orally daily in two equal doses at 9 a.m. and 5 p.m. for from three to six days. During a third period of study of each patient, PGA was continued in the dosage stated and in addition, 1 gm. of ascorbic acid was administered daily for from six to nine days. The ascorbic acid was given orally in the cases of T. C. and J. O'C., and intramuscularly as sodium ascorbate in the case of F. O'D. in four equal doses at 9 a.m., 1 p.m., 5 p.m., and 9 p.m. PGA was then discontinued, but the basal diets and ascorbic acid continued for from six to nine days. In the case of the scorbutic patients, PGA was administered orally on a second occasion according to the same dosage schedule previously employed while ascorbic acid therapy and the basal diets were continued unchanged. Thus, the patients served as their own controls, since observations on the urinary excretion of CF following PGA taken orally were made before and after the alleviation of the scorbutic state by adequate therapy with ascorbic acid.

A final period of study in F. O'D. consisted of the oral supplementation of his diet with 10 mg. of PGA daily for six days without supplemental ascorbic acid. The final period of study in J. O'C. consisted of six days on the constant diet and was therefore identical to the initial study period in this patient.

During these studies the urine specimens were collected and immediately placed in a refrigerator. After completion of each 24-hour urine collection the daily urine volumes were measured, and aliquots stored in a deep freeze at − 20° C. for subsequent microbiologic assay for PGA and CF activity. The microbiologic assays for CF were done according to the method of Sauberlich (1, 12) using a synthetic crystalline material with *L. citrovorum* activity (leucovorin) as the reference standard, and for PGA activity by the growth response of *Lactobacillus casei* (25). Buffy coat ascorbic acid concentrations were measured by the method of Butler and Cushman (26).

[4] The pteroylglutamic acid (folvite) and the crystalline citrovorum factor (leucovorin) used in these studies was generously provided by the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

RESULTS

The results of these studies are presented in Figures 1, 2, and 3. T. C., Figure 1, a patient with scurvy, excreted about 1.2 gamma of urinary CF daily during three initial days of study. When 10 mg. of PGA were administered orally daily for three days, urinary CF excretion ranged from 9.1 to 13.3 gamma daily. One gram of ascorbic acid was then given orally daily for seven days, in addition to PGA. Urinary CF excretion was 16.9 gamma on the first day of ascorbic acid therapy and gradually increased to 110.4 gamma by the seventh day. PGA was then omitted but ascorbic acid continued. Urinary CF returned within four days to initial levels. After six days PGA was given again in addition to ascorbic acid. On this occasion on the first day of PGA administration urinary CF was 93.9 gamma, and averaged 132.6 gamma daily during the three additional days of the final study period.

The second patient with scurvy, F. O'D., Figure 2, excreted about 0.4 gamma of urinary CF

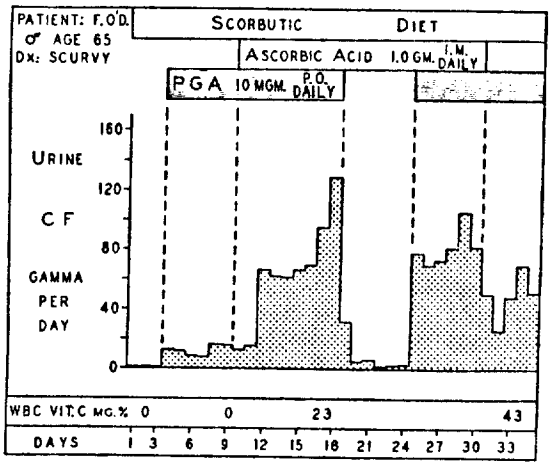

FIG. 2. URINARY EXCRETION OF CF FOLLOWING ORAL PGA IN A PATIENT WITH SCURVY BEFORE AND AFTER ASCORBIC ACID THERAPY

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National Research Council, National Academy of
Sciences, Washington, D.C.

The ascorbic acid content (micrograms/g) is less in the poisoned groups in comparison with the controls (fig. 2/c), the differences being, however, statistically insignificant.

2. The administration of vitamins in the incipient phase of poisoning was followed by significant prolongation of duration of life both for the group with vitamin C alone and for the group which received the two vitamins together (fig. 2/a).

Tabelul II Table II

Durata de supraviețuire (ore) a mormolocilor în cazul administrării vitaminelor după apariția semnelor de intoxicație (concentrația fenosept: 0,1 mg/l).

Duration of survival (hours) of tadpoles in the case of administration of vitamins after appearance of signs of poisoning (phenosept concentration 0.1 mg/l)

Lotul Group	N obs.	M. ± E.S.	P.
Fenosept Phenosept	25	180,2 ± 22,62	**
Fenosept + vitamina C	25	321,3 ± 56,03	0,05 > P > 0,02
Fenosept + pantotenat	25	167,5 ± 45,66	P > 0,50
Fenosept + vitamina C + pantotenat	25	304,1 ± 56,86	P = 0,05
Apă	25	265,8 ± 39,74	0,10 > P > 0,05

DISCUSSION OF THE RESULTS

The data presented show that the antitoxic effect of the vitamin is manifested in the zone of small concentrations of phensept, for which the reactions are considered reversible and thus subject to the influence of vital substances.

Of the two vitamins studied, ascorbic acid shows a strong antitoxic effect expressed both in the case of the concomitant administration of vitamin C with the toxic element and with regard to its effect of restoring the poisoned organism.

ascorbic acid greatly facilitates the conversion of PGA to CF, and suggest that one of the biochemical functions of ascorbic acid in man is to take part in the biosynthesis of CF from PGA.

SUMMARY AND CONCLUSIONS

The urinary excretion of CF was determined microbiologically following PGA administered orally to two men with scurvy and to one non-scorbutic adult before, during and after ascorbic acid therapy.

A small amount of CF was excreted by the patients with scurvy and by the non-scorbutic subject during the initial control period. Urinary CF was increased slightly when PGA was administered orally to the patients during the scorbutic state. However, maximal CF excretion, comparable to that observed in the non-scorbutic individual, occurred in the patients with scurvy only after adequate therapy with ascorbic acid.

The data indicate that CF is metabolically related to PGA, and that one role of ascorbic acid in man is to provide for the conversion of PGA to CF.

ACKNOWLEDGMENTS

The authors wish to thank the Misses Alice N. Ballou, Leonora De Carli, Elaine Hirschberg, and Virginia Olsson for technical assistance.

REFERENCES

1. Sauberlich, H. E., and Baumann, C. A., A factor required for the growth of Leuconostoc citrovorum. J. Biol. Chem., 1948, **176**, 165.
2. Sauberlich, H. E., and Baumann, C. A., Further studies on the factor required by Leuconostoc citrovorum 8081. J. Biol. Chem., 1949, **181**, 871.
3. Broquist, H. P., Stokstad, E. L. R., and Jukes, T. H., Some biological and chemical properties of the citrovorum factor. J. Biol. Chem., 1950, **185**, 399.
4. Shive, W., Bardos, T. J., Bond, T. J., and Rogers, L. L., Synthetic members of the folinic acid group. J. Am. Chem. Soc., 1950, **72**, 2817.
5. Brockman, J. A., Jr., Roth, B., Broquist, H. P., Hultquist, M. E., Smith, J. M., Jr., Fahrenbach, M. J., Cosulich, D. B., Parker, R. P., Stokstad, E. L. R., and Jukes, T. H., Synthesis and isolation of a crystalline substance with the properties of a new B vitamin. J. Am. Chem. Soc., 1950, **72**, 4325.
6. Broquist, H. P., Stokstad, E. L. R., and Jukes, T. H., Comparative biological activity of crystalline citrovorum factor and pteroylglutamic acid. Federation Proc., 1951, **10**, 167.
7. Waisman, H. A., Green, M., Munoz, J. C., Ramenchik, A., and Richmond, J. B., Role of aureomycin and citrovorum factor in "folic acid" deficiencies. Proc. Soc. Exper. Biol. & Med., 1951, **76**, 384.
8. Nichol, C. A., and Welch, A. D., On the mechanism of action of aminopterin. Proc. Soc. Exper. Biol. & Med., 1950, **74**, 403.
9. Schoenbach, E. B., Greenspan, E. M., and Colsky, J., Reversal of aminopterin and amethopterin toxicity by citrovorum factor. J. A. M. A., 1950, **144**, 1558.
10. Burchenal, J. H., Babcock, G. M., Broquist, H. P., and Jukes, T. H., Prevention of chemotherapeutic effects of 4-amino-N^{10}-methyl-pteroylglutamic acid on mouse leukemia by citrovorum factor. Proc. Soc. Exper. Biol. & Med., 1950, **74**, 735.
11. Cravens, W. W., and Snell, E. E., Reversal of aminopterin inhibition in the chick embryo with the Leuconostoc citrovorum factor. Proc. Soc. Exper. Biol. & Med., 1950, **75**, 43.
12. Sauberlich, H. E., The effect of folic acid upon the urinary excretion of the growth factor required by Leuconostoc citrovorum. J. Biol. Chem., 1949, **181**, 467.
13. Anker, R. M., Boehne, J. W., and Welch, A. D., A folic acid-derived growth factor for Leuconostoc citrovorum in human urine. Federation Proc., 1950, **9**, 351.
14. Broquist, H. P., Stokstad, E. L. R., and Jukes, T. H., Biochemical studies with the "citrovorum factor." J. Lab. & Clin. Med., 1951, **38**, 95.
15. Welch, A. D., Nichol, C. A., Anker, R., and Boehne, W., Urinary excretion of a biologically active metabolic alteration product derived from folic acid. J. Pharm. & Exper. Therap., 1951, **101**, 37.
16. Welch, A. D., Nichol, C. A., Anker, R. M., and Boehne, J. W., III, The effect of ascorbic acid on the urinary excretion of citrovorum factor derived from folic acid. J. Pharm. & Exper. Therap., 1951, **102–3**, 403.
17. Meyer, L. M., Brahin, C. M., and Sawitsky, A., Treatment of pernicious anemia with citrovorum factor. Proc. Soc. Exper. Biol. & Med., 1951, **76**, 86.
18. Ellison, R. R., Wolfe, S., Lichtman, H., Ginsberg, V., and Watson, J., Effect of citrovorum factor in pernicious anemia. Proc. Soc. Exper. Biol. & Med., 1951, **76**, 366.
19. Jarrold, T., Horrigan, D., Thompson, C., and Vilter, R. W., The hematologic effect of folinic acid (citrovorum factor) in persons with pernicious anemia. Science, 1951, **113**, 688.
20. Davidson, L. S. P., and Girdwood, R. H., Treatment of pernicious anemia with leuconostoc citrovorum factor. Lancet, 1951, **1**, 722.
21. Spies, T. D., Lopez, G. G., Milanes, F., Toca, R. L., Reberedo, A., and Stone, R. E., The response of patients with pernicious anemia with nutritional macrocytic anemia and with tropical sprue to folinic

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acid or citrovorum factor. South. M. J., 1950, 43, 1076.

22. Woodruff, C. W., Peterson, J. C., and Darby, W. J., Citrovorum factor and folic acid in treatment of megaloblastic anemia in infancy. Proc. Soc. Exper. Biol. & Med., 1951, 77, 16.

23. May, C., Sundberg, R. D., and Scharr, F., Comparison of effects of folic acid and folinic acid in experimental megaloblastic anemia. J. Lab. & Clin. Med., 1950, 36, 963.

24. Nichol, C. A., and Welch, A. D., Synthesis of citrovorum factor from folic acid by liver slices; augmentation by ascorbic acid. Proc. Soc. Exper. Biol. & Med., 1950, 74, 52.

25. Teply, L. J., and Elvehjem, C. A., The titrimetric determination of "Lactobacillus casei factor" and "folic acid." J. Biol. Chem., 1945, 157, 303.

26. Butler, A., and Cushman, M., Distribution of ascorbic acid in the blood and its nutritional significance. J. Clin. Invest., 1940, 19, 459.

Anal. Chem. 32, 1415-1417

2703

Paper Chromatography of Certain Vitamins in Phenol and Butanol–Propionic Acid–Water Solvents

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▶ Separation and detection of vitamins using two-dimensional paper chromatography with phenol and a butanol–propionic acid solvent system have been investigated. The water-soluble vitamins were successfully separated and, depending upon the mixture, can be located following the application of a selected order of detecting agents. Of the fat-soluble vitamins, only α-tocopherol can be identified by the procedures studied.

Though paper chromatographic techniques are now used in studies of amino acids, organic acids, and many other organic compounds, application of this method to vitamins has not been extensive. Many of the procedures now available necessitate the use of specially treated paper (*13, 17, 40*), or estimation of the vitamin by bioautographic techniques (*16, 38, 41*).

Paper chromatography has been an important analytical tool in this laboratory in the study of the metabolism of the amino acid, methionine, by the adult rat. For this investigation it was necessary to determine the R_f values of pure amino acids using two solvents which were most satisfactory for our purposes (phenol and butanol–propionic acid–water). It was discovered that many of the vitamins migrate to the same general location as the amino acids on paper chromatograms. However, a review of the literature revealed few R_f values in these solvents.

The estimation of vitamin A and related compounds on alumina-impregnated paper has been described by Datta, Overell, and Stack-Dunne (*13*). Rossi (*33*) has reported the paper chromatography of vitamin D_2. Brown and Blaxter (*7*) have described the separation of α-, β-, and γ-tocopherols by reversed phase paper chromatography on vaseline-coated paper. Procedures for this vitamin have also been described by others (*8, 9, 15, 17, 18*).

The paper chromatography of thiamine and some of its derivatives has been reported by several workers (*20, 22, 23, 30, 34, 36*). Crammer (*10*), Hais and Pecakova (*19*), Yagi (*42*), and Blair and Graham (*4*) have described the use of this technique in the separation of riboflavin, its phosphate and nucleotides. Ackermann and Kirby (*1*), Baddiley and Thain (*2*), and Crokaert (*11*) have studied pantothenic acid. The paper chromatography of nicotinic acid has been reported by several workers (*23–26*). Procedures for the estimation of pyridoxines (*12, 35*), folic acid and its derivatives (*16, 31*), and choline (*36*) have also appeared.

Bowden and Peterson (*6*) have investigated the resolution of biotin components in liver by paper chromatography. Winsten and Eigen (*39*), Woodruff and Foster (*40*), and Harrison (*21*) have reported studies of vitamin B_{12}. Patschky (*32*), Mapson and Partridge (*29*), Weygand (*37*), and several other investigators have described the paper chromatographic behavior of vitamin C.

The present study was to investigate the migration characteristics of certain water- and fat-soluble vitamins in water-saturated phenol and butanol–propionic acid–water on untreated filter paper.

EXPERIMENTAL PROCEDURES

Solutions of vitamins were made up in a concentration of 5 mg. per ml., with the exception of *p*-aminobenzoic acid and α-tocopherol. Because of its lower solubility, *p*-aminobenzoic acid was made up in a concentration of 2.5 mg. per ml. and α-tocopherol was diluted 1 to 10 with absolute alcohol. Distilled water was used as the solvent for the water-soluble vitamins; absolute alcohol as the solvent for the fat-soluble compounds.

The vitamin solutions were individually applied to the lower right-hand corner of 18 × 22 inch Whatman No. 1 filter paper sheets. Buffered water-saturated phenol was used as the first solvent. The aqueous buffer solution contained 6.3% sodium citrate and 3.7% potassium dihydrogen phosphate (100 ml. of phenol per 25 ml. of salt solution). These salts inhibit the diffusion of spots and prevent the migration of a contaminant in the paper. A mixture of 1-butanol, propionic acid, and water was used for the second dimension. Fresh solvent was prepared from equal volumes of two solutions: A (1246 ml. of 1-butanol and 84 ml. of water) and B (620 ml. of propionic acid and 790 ml. of water).

The papers were run by the descending technique in paraffin-coated wooden Chromatocabs housed in a constant temperature room maintained at 24° ± 0.5° C. A separate cabinet was used for each solvent. A small amount of solvent was kept in a container at the bottom of the cabinets to bring the atmosphere more quickly to equilibrium with the solvent.

After the phenol had migrated down the papers (about 18 to 22 hours), they were removed from the cabinet. The solvent front was outlined with a lead pencil and the papers were dried in a fume hood overnight. They were then turned at a 90° angle counterclockwise and butanol–propionic acid–water was allowed to descend the papers (14 to 16 hours). The solvent front was outlined again and the papers were dried overnight in a fume hood.

Various color reagents were required to detect the different vitamins.

RESULTS AND DISCUSSION

Table I presents the method of identification, R_f values, and concentrations of the vitamins chromatographed. No attempt was made to determine the minimum quantities which could be detected by paper chromatography. The quantities used were large, as we were primarily interested in determining the R_f values of the vitamins.

Of the fat-soluble vitamins, only α-tocopherol was successfully detected by a color reagent after chromatography. Vitamin A and Menadione (vitamin K) were yellow in solution and migrated as a yellow spot during the phenol run; therefore, their R_f values in this solvent could be calculated. Their yellow color, however, was lost during butanol–propionic acid migration, and they could not be detected with color reagents. We were also unsuccessful in detecting vitamin D and thioctic acid. Antimony trichloride is listed by some investigators as a reagent for vitamins A and D (*5*). Discrete spots were not obtained with this reagent. Many investigators working with the chromatography of fat-soluble vitamins have impregnated their filter papers with alumina [$Al_2(SO_4)$], coated them with vaseline or paraffin, or treated them in other ways (*5, 27*). There was no attempt to treat the papers specially, as we wanted to secure R_f values for the vitamins using the same procedures as were applied in the two-dimensional chroma-

tography of other compounds in our experiments (*14*). Our failure to obtain discrete spots may have been due to this reason.

The water-soluble vitamins were easily separated using phenol and butanol-propionic acid-water. Detection was as follows: The B complex group, riboflavin, pyridoxine, and folic acid fluoresce under ultraviolet light without any special treatment. (A short-wave ultraviolet lamp was used.) Thiamine fluoresces after being sprayed with an alkaline ferricyanide-nitroprusside reagent (*3*). Niacin and nicotinamide may be detected by exposure to cyanogen bromide, followed by spraying with 2% *p*-aminobenzoic acid (*5*). Vitamin B_{12}, which is pink in solution, migrates as a pink spot. Many organic compounds react with iodine vapors to yield a brown spot. Niacin, nicotinamide, choline, and biotin were detected by this method.

The R_f values shown in the last column were obtained by calculating the ratio of the distance traveled by the compound to the distance traveled by the solvent front. The values listed are for vitamins run as individual compounds. We have, however, also run a mixture of vitamin solutions, separating and identifying the various vitamins by the methods listed above.

Because several different methods and color reagents are required to detect the vitamins, it was necessary to determine the order in which these reagents could be used on a single paper chromatogram. Those compounds which retain their color are identified first. Papers are then viewed under ultraviolet light to determine which compounds fluoresce. Ninhydrin, when used as the first color reagent, appears to have little or no effect on the reaction of subsequent color reagents. Papers sprayed with ninhydrin may be exposed to iodine vapors with satisfactory results. After the iodine is allowed to vaporize off the paper, it may be sprayed with ferric chloride or ammonical silver nitrate. Ferric chloride interferes with the subsequent reaction of ammonical silver nitrate and cannot be successfully used before or after this solution. A map giving the relative positions of the vitamins is shown in Figure 1.

Thus, a single procedure which can be applied to several water-soluble vitamins and one fat-soluble vitamin has been developed. It should be helpful in identifying qualitatively the water-soluble vitamins present in a mixture.

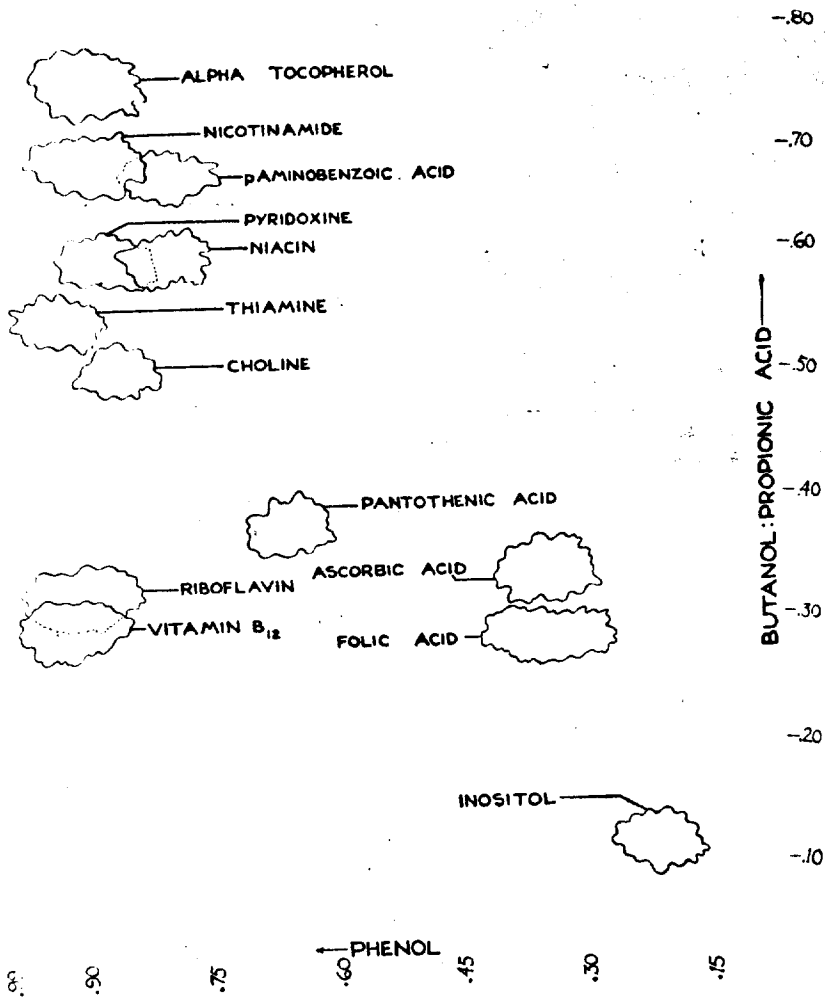

Figure 1. Chromatomap of vitamins and related compounds showing migration in phenol and butanol–propionic acid

Positions of spots obtained by plotting R_f values given in Table I

Table I. Paper Chromatographic Behavior of Vitamins and Related Compounds

Vitamins and Related Compounds	Quantity Used, μg.[a]	Method of Identification: Color reagent[b]	Color	UV lamp	R_f Values: Phenol	Butanol–propionic acid–water
Vitamin A	250	...	Yellow	...	0.90	...
α-Tocopherol	250	AmAg	Black	...	0.89	0.77
Menadione	250	...	Yellow	...	0.94	...
Thiamine	250	FCNP	None	Fluorescent	0.93	0.55
		Nin	Yellow			
Riboflavin	20	...	Yellow	Fluorescent	0.91	0.32
Niacin	250	I_2	Brown	...	0.83	0.68
		DCPI	Pink			
		CNBr	Orange			
Nicotinamide	250	I_2	Brown	...	0.85	0.69
		CNBr	Yellow			
		FCNP	Yellow			
Pyridoxine	250	$FeCl_3$	Brown	Fluorescent	0.87	0.60
Pantothenic acid	250	Nin	Purple	...	0.66	0.38
Biotin	250	I_2	Brown	...	0.79	0.78
Inositol	50	AmAg	Brown	...	0.21	0.12
Choline	250	I_2	Brown	...	0.87	0.52
p-Aminobenzoic acid	250	$FeCl_3$	Brown	...	0.80	0.69
		PHC	Brown			
Folic acid	12.5	...	...	Fluorescent	0.34	0.30
Vitamin B_{12}	50	...	Pink	...	0.92	0.31
Vitamin C	250	AmAg	Brown	...	0.34	0.34

[a] α-Tocopherol in mg.
[b] AmAg = ammonical silver nitrate (*5*); FCNP = ferricyanide-nitroprusside (*3*); Nin = ninhydrin; I_2 = iodine vapor; DCPI = 2,6-dichlorophenol-indophenol (*3*); CNBr = cyanogen bromide (*5*); $FeCl_3$ = ferric chloride reagent (*3*); PHC = phenol-hypochlorite reagent (*3*).

LITERATURE CITED

(1) Ackermann, W. W., Kirby, H., *J. Biol. Chem.* **175**, 483 (1948).

(2) Baddiley, J., Thain, E. M., *J. Chem. Soc.* **1951**, 2253.
(3) Biochemical Institutes Studies, IV, University of Texas, Publ. 5109 (1951).
(4) Blair, J. A., Graham, J., *J. Biochem.* **56**, 286 (1954).
(5) Block, R. J., Durrum, E. L., Zweig, G., "Manual of Paper Chromatography and Paper Electrophoresis," pp. 398–409, Academic Press, New York, 1958.
(6) Bowden, J. P., Peterson, W. H., *J. Biol. Chem.* **178**, 533 (1949).
(7) Brown, F., Blaxter ,K. L., *Chem. & Ind. (London)* **1951**, 633.
(8) Brown, J. A., ANAL. CHEM. **25**, 774 (1953).
(9) Brown, J. A., March, M. M., *Ibid.*, **24**, 1952 (1952).
(10) Crammer, J. L., *Nature* **161**, 349 (1948).
(11) Crokaert, R., *Arch. Intern. Physiol.* **56**, 189 (1948).
(12) Dalgiesh, C. E., *J. Biochem.* **52**, 3 (1952).
(13) Datta, S. P., Overell, B. G., Stack-Dunne, M., *Nature* **164**, 673 (1949).
(14) Edwards, C. H., Gadsden, E. L., Edwards, G. A., *J. Chromatog.* **2**, 188 (1959).
(15) Eggitt, P. W. R., Ward, L. D., *Sci. Food Agr.* **4**, 176 (1953).
(16) Fountain, J. R., Hutchison, D. J., Waring, G. B., Burchenal, J. H., *Proc. Soc. Exptl. Biol. Med.* **83**, 369 (1953).
(17) Green, J., Marunkiewicz, S., *Nature* **176**, 1172 (1955).
(18) Guerillot, J., Guerillot-Vinet, A., Delmas, L., *Compt. rend.* **235**, 1295 (1952).
(19) Hais, I. M., Pecakova, L., *Nature* **163**, 768 (1949).
(20) Hanes, C. S., Isherwood, F. A., *Ibid.*, **164**, 1107 (1949).
(21) Harrison, J. S., *Analyst* **76**, 77 (1951).
(22) Heyndrickx, A., *J. Am. Pharm. Assoc., Sci. Ed.* **42**, 680 (1953).
(23) Holman, W. I. M., *J. Biochem.* **56**, 513 (1954).
(24) Huebner, H. F., *Nature* **167**, 119 (1951).
(25) Johnson, B. C., Lin, P. H., *J. Am. Chem. Soc.* **75**, 2971 (1953).
(26) Kodicek, E., Reddi, K. K., *Nature* **168**, 475 (1951).
(27) Lederer, E., Lederer, M., "Chromatography," pp. 256–7, Elsevier, Amsterdam, 1953.
(28) Malyoth, G., Stein, H. W., *Biochem. Z.* **323**, 265 (1952).
(29) Mapson, L. W., Partridge, S. M., *Nature* **164**, 479 (1949).
(30) Miyaki, K., Momiyama, H., Hayashi, M., *J. Pharm. Soc. Japan* **72**, 688 (1952).
(31) Nichol, C. A., Zakrzewski, S. F., Welch, A. D., *Proc. Soc. Exptl. Biol. Med.* **83**, 272 (1953).
(32) Patschky, A., *Angew. Chem.* **62**, 50 (1950).
(33) Rossi, C. A., *Boll. soc. ital. sper.* **26**, 1563 (1950).
(34) Siliprandi, D., Siliprandi, N., *Biochim. et Biophys. Acta* **14**, 52 (1954).
(35) Snyder, J. I., Wender, S. H., *Arch. Biochem. Biophys.* **46**, 465 (1953).
(36) Viscontini, M., Bonetti, G., Ebnother, C., Karrer, P., *Helv. Chim. Acta* **34**, 1384 (1951).
(37) Weygand, F., *Arkiv Kemi* **3**, 11 (1950).
(38) Winsten, W. A., Eigen, E., *J. Biol. Chem.* **177**, 989 (1949).
(39) *Ibid.*, **181**, 109 (1949).
(40) Woodruff, H. B., Foster, J. C., *Ibid.*, **183**, 569 (1950).
(41) Wright, L. D., Cresson, E. L., Driscoll, C. A., *Proc. Soc. Exptl. Biol. Med.* **86**, 480 (1954).
(42) Yagi, K., *J. Biochem. (Japan)* **38**, 161 (1951).

RECEIVED for review March 1, 1960. Accepted June 21 1960.

THE ANTI-TOXIC EFFECT OF ASCORBIC ACID IN EXPERIMENTAL POISONING WITH PHENYLMERCURIBORATE

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Summary

Experiments were carried out with ascorbic acid and calcium pantothenate on tadpoles of Rana temporaria, using phenylmercuriborate solutions in six concentrations (between 2.5 x 10^{-6}M/1 and 7.8 x 10^{-9}M/1), with and without the addition of vitamins.

Studies were made on the anti-toxic effect, with the administration of vitamins both as concomitant as well as after the appearance of the first signs of poisoning.

The observations show that vitamin C increases the resistance of tadpoles to the toxic effect of the organo-mercuric compound, both in the case of the application of the concomitant phenosept solution as well as after the appearance of the first sign of poisoning. Calcium pantothenate did not significantly influence the reaction of the tadpoles.

The relationship between vitamins and the various chemical substances with a harmful effect upon the organism has been the object of numerous experimental studies.

In this activity ascorbic acid occupies an important place, it being a vital component with positive effects in many cases of exposure to organic (1,2,8,21) and mineral (3,4,7,15,18,20,22,25,32)

toxic substances. Our observation on man (3) or experimentally on animals have confirmed the importance of vitamin C as an anti-toxic factor. On the other hand, pantothenic acid, a component of the co-enzyme A, being a part of the process of biological acetylization, is presumed to have a positive role as well (19,27,31).

Special ~~Different~~ attention is given recently to ~~the~~ interaction among the vitamins, a problem concerning the defense mechanisms of the organism, these interactions being inadequately explained and presenting numerous contradictory opinions.

One of the much disputed problems is the co-relation between pantothenic acid and vitamin C. Some authors indicate favorable interrelationships among the effects of the two vitamins (5,24,28, 29,30). Under other experimental conditions, however, these observations could not always be confirmed (6,10,17,23).

In the present work, we have primarily been interested in the degree to which vitamin C plays an anti-toxic role and secondarily whether pantothenate really favorably influences the effect of vitamin C or whether there exists reciprocal influences on the anti-toxic mechanisms.

We have caused poisoning with an organo-mercuric compound, because we have considered it unimportant in which form mercury is administered, it forms in the organism organo-mercuric compounds, but the substance used by us can be more precisely studied. The mechanism of effect of the mercuric compound is of special interest to the substances with the thiolic groups (for example, glutation), the enzymatic systems also belonging here (transaminase, succindehydrogenase, lactico-dehydrogenase, Co.A,

pyruvate-oxidase, lipoic acid, etc.), which intervene in important stages of the intermediary metabolism (19,11,12,13,14,16). The constancy of stability of the compounds with mercury is greater when two groupings enter the reaction -- the proximate groupings SH, so that a good part of the monothiolic combinations can be reestablished. On the other hand, ascorbic acid having the property of empowering the thiolic groupings ~~from~~ of the tissues, all the premisses exist to allow an increase of ~~enable a great~~ tolerance of the organism to small concentrations of mercury through the rational administration of ~~some~~ doses of ascorbic acid. In our experiment, we have used these two vitamins and in particular ~~especially~~ ascorbic acid and for its general stimulative effect.

Procedure

Research was done on the on the frog tadpoles - Rana Temporaria - with ages between 3 and 6 weeks, they being divided into experiemntal groups of 25-30 individuals of the same age and size, in solutions of phenylmercuriborate in tap water (30 ml/one tadpole). 6 concentrations of phenosept were used ranging from 0.78 mg/1 (2.5×10^{-6} M/1) to 0.025 mg./1 (7.8×10^{-9} M/1) with and without the concomitant addition of ascorbic acid (100 mg.%) or calcium pantothenate (1 mg./1 and 25 mg./1), or both in a mixture. The following aspects were studied:

1. The antitoxic effect in the case of concomitant administration of the vitamins with the toxic solution observing ~~following~~: a) duration of life (in hours) of the tadpoles; b) the content of ascorbic acid [micrograms/gram, according to the Roe and Kuether method (26)], and c) the development in length (in the concentration of 0.025 mg/1) after 24 days of observation,

forming 5 groups with 7 tadpoles each (1. controls in water; 2. phenosept; 3. phenosept + vitamin C; 4. phenosept + pantothenate; 5. phenosept + vitamin C + pantothenate), each individual having the same initial length of 20 mm.

2. The effect of restoring the organism (with the concentration of 0.1 mg/l phenosept), in the case of administering the vitamin at the moment of the appearance of the first signs of poisoning, replacing the phenosept solution with aqueous solutions of vitamins.

RESULTS

1. In the case of concomitant administration of the vitamins and of the toxic solution, there was observed a significant prolonging (0.01 > P > 0.001) of the duration of life of the group with ascorbic acid alone, in comparison with the other poisoned groups, the effect being more obvious in the first 4 concentrations (ranging from 0.78 to 0.1 mg/l). The pantothenate alone or in combination with vitamin C did not have an obvious effect (Fig. 1).

The development in length after 24 days vis-à-vis the controls, the poisoned groups showed a delayed development. There was also observed in this case a discreet positive effect but statistically insignificant for vitamin C (fig. 2/b).

Table I

Tabelul I

Dezvoltarea în lungime (mm) după 24 zile de intoxicare (concentrație fenosept 0,025 mg/l)	Lotul	No. obs.	M. ± E.S.	P
Concentrație de vitamine	Fenosept	7	24,9 ± 1,18	**
	Fenosept + vitamina C	7	29,4 ± 1,06	0,10 > P > 0,05
Acid ascorbic 100 mg %	Fenosept + pantotenat	7	25,5 ± 2,11	P > 0,50
	Fenosept + vitamina C + pantotenat	7	26,1 ± 1,71	P > 0,50
Pantotenat de Ca : 1 mg/l	Apă	7	35,8 ± 2,02	0,01 > P

values varied independently from the constant intakes of PGA, it is unlikely that the urinary excretion of PGA in large amounts following its oral administration accounts for the observed alterations in urinary CF excretion. This is obvious from comparing the urine CF values obtained in the patients with scurvy before and during ascorbic acid therapy. Likewise, the urine CF values were not related directly to ascorbic acid administration. However, in order further to validate the microbiologic assay of the urines studied for CF activity content, all urine specimens were assayed for PGA and ascorbic acid content. Although the urines obtained during periods of PGA administration contained very high concentrations of PGA, in no case was this of sufficient magnitude, taking into account the dilution factors used in the assays for CF, to allow for growth stimulation of *L. citrovorum*. Likewise, ascorbic acid alone or in combination with PGA in the concentrations encountered in the urines studied failed to replace CF for the growth of *L. citrovorum*.

The urinary CF excretion pattern observed in patient T. C., Figure 1, might suggest that initial saturation with PGA may be necessary before maximal urinary CF excretion occurs. The urinary PGA excretion data do not, however, indicate an initial retention of PGA. In fact, the quantities of PGA excreted were comparable before and after ascorbic acid therapy. Illustrative data obtained in one of the patients studied are presented in Table I. The failure to observe a gradually increasing CF excretion in patient F. O'D., Figure 2, during six days of PGA administration before ascorbic acid therapy also makes it unlikely that the gradually increasing urinary CF excretion noted in patient T. C., Figure 1, would have occurred even if ascorbic acid had not been given.

The results of the PGA assays on the urines also demonstrate that the failure of maximal urinary CF excretion to occur in the scorbutic state is not related to a failure of absorption of the orally administered PGA in the absence of ascorbic acid since in both patients with scurvy, large amounts of urinary PGA were excreted daily before ascorbic acid therapy (Table I).

The relatively small but significant increase in urinary CF excretion noted in the patients with scurvy given PGA orally is not readily explained.

TABLE I

Urinary PGA and CF excretion following oral PGA before and during ascorbic acid therapy in a patient with scurvy (T. C.)

Day of study	Oral medication		Urinary excretion	
	PGA	Ascorbic acid	PGA	CF
	mg.	gm.	γ	γ
1			8.0	1.1
2			4.4	1.3
3			6.9	1.1
4	10.0		5,400	9.1
5	10.0		5,500	8.9
6	10.0		7,200	13.3
7	10.0	1.0	8,400	16.9
8	10.0	1.0	8,500	33.4
9	10.0	1.0	6,800	57.6
10	10.0	1.0	5,600	62.4
11	10.0	1.0	6,600	89.1
12	10.0	1.0	6,800	94.5
13	10.0	1.0	5,900	110.4
14		1.0	190	18.1
15		1.0	15.7	6.2
16		1.0	10.2	4.6
17		1.0	2.7	0.9
18		1.0	3.7	1.2
19		1.0	8.4	3.6
20	10.0	1.0	6,600	93.9
21	10.0	1.0	6,100	123.9
22	10.0	1.0	8,000	111.4
23	10.0	1.0	8,100	162.5

This may be related to the large doses of PGA used. Other possibilities are that although ascorbic acid was not found in the buffy coats of the patients studied, they were not totally deficient in ascorbic acid, or perhaps that reducing substances other than ascorbic acid may contribute to the biosynthesis of CF from PGA. In this regard, glucoascorbic acid as well as ascorbic acid markedly increased the urinary excretion of CF in rats given PGA, while cysteine and glutathione were without effect (16).

Urinary CF did not increase promptly in the patients with scurvy after ascorbic acid was given. Several days of ascorbic acid therapy were required before outputs of CF comparable to those observed in normal individuals occurred. This delay in CF excretion may be related to the quantity of ascorbic acid required to restore adequate tissue concentrations of this substance, or it may indicate tissue storage of CF occurring before excretion of this material commences.

The results of these studies indicate that PGA is closely related metabolically to CF in adult man, in confirmation of similar findings by others (12–16). In addition, the data presented indicate that

This effect can be explained by the important rôle of this vitamin in all the processes of oxido-reduction of the organism, by its influence on the cellular respiration both directly and through its protective effect exerted on the enzymatic systems. Calcium pantothenate had no favorable effect at all; only in the case of its application associated with vitamin C was it possible to observe a not insignificant restorative effect on the poisoned organism (table II).

As is seen from fig. 2/c, in the sub-acute experiment, the pantothenic acid administered did not show significant correlations with vitamin C; the ascorbic acid content of the organism of the tadpoles remains at the same level both in the poisoned group -- not having received vitamins, as well as in the poisoned group which received pantothenic acid, which also agrees with the obervations of the authors, Fidanza, Constable and collaborators (6) and Pudelkewitz and collaborators (23).

Fig. 3 shows the difference of individual reactivity of the tadpoles; as the concentration is decreased, the values are more dispersed around the average (mean), thus as the subjects react more distantly in time. This fact added to the age of the subjects, the conditions of variation of the temperatures of the various days of the experiment, etc., causes the quantitative evaluations to be difficult; therefore, in our experiment, the tests on the tadpole gave us the possibility of arriving at interpretations, studying the tendances rather than absolute values.

CONCLUSIONS

1. Ascorbic acid has a general effect of restoring the organism, increasing the resistance of tadpoles to the toxic effect of the organo-mercuric compound, both in the case of applying them concomitantly with phenosept solution as well as after the beginning of the toxic effect.

2. Pantothenic acid administered alone did not show any antitoxic effect at all, but in the case of its application in combination with vitamin C in poisoning with small concentrations of phenosept, the correlation of these two vitamins was insignificant; the ascorbic acid content of the organism of the poisoned tadpoles was decreased identically, both in the group that did not receive vitamins and in the group that received a supplement of pantothenate.

3. The antitoxic effect of the vitamins is shown in the zone of small concentrations of toxic substance, in which the reactions are considered reversible, subject to the influence of vital substances.

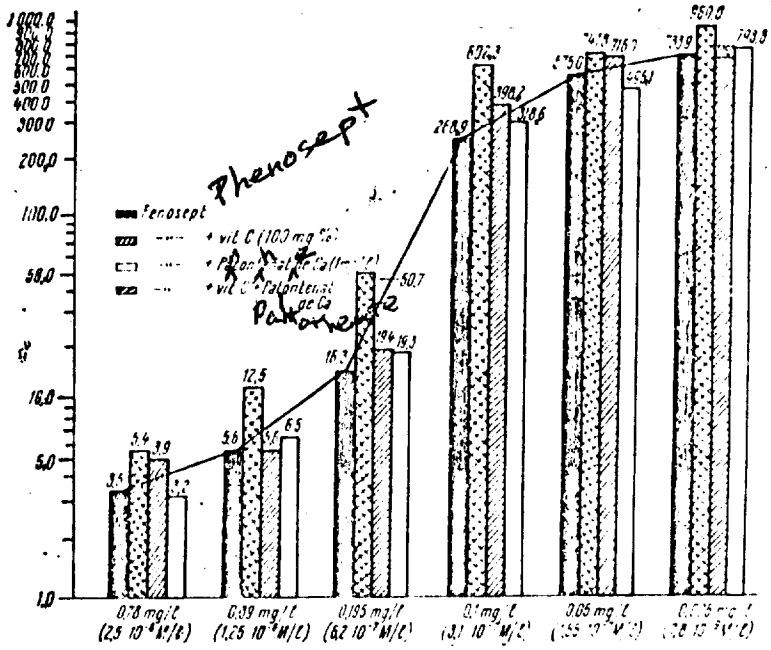

Fig. 1. — Durata de viaţă a mormolocilor (în ore) la diferite concentraţii de fenilmercuriborat, în cazul aplicării concomitente a vitaminelor şi a soluţiilor toxice.

Fig. 1 — The duration of life of tadpoles (in hours) related to the various concentrations of phenylmercuiborate in the case of concomitent application of vitamins and of toxic solutions.

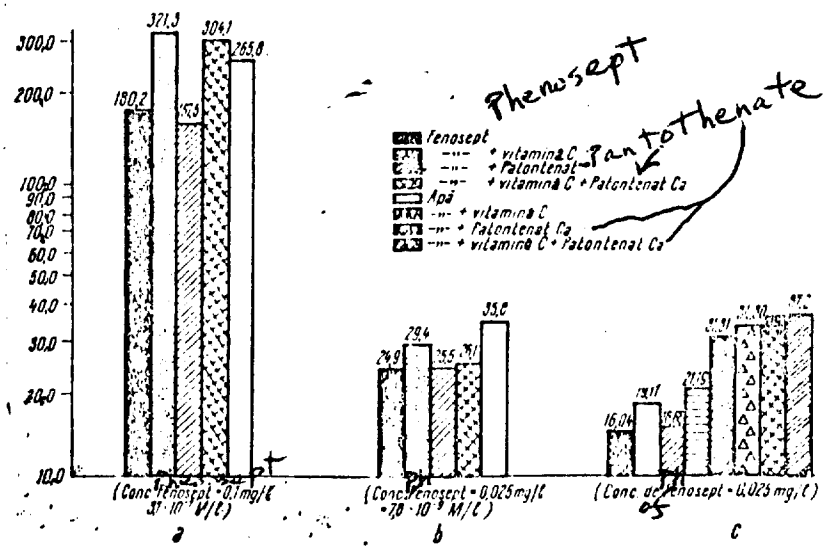

Fig. 2 *a)* Acţiunea terapeutică (aplicînd vitaminele) după apariţia primelor simptome de intoxicaţie. b) Dezvoltarea în lungime (mm) după 24 de zile de observaţie. *c)* Conţinutul de vitamină C (mg/g) din organismul mormolocilor.

Fig. 2 — a) the therapeutic effect (applying vitamins) after the appearance of the first symptoms of poisoning; b) Development in length (mm) after 24 days of observation; c) vitamin C content (mg/g) of the organism of the tadpoles.

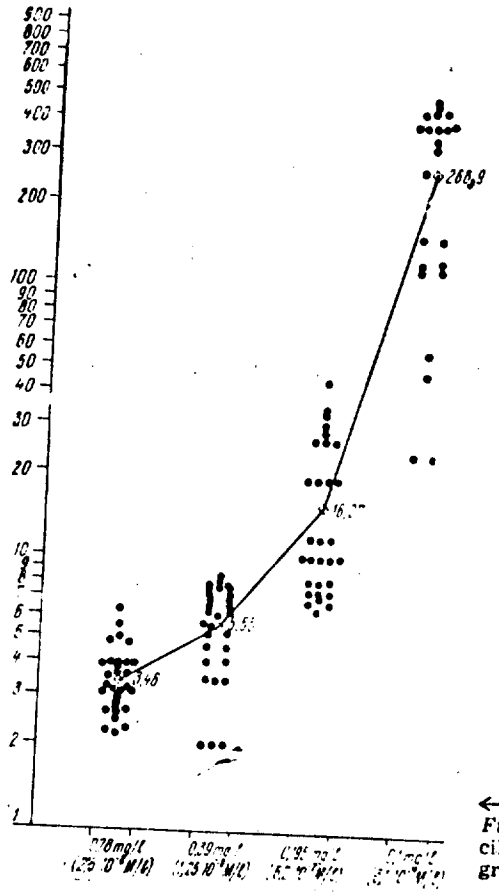

Fig. 3. — Durata de viață a mormolocilor intoxicați cu fenilmercuriborat; gradul dispersie a valorilor individuale la diferite concentrații.

Fig. 3 Duration of life of tadpoles poisoned with phenylmercuriborate; the degree of dispersion of individual values at different concentrations.

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IGIENA
vol. XIX, nr. 2, anul 1970

Acțiunea antitoxică a acidului ascorbic în intoxicația experimentală cu fenilmercuriborat

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REZUMAT

S-a experimentat cu acidul ascorbic și pantotenatul de calciu pe mormolocii *Rana temporaria*, întrebuințînd soluții de fenilmercuriborat în 6 concentrații (între $2,5 \times 10^{-6}$ M/1 și $7,8 \times 10^{-9}$ M/1), cu și fără adaos de vitamine.

S-a cercetat efectul antitoxic, administrînd vitaminele atît concomitent, cît și după apariția primelor semne de intoxicație.

Din observații rezultă că vitamina C mărește rezistența mormolocilor la acțiunea toxică a compusului organo-mercuric, atît în cazul aplicării lui concomitent cu soluția de fenosept, cît și după apariția primelor semne de intoxicație. Pantotenatul de calciu nu a influențat semnificativ reacția mormolocilor.

Relația dintre vitamine și diferitele substanțe chimice cu acțiune nocivă asupra organismului, a constituit obiectul a numeroase lucrări experimentale.

Un loc important îl ocupă în această privință acidul ascorbic, care este un component vital cu efecte pozitive în multe cazuri de expunere la substanțe toxice organice (1, 2, 8, 21) și minerale (3, 4, 7, 15, 18, 20, 22, 25, 32). Observațiile noastre la om (3) sau experimental la animale, au confirmat însemnătatea vitaminei C ca factor antitoxic. Pe de altă parte, acidul pantotenic, component al coenzimei A, fiind implicat în procesele de acetilare biologică, se presupune că prezintă și el un rol pozitiv (19, 27, 31).

O atenție deosebită se acordă în ultimul timp interacțiunii dintre vitamine, problemă care interesează mecanismele de apărare ale organismului, insuficient clarificate și prezentînd numeroase păreri contradictorii.

Una dintre problemele mult disputate este corelația dintre acidul pantotenic și vitamina C. Unii autori semnalează interrelații favorabile între acțiunea celor două vitamine (5, 24, 28, 29, 30). În alte condiții experimentale, însă, aceste observații nu au putut fi întotdeauna confirmate (6, 10, 17, 23).

În lucrarea de față în primul rînd ne-a interesat măsura în care vitamina C îndeplinește un rol antitoxic și în al doilea rînd, dacă într-adevăr pantotenatul influențează favorabil acțiunea vitaminei C, sau dacă există influențe reciproce în mecanismele antitoxice.

Intoxicația am provocat-o cu un compus organo-mercuric, deoarece, am considerat că indiferent sub ce formă se administrează mercurul, el formează în organism compuși organo-mercurici, iar substanța folosită

de noi, poate să fie mai precis urmărită. Mecanismul de acţiune a compuşilor mercurici interesează în special substanţele cu grupe tiolice (de exemplu glutation), aparţinînd aici şi sisteme enzimatice (transaminaze, succindehidrogenaza, lactico-dehidrogenaza, Co. A, piruvat-oxidaza, acidul lipoic etc.), care intervin în etape importante ale metabolismului intermediar (19, 11, 12, 13, 14, 16). Constanta de stabilitate a compuşilor cu mercur este mai mare cînd intră în reacţie două grupări — SH învecinate, încît o bună parte din combinaţiile monotiolice pot să fie restabilite. Pe de altă parte, acidul ascorbic, avînd proprietatea de a potenţa grupele tiolice din ţesuturi, există toate premisele pentru a putea mări toleranţa organismului la concentraţii mici de mercur, prin administrarea raţională a unor doze de acid ascorbic. În experimentul nostru am utilizat cele două vitamine şi în special acidul ascorbic şi pentru efectul lui general de stimulare.

METODA DE LUCRU

S-a lucrat cu mormolocii de broască — *Rana Temporaria* — cu vîrsta între 3—6 săptămîni, împărţindu-i în loturi experimentale a cîte 25—30 indivizi de aceeaşi vîrstă şi dimensiune, în soluţii de fenilmercuriborat în apă de robinet (30 ml/ un mormoloc). S-au utilizat 6 concentraţii de fenosept între 0,78 mg/l ($2,5 \times 10^{-6}$ M/l) şi 0,025 mg/l ($7,8 \times 10^{-9}$ M/l) cu şi fără adaos concomitent de acid ascorbic (100 mg%) sau pantotenat de calciu (1 mg/l şi 25 mg/l), sau amîndoi în amestec. S-au cercetat următoarele aspecte :

1. Acţiunea antitoxică, în cazul administrării *concomitente* a vitaminelor cu soluţie toxică urmărindu-se : a) *durata de viaţă* (în ore) a mormolocilor ; b) *conţinutul de acid ascorbic* [micrograme/gram, după metoda Roe şi Kuether (26)] şi c) *dezvoltarea în lungime* (la concentraţia de 0,025 mg/l) după 24 zile de observaţie, alcătuind 5 loturi cu cîte 7 mormoloci (1. martori în apă ; 2. fenosept ; 3. fenosept + vitamina C ; 4. fenosept + pantotenat ; 5. fenosept + vitamina C + pantotenat), fiecare individ avînd aceeaşi lungime iniţială de 20 mm.

2. Acţiunea de restabilire a organismului (la concentraţie de 0,1 mg/l fenosept), în cazul administrării vitaminelor în momentul apariţiei primelor semne de intoxicare, înlocuindu-se soluţia de fenosept cu soluţiile apoase ale vitaminelor.

REZULTATE

1. În cazul administrării concomitente a vitaminelor şi a soluţiei toxice, s-a constatat prelungirea semnificativă ($0,01 > P > 0,001$) a *duratei de viaţă* la lotul cu acid ascorbic singur, în comparaţie cu celelalte grupe intoxicate, efectul fiind mai evident la primele 4 concentraţii (între 0,78 şi 0,1 mg/l). Pantotenatul singur sau în combinaţie cu vitamina C, nu a avut o acţiune evidentă (fig. 1).

Dezvoltarea în lungime după 24 zile faţă de martori, grupurile intoxicate au prezentat o dezvoltare întîrziată. S-a constatat şi în acest caz un discret efect pozitiv dar statistic nesemnificativ, al vitaminei C (fig. 2/b).

Tabelul I

Dezvoltarea în lungime (mm) după 24 zile de intoxicare (concentraţie fenosept 0,025 mg/l)	Lotul	Nr. obs.	M.±E.S.	P
	Fenosept	7	24,9±1,18	**
Concentraţie de vitamine	Fenosept + vitamina C	7	29,4±1,96	0,10>P>>0,05
Acid ascorbic : 100 mg %	Fenosept + pantotenat	7	25,5±2,11	P>0,50
	Fenosept + vitamina C + pantotenat	7	26,1±1,71	P>0,50
Pantotenat de Ca : 1 mg/l	Apă	7	35,8±2,02	0,01>P

Conținutul de acid ascorbic (micrograme/g) este mai scăzut la loturile intoxicate în comparație cu martorii (fig. 2/c), diferențele fiind însă statistic nesemnificative.

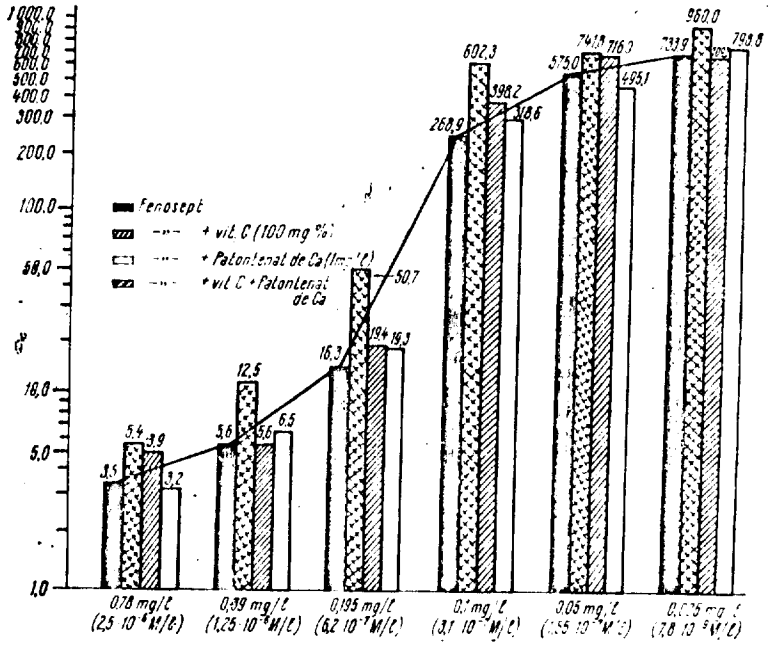

Fig. 1. — Durata de viață a mormolocilor (în ore) la diferite concentrații de fenilmercuriborat, în cazul aplicării concomitente a vitaminelor și a soluțiilor toxice.

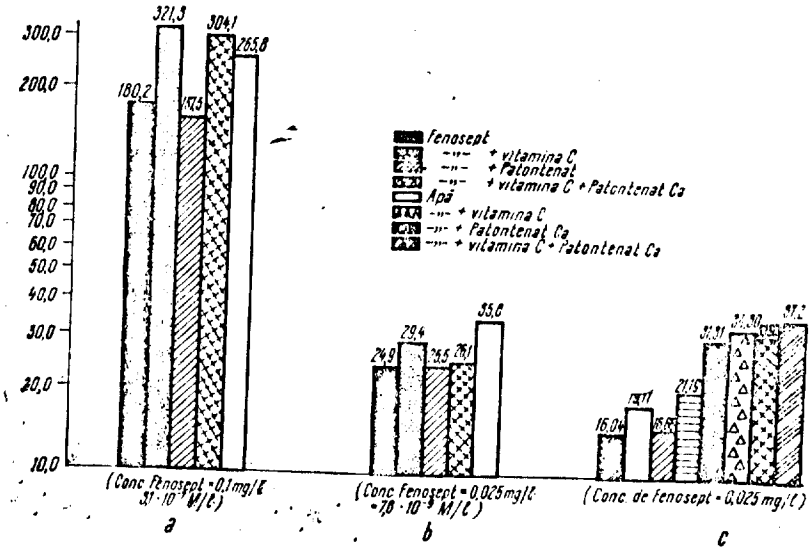

Fig. 2 *a)* Acțiunea terapeutică (aplicînd vitaminele) după apariția primelor simptome de intoxicație. b) Dezvoltarea în lungime (mm) după 24 de zile de observație. *c)* Conținutul de vitamină C (mg/g) din organismul mormolocilor.

2. Administrarea vitaminelor în faza incipientă a intoxicației a fost urmată de prelungirea semnificativă a duratei de viață atît a lotului numai cu vitamina C, cît și a grupului care a primit cele două vitamine asociate (fig. 2/a).

Tabelul II

Durata de supraviețuire (ore) a mormolocilor în cazul administrării vitaminelor după apariția semnelor de intoxicație (concentrația fenosept: 0,1 mg/l).	Lotul	Nr. obs.	M. ± E.S.	P.
	Fenosept	25	180,2 ± 22,62	**
	Fenosept + vitamina C	25	321,3 ± 56,03	0,05 > P > 0,02
	Fenosept + pantotenat	25	167,5 ± 45,66	P > 0,50
	Fenosept + vitamina C + pantotenat	25	304,1 ± 56,86	P = 0,05
	Apă	25	265,8 ± 39,74	0,10 > P > 0,05

DISCUTAREA REZULTATELOR

Din cele prezentate, rezultă că acțiunea antitoxică a vitaminelor se manifestă în zona concentrațiilor mici de fenosept, la care reacțiile sînt considerate reversibile și deci influențabile prin substanțe vitale.

Dintre cele două vitamine studiate, *acidul ascorbic* se evidențiază printr-o acțiune antitoxică foarte exprimată, atît în cazul administrării concomitente a vitaminei C cu toxicul, cît și privind efectul său de refacere a organismului intoxicat. Această acțiune este explicabilă prin rolul important al acestei vitamine în toate procesele de oxido-reducere din organism, prin influența ei asupra respirației celulare atît direct, cît și prin acțiunea sa de protecție exercitată asupra sistemelor enzimatice.

Pantotenatul de calciu nu a avut nici un efect favorizant; numai în cazul aplicării asociate cu vitamina C s-a putut constata o acțiune neînsemnată de refacere a organismului intoxicat (tabelul II).

După cum reiese din fig. 2/c, în experimentul subacut, acidul pantotenic administrat nu a arătat corelații semnificative cu vitamina C; conținutul de acid ascorbic din organismul mormolocilor se situează la același nivel, atît la lotul intoxicat — nevitaminizat, cît și la cel intoxicat și suplimentat cu acid pantotenic, ceea ce concordă și cu observațiile autorilor Fidanza, Constable și colab. (6) și Pudelkewitz și colab. (23).

Din fig. 3 reiese diferența de reactivitate individuală a mormolocilor; cu cît se micșorează concentrația, cu atît valorile sînt mai dispersate în

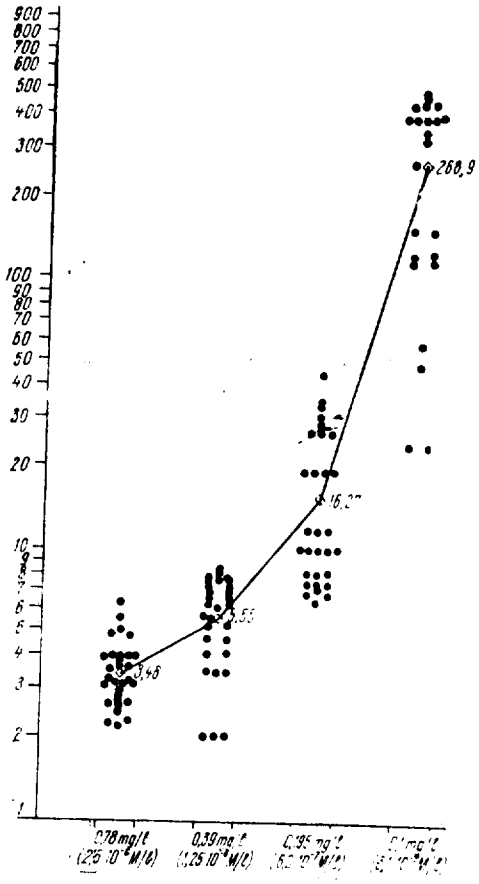

Fig. 3. — Durata de viață a mormolocilor intoxicați cu fenilmercuriborat; gradul dispersie a valorilor individuale la diferite concentrații.

jurul mediei, deci cu atît subiecţii reacţionează mai distanţat în timp. Acest fapt, adăugat la vîrsta subiecţilor, la condiţiile de variaţii ale temperaturii din diverse zile ale experimentului şi altele, face ca evaluările cantiative să fie dificile; de aceea, în experimentul nostru, testul mormoloci ne-a dat posibilitatea de a ajunge la interpretări, urmărind mai mult tendinţele decît valorile absolute.

CONCLUZII

1. Acidul ascorbic are o acţiune generală de restabilire a organismului, mărind rezistenţa mormolocilor la acţiunea toxică a compusului organo-mercuric, atît în cazul aplicării ei *concomitente* cu soluţia de fenosept, cît şi *după* începutul instalării efectului toxic.

2. Acidul pantotenic administrat izolat nu a prezentat nici un efect antitoxic, iar în cazul aplicării asociate cu vitamina C în intoxicaţie cu concentraţii mici de fenosept, corelaţiile dintre aceste două vitamine au fost nesemnificative; conţinutul de acid ascorbic din organismul mormolocilor intoxicaţi a scăzut în mod identic, atît la lotul nevitaminizat, cît şi la cel cu supliment de pantotenat.

3. Acţiunea antitoxică a vitaminelor se manifestă în zona concentraţiilor mici de substanţă toxică, la care reacţiile sînt considerate reversibile, influenţabile prin substanţe vitale.

BIBLIOGRAFIE

1. BROWNING E. — *Bull. Hyg. (Lond.)*, 1966, vol. 41, nr. 6, p. 517—518. — 2. CASTROVILLI G. — *Med. d. Lavoro* 1937, vol. 28, p. 106. — 3. COMES V., GALEA V., MARGARETA WEINLICH — *Stud. Cercet. Biochim.*, 1965, vol. 8, nr. 1, p. 33—41. — 4. DAVIDOVA G. N. — *Tr. Leningr. sanit. — gig. med. Inst.*, 1953, vol. 14, p. 66—73. — 5. EVERSON GLADYS, NORTHROOP L., NAM JOUNG CHUNG, GETTY R. — *J. Nutr.*, 1954, vol. 54, p. 305—311. — 6. FIDANZA A., CONSTABLE B. — *J. Acta Vitaminol*, 1963, vol. 17, nr. 3, p. 95—99. — 7. GONŢEA I., PARASCHIVA ŞUŢESCU — *Igiena*, 1964, nr. 6, p. 501—511. — 8. GROSMAN IU. S., NAZAROVA Z. A. — *Farmakol. i. Toksikol.*, 1957, vol. XX, nr. 3, p. 82—86. — 9. GRUBER W., WARZECKA K., PFLEIDERER G. — *Biochem. Z.*, 1962, vol. 336, nr. 2, p. 107—117. 10. HURLEY S. LUCILLE, MACKENZIE B. IULIA — *J. Nutr.*, 1954, vol. 54, p. 403—415. — 11. IONEK I. — *Int. Arch. f. Gewerbepath.*, 1964, vol. 21, p. 1—10. — 12. IONEK I., GRZYBEK H. — *Int. Arch. F. Gewerbepath.*, 1964, vol. 2, nr. 6, p. 572—579.—13. IONEK I., PAACHOLEK A.—*Int. Arch.f. Gewerbepath.*, 1966, vol. 20, nr. 6, p. 562—572—14. IONEK I., VACLAWCZYK H. — *Zbl. Arbeitsmed.*, 1964, vol 14 nr. 9, p. 205—210.—15. KLIMENTOVSKAYA A. E.—*Chem. Abstr.*, 1963 vol.60, 16691 d, e.— 16. KOSMIDER ST.— *Int. Arch. f. Gewerbepath.*, 1965, vol. 21, nr. 3, p. 282—314. — 17. LENTI L., DE CICCO A., GAMBERINI E. — *Boll. Soc. ital. Biol. sper.*, 1965, vol. 41, p. 583—585. — 18. MATUSEVICH I. Z. — *Chem. Abstr.*, 1956, vol. 50, 5916, b, c , d, e. — 19. MEYER D. I. — Literatur-Eildienst „Roche", 1961, vol. 29, nr. 11, p. 41—44. — 20. MILENKO V., SUSCIC B. — *Chem. Abstr.*, 1956, vol. 50, 9599 f. — 21. MIRZABEKJAN G. J., NIGOGOSJAN S. V. — *Zbl. Arbeitsmed.*, 1962, vol. 12, nr. 11, p. 280. — 22. MOMCILO S. MOKRANJAK, CEDA PETROVIC — Compt. rend., 1964, vol. 258, nr. 4, p. 1 341— 1 342. — 23. PUDELKEWITZ CECILIA, RODERUCK CHARLOTTE — *J. Nutr.* 1963, vol. 81, nr. 4, p. 415—418. — 24. RALLI P. ELAINE — *Chem Abstr.*, 1955, vol. 49, p. 5 604 b. — 25. REMY R. — *Dtsch. Tierärzt. Wschr.*, 1965, vol. 63, p. 405—408. — 26. ROE J. H., KUETHER C. A. — *J. Biol. Chem.*, 1943, vol. 147, p. 399. — SHYAMALA M. B., BHAT J. V. — *Int. Z. Vitamin-forsch.*, 1963, vol. 1, nr. 33, p. 102. — 28. TERROINE THÉRÈSE — Compt. rend., 1958, vol. 246, p. 3677—3680. — 29. TERROINE THÉRÈSE — *Chem. Abstr.*, 1960, vol. 54, 10077 e. — 30. TERROINE THÉRÈSE — *Chem. Abstr.*, 1960, vol. 54, 10043 g. — 31. WEBER T. M. — *Int. Z. Vitamin-forsch.*, 1964, vol. 1, nr. 34, p. 165. — 32. WU H. W., LU S. H., CHANG M. C. — *Chem. Abstr.*, 1963, vol. 58, 10647 g.h.

Articol intrat în redacţie la 5.IX.1969

Indicele de clasificare: 615.9—092.259:547.475.2

RÉSUMÉ

V. Galea, Margareta Weinlich, Zoe Piersica — L'ACTION ANTITOXIQUE DE L'ACIDE ASCORBIQUE DANS L'INTOXICATION EXPÉRIMENTALE PAR PHÉNYLMERCURIBORATE

On a effectué des expériences avec l'acide ascorbique et le pantothénate de calcium sur des têtards de *Rana temporaria* en utilisant des solutions de phénylmercuriborate à 6 concentrations (entre $2,5 \times 10^{-6}$ M l et $7,8 \times 10^{-9}$ M/l), avec et sans adjonction de vitamines. On a étudié l'effet antitoxique, en administrant les vitamines simultanément, et après l'apparition des premiers signes d'intoxication. Les observations mettent en évidence que la vitamine C augmente la résistance des têtards à l'action toxique du composé organo-mercurique, aussi bien dans les cas de son application concomitante avec la solution de phénosept, qu'après l'apparition des premiers signes d'intoxication. Le pantothénate de calcium n'a pas influencé significativement la réaction des têtards.

ZUSAMMENFASSUNG

V. Galea, Margareta Weinlich, Zoe Piersica — DIE GIFTBEKÄMPFENDE WIRKUNG VON ASKORBINSÄURE BEI PHENYLMERKURIBORATVERGIFTUNG IM TIERVERSUCH

Verff. unternahmen Versuche mit Askorbinsäure und Calcium pantothenicum an Kaulquappen von *Rana temporaria*, bei denen sie Phenylmerkuriborat-Lösungen in 6 Konzentrationen (zwischen $2,5 \times 10^{-6}$ M/l und $7,8 \times 10^{-9}$ M/l) mit und ohne Vitaminzusatz benuntzten. Es wurde die giftbekämpfende Wirkung untersucht, indem die Vitamine gleichzeitig oder nach Auftreten der ersten Vergiftungserscheinungen zugeführt wurden. Aus den Beobachtungen geht hervor, dass C-Vitamin den Widerstand der Kaulquappen gegen die giftige Wirkung des organischen Quecksilberpräparats sowohl im Falle seiner gleichzeitigen Applikation mit Fenoseptlösung als auch nach Auftreten der ersten Vergiftungserscheinungen erhöht. Pantothenatkalzium hat die Reaktion der Kaulquappen nicht signifikant erhöht.

SUMMARY

V. Galea, Margareta Weinlich, Zoe Piersica — THE ANTITOXIC ACTION OF ASCORBIC ACID IN EXPERIMENTAL INTOXICATION WITH PHENYLMERCURIBORATE

Experiments were carried out with ascorbic acid and calcium pantothenate on *Rana temporaria* tadpoles, using phenylmercuriborate solutions in 6 different concentrations, ranging from 2.5×10^{-6} M l to 7.8×10^{-9} M l, with and without vitamins. The antitoxic effect of ascorbic acid was tested by administering the vitamins concomitantly and after the first signs of intoxication appeared. Vitamin C increased the resistance of the tadpoles to the toxic action of the organo-mercuric compound, both when applied concomitantly with the phenosept solution and when applied after the onset of the first signs of intoxication. Calcium pantothenate did not significantly influence the reaction of the tadpoles.

РЕЗЮМЕ

В. Галя, Маргарета Вейнлих, Зое Пиерсика — АНТИТОКСИЧЕСКОЕ ДЕЙСТВИЕ АСКОРБИНОВОЙ КИСЛОТЫ ПРИ ЭКСПЕРИМЕНТАЛЬНОЙ ИНТОКСИКАЦИИ ФЕНИЛМЕРКУРИБОРАТОМ

Авторы подвергли экспериментальному исследованию действие аскорбиновой кислоты и пантотената кальция на головастиков лягушки *Rana temporaria* применяя растворы фенилмеркурибората в 6 концентрациях (от $2,5 \times 10^{-6}$ М/л до $7,8 \times 10^{-9}$ М/л) с добавлением и без добавления витаминов. Исследовалось антитоксическое действие с добавлением витаминов как одновременно, так и после появления первых признаков отравления. Из осуществленных наблюдений следует, что витамин С повышает резистентность головастиков к токсическому действию фенилмеркурибората как в случае его одновременного применения с раствором феносепта, так и после появления первых признаков отравления. Пантотенат кальция не оказывал значительного действия на реакцию со стороны головастиков.

J. Amer. Med. Assoc. 211: 105

Effect of Ascorbic Acid on Endurance Performance and Athletic Injury

Capt George O. Gey, MC, USAF; Lt Col Kenneth H. Cooper, MC, USAF; and Robert A. Bottenberg, PhD

It has been postulated that supplemental doses of ascorbic acid (vitamin C) may improve endurance performance and perhaps reduce the severity and morbidity of athletic injuries.[1-4] The present study was designed to answer these questions and to determine the long-term effects of a large supplemental dose of ascorbic acid in contrast to those of a placebo.

Materials and Methods

Two hundred and eighty-six male officers (average age, 28.0 years) participated in this study while attending the 12-week Squadron Officers School, Maxwell Air Force Base, Ala. Each officer ran the 12-minute field test[5] at the beginning of training and then was given a bottle containing either 500-mg tablets of ascorbic acid or a placebo. The bottles were numbered at random with a code that was kept in strict secrecy until completion of the study. The subjects were instructed to take two pills each day throughout the training period. Also, they were asked to keep a detailed record of all injuries sustained during the training period, which consisted of three weeks of preconditioning, and then nine weeks of competitive soccer, volleyball, and flickerball. The 12-minute field test was repeated at the end of training in order to document any change in endurance performance. No attempt was made to standardize the diet since the subjects lived and ate in the same general environment.

Results

The majority of the 286 subjects in both the ascorbic acid and placebo groups took all the pills as directed. After 12 weeks of training neither the endurance performance nor the overall improvement as measured by the average distance on the 12-minute, walk-run test were significantly different (Table). Likewise, when the number and rate of injuries were compared, neither the total injury rates in the two ascorbic acid groups nor the total injury rates for the ascorbic acid and placebo groups were significantly different. Minor injuries were most common, but the percentage of injuries in both the ascorbic acid and placebo groups were remarkably comparable. The majority of the injuries persisted for eight days or longer, but there was no significant difference between the groups taking ascorbic acid or placebo when the duration of injuries was compared.

Performance on 12-Minute, Walk-Run Test

		At Beginning of Training		After 12 Weeks of Training	
Pills Taken		No. of Subjects	Distance (Miles)	No. of Subjects*	Distance (Miles)
All	Ascorbic acid	112	1.32 ± 0.23	111	1.65 ± 0.16
	Placebo	100	1.30 ± 0.24	96	1.62 ± 0.17
Less than one half	Ascorbic acid	29	1.20 ± 0.22	27	1.60 ± 0.15
	Placebo	45	1.34 ± 0.26	44	1.62 ± 0.17
Total	Ascorbic acid	141	1.30 ± 0.23	138	1.65 ± 0.16
	Placebo	145	1.30 ± 0.24	140	1.62 ± 0.17

*Five subjects were unable to take the final running test because of athletic injuries.

Comment.—The effect of a daily dose of 1,000 mg of ascorbic acid on endurance perforance, and the rate, severity, and duration of athletic injury was studied. The effect was negligible when compared to that of a placebo. Perhaps the results would have been different if the subjects had been older or the physical activity had not included contact sports. Such questions remain unanswered, but the need for large, double-blind studies to answer such questions is apparent. Only in this manner can the true value of dietary supplements are ascertained.

The ascorbic acid and placebo tablets were prepared by Eli Lilly and Co.

From the Aerospace Medical Laboratory (Clinical), Wilford Hall USAF Medical Center (Capt Gey and Lt Col Cooper), and the Personnel Research Laboratory (Dr. Bottenberg), Aerospace Medical Division (AFSC), Lackland Air Force Base, Tex. Dr. Gey is now at Baltimore City Hospital.

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References

1. Robertson, W.V.B.: The Biochemical Role of Ascorbic Acid in Connective Tissue, *Ann NY Acad Sci* 92:159-167 (April 21) 1961.

2. Abt, A.F., and Von Schuching, S.: Catabolism of L-Ascorbic-1-C-14 Acid as a Measure of Its Utilization in the Intact and Wounded Guinea Pig on Scorbutic, Maintenance, and Saturation Diets, *Ann NY Acad Sci* 92:148-158 (April 21) 1961.

3. Moriguand, G.: Nutritional Problems Posed by the Behavior of a Gravid Terrain in the Presence of Avitaminosis and Hypervitaminosis C (Abortive Stage, Parturition, Fetomaternal Protection, Partial Deficiencies and Dietotoxic Effect), Hypervitaminosis C and Infecundity: Experimental Bioclinical Research, *Presse Med* 70:451-453 (Feb 24) 1962.

4. Margaria, R.; Aghemo, P.; and Rovelli, E.: The Effect of Some Drugs on Maximum Capacity of Athletic Performance in Man, *Int Z Angew Physiol* 20:281, 1964.

5. Cooper, K.H.: A Means of Assessing Maximal Oxygen Intake: Correlation Between Field and Treadmill Testing, *JAMA* 203:201-204 (Jan 15) 1968.

ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 70, 129–140 (1957)

Fatty Acid Oxidation by Means of Ascorbic Acid and Oxygen[1]

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Received September 27, 1956

Introduction

In a previous report (1) the oxidation of long-chain saturated fatty acids by means of oxygen and ascorbic acid was shown to occur under conditions normally used in physiological experiments. This was in marked contrast to the drastic conditions and potent reagents usually employed to accomplish this reaction. Thus, high concentrations of hydrogen peroxide and elevated temperatures were used by Dakin (2, 3), by Clutterbuck and Raper (4), and by Smedley-MacLean and Pearce (5). At a temperature of 100°C. considerable oxidation of the soaps of long-chain fatty acids by means of oxygen and catalysts was reported by Paquot and de Goursac (6). Whereas hydrogen peroxide caused the oxidation of both long- and short-chain acids (3), the latter formed no $C^{14}O_2$ when oxidized with the ascorbic acid–oxygen system (1). It is of interest that in contrast to the β-oxidation of fatty acids in animal tissues (7), a peanut cotelydon system has been reported that reacts *only* with long-chain fatty acids (8).

In the present paper the results of further studies concerning the ascorbate-catalyzed oxidation of fatty acids are given. These deal with such aspects as the effects of ions and chelating agents, and the influences of the concentration of reactants, the duration of the incubation period, and pH. The results of studies with carboxyl-, α- and β-labeled stearic acids and with oleic acid-1-C^{14} and elaidic acid-1-C^{14} are also given.

[1] Supported in part by grants-in-aid from the National Cancer Institute (C-722), National Institutes of Health, U. S. Public Health Service, Bethesda, Maryland; and a contract between Harvard University and the U. S. Air Force (AF-18(600)-455) monitored by the Arctic Aeromedical Laboratory, Ladd Air Force Base, Alaska; the American Cancer Society through an Institutional Grant to Harvard.

Experimental

Octanoic acid-1-C^{14}, palmitic acid-1-C^{14}, stearic acid-1-C^{14}, and stearic acid-2-C^{14} were from commercial sources.[2] Stearic acid-3-C^{14} was prepared from palmitic acid-1-C^{14} via the malonic acid synthesis.[3] Oleic acid-1-C^{14} was prepared as follows: 1-bromo-8,9-heptadecanediol was prepared from purified oleic acid by the procedure of Bergstrom *et al.* (9). By means of HBr and subsequent treatment with zinc, 1-bromo-8-heptadecene was obtained. From this, carboxyl-labeled oleic acid was prepared via the Grignard synthesis. A portion of the oleic acid was converted to elaidic acid (m.p. 44.1–44.5°C.) by isomerization with Se. Infrared analysis of the oleic acid showed the presence of approximately 5% of the *trans* isomer. All of the radio fatty acids used had specific activities of approximately 1 mc./mmole of acid and were used as their sodium salts. Where necessary, the solutions were heated prior to use.

Ascorbic acid[4] was always freshly prepared and neutralized just before use, and, in all experiments, it was the last addition to the Warburg flasks. Solutions of other unstable compounds such as cysteine were also freshly prepared. Ethylenediaminetetraacetic acid (EDTA) was used as its neutralized sodium salt.[5] Hydrogen peroxide solutions were prepared from 30% material.[6] Solutions of various metallic salts were prepared in carbonate-free water prior to use. Double-distilled water was used throughout these experiments.

The experiments and analyses for $C^{14}O_2$ were conducted as previously described (1). Except where otherwise designated, a 100% O_2 gas phase was employed. Ascorbic acid analyses were done by the method of Bessey (10). Pertinent data and results are given in the appropriate tables and figures. Throughout the paper the term "oxidation" when applied to fatty acids refers to the conversion of the carboxyl carbon to carbon dioxide.

Results and Discussion

In the presence of ascorbic acid and oxygen and at a temperature of 38°C., appreciable radiocarbon dioxide was formed from carboxyl-labeled long-chain saturated fatty acids. As shown by the data in Fig. 1, approximately two-thirds of the $C^{14}O_2$ was formed from palmitate-1-C^{14} within the first 60 min. Little additional $C^{14}O_2$ was formed after the second hour even though oxygen uptake was still rapid, and analyses by the method of Bessey (10) showed that ascorbic acid was still present. When the

[2] Carboxyl-labeled saturated acids were obtained from Tracerlab, Inc., Boston Mass. Stearic acid-2-C^{14} was obtained from Nuclear, Inc., Chicago, Illinois.

[3] The stearic acid-3-C^{14}, oleic acid-1-C^{14}, and the elaidic acid-1-C^{14} were prepared by Dr. Walter J. Gensler, Chemistry Department, Boston University Boston, Massachusetts.

[4] Generously supplied by Merck and Co., Rahway, New Jersey.

[5] Generously supplied by Versenes, Inc., Framingham, Massachusetts, now a subsidiary of Dow Chemical Corporation, Midland, Michigan.

[6] Superoxol. Merck and Co., Rahway, New Jersey.

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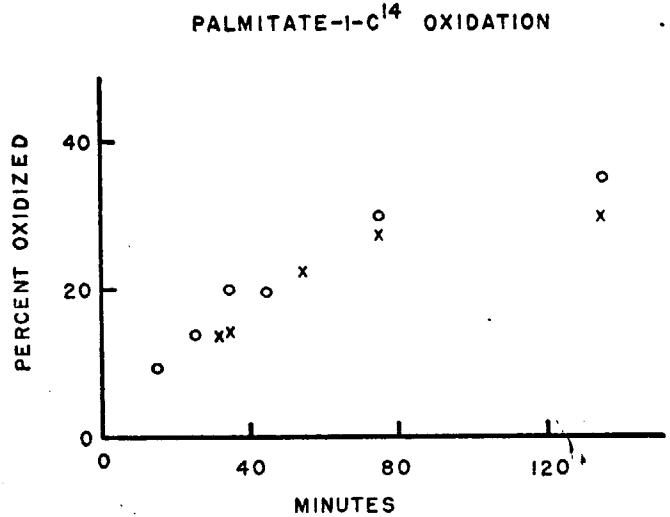

FIG. 1. Each flask contained 54.7 μmolar palmitate-1-C^{14}, 47 mmolar ascorbic acid, 0.1 *M* phosphate buffer (pH 7.1), and a total volume of 3 ml. An oxygen gas phase and a temperature of 38°C. were used. Typical results of two experiments are shown.

oxidation rate of the fatty acid had decreased, the addition of fresh radiopalmitate with or without the addition of fresh ascorbate caused appreciable $C^{14}O_2$ formation (Table I). This indicated that the decreased rate of fatty acid oxidation was not due to inhibition caused by reaction prod-

TABLE I

Effect of Sequence of Addition of the Various Reactants on the Extent of Oxidation of Palmitate-1-C^{14}

The total volume in each flask was 3 ml. and the final concentration of the components was as follows: 54.7 μmolar palmitate-1-C^{14}, 0.1 *M* phosphate buffer, 53.3 mmolar ascorbic acid, and 31 μmolar $FeCl_3$. The ascorbic acid and palmitate-1-C^{14} solutions added in the second period were freshly prepared. The total counts per minute added to each flask was 24,600, and the gas phase was air. Ascorbic acid, $FeCl_3$, and palmitate-1-C^{14} are designated as AA, Fe, and P, respectively.

Additions at start of first 30-min. period	Additions at start of second 30-min. period	Total radioactivity of carbon dioxide *counts/min.*
AA, Fe, P		4,960
Fe, P	AA	454
P	AA, Fe	237
AA, Fe	P	3,490

TABLE II

Effect of Ascorbic Acid Concentration on $C^{14}O_2$ Formation from Palmitate-1-C^{14}

All flasks contained 0.1 *M* phosphate buffer (pH 7.2), 54.7 μmolar palmitate-1-C^{14}, and a total volume of 3 ml. The gas phase was O_2 and the temperature was 38°C.

Ascorbic acid concentration *mM*	Per cent of C^{14} recovered as $C^{14}O_2$ in one hour
1.33	9.2
3.33	11.5
6.66	13.5
19.98	24.3
46.62	29.6
106.56	33.9
173.16	33.6

ucts, but rather that the residual palmitate was not readily available. That this was the case was shown by first incubating the radioacid in air or O_2 in the absence of ascorbate or in N_2 in its presence, and then incubating in air or O_2 in the presence of ascorbate. As shown in Table I, preincubation of the palmitate resulted in a decided decrease in the formation of $C^{14}O_2$. Similar results were obtained when the preincubation was carried out in N_2. Since radiocarbon recovery experiments have shown that the unoxidized C^{14} can be accounted for almost entirely in the form of the original substrate, these data strongly suggest that the physical state of the fatty acid was a critical factor in these experiments.

Increasing the ascorbate concentration beyond 46.6 mmolar did little to increase the amount of oxidation (Table II). An eightfold increase in fatty acid concentration resulted in a greater absolute oxidation of palmi-

TABLE III

Effect of Concentration of Palmitate-1-C^{14} on $C^{14}O_2$ Formation

All flasks contained 0.1 *M* phosphate buffer (pH 7.2), and 53.3 mmolar ascorbic acid, and a total volume of 3 ml.

Palmitate-1-C^{14} concentration *μM*	Palmitate-1-C^{14} oxidized in one hour *μmoles* × 10^{-3}
10.9	1.96
27.8	4.84
54.7	27.8
109.4	53.1
218.8	104.0
437.6	229.0
875.2	337.0

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tate-1-C^{14} (Table III), but the per cent oxidized remained practically unchanged. A 16-fold increase in palmitate concentration caused a decrease in $C^{14}O_2$ formation on a percentage basis, probably stemming from the limited solubility of this substrate. Although a higher pH would have favored the solubility of the fatty acid, it would also have favored the destruction of the ascorbic acid. It is apparent that the addition of the sodium palmitate to the reaction mixture at any pH not conducive to soap formation would result in loss of solubility of the fatty acid moiety even at the very high dilutions used. Since sodium soaps of saturated fatty acids are very insoluble, potassium and ammonium palmitate-1-C^{14} were also used. Results with the potassium soap were no higher than with the sodium soap, while very little $C^{14}O_2$ was formed from the ammonium soap. In some experiments potassium and ammonium ascorbate were used with the respective radiopalmitate soaps. Again, the potassium compounds were as effective as the sodium salts, but the ammonium compounds were very inferior.

As previously shown (1), little $C^{14}O_2$ was formed from short-chain acids by the action of the ascorbate–oxygen system. In order to determine whether or not the labeled short-chain acids would form $C^{14}O_2$ in the presence of a long-chain acid, octanoate-1-C^{14} was incubated in the presence of nonradioactive palmitic acid, ascorbic acid, and oxygen. Under these conditions no significant amount of $C^{14}O_2$ was formed. In related experiments in which nonradioactive octanoate was added to palmitic acid-1-C^{14}, no influence on $C^{14}O_2$ formation was found. Thus, the failure of short-chain acids to form carbon dioxide from the carboxyl carbon was not due to an inhibitory action of such acids.

The participation of metallic-ion catalysis was indicated by the decided reduction in both oxygen uptake and $C^{14}O_2$ production from palmitic acid-1-C^{14} occasioned by the addition of EDTA (Table IV). Therefore, a number of ions were tested for effects on the oxidation of carboxyl-labeled palmitate. At final concentrations of 0.02–0.05 m*M*, Zn^{++} and Ca^{++} almost completely blocked $C^{14}O_2$ formation; Cu^{++}, Sn^{++++}, and Mn^{++} halved $C^{14}O_2$ formation; whereas, Co^{++}, Ni^{++}, and Mg^{++} had no effect. The difference between the action of Ca^{++} and Mg^{++} is of interest because the physical and chemical properties of their saturated fatty acid salts are quite similar. Copper ions are known to be a potent accelerator of ascorbate oxidation, and their effect has been extensively studied (11). In the present experiments cupric ions speeded ascorbate oxidation, but $C^{14}O_2$ formation was always decreased.

Unlike the other ions tested, Fe^{++} and Fe^{+++} caused a twofold in-

TABLE IV

Effect of pH, Fe^{+++} and EDTA on Palmitate-1-C^{14} Oxidation[a]

pH		Per cent of C^{14} recovered as $C^{14}O_2$			
Initial	Final	Control	Fe^{+++} 0.042 μ*M*	EDTA 0.039 μ*M*	Fe^{+++} + EDTA 0.042 μ*M* 0.039 μ*M*
4.60	4.97	3.1	8.9	0.1	3.1
5.03	5.95	5.5	18.8	0.3	9.6
5.70	6.19	5.9	23.1	0.2	8.4
6.15	6.44	5.3	20.5	0.3	11.8
6.65		9.0	35.2	0.8	15.6
7.60	7.30	10.4	33.5	0.7	17.7
8.55[b]	7.25	11.5	48.4	1.1	27.9
9.61[b]	8.70	2.5	10.2	1.0	9.7

[a] Each flask contained 54.7 μmolar palmitate-1-C^{14}, 0.1 *M* phosphate buffer, 53.3 mmolar ascorbic acid, and a total volume of 3 ml. Incubation time was 1 hr.
[b] Borate buffer (13) used in place of phosphate buffer.

crease in palmitic acid-1-C^{14} oxidation (Table IV). These are the same ions found by Udenfriend *et al.* (12) to function in the hydroxylation of aromatic compounds by means of ascorbic acid and oxygen. Of particular interest is the fact that these authors reported that EDTA enhanced the hydroxylation reaction (12). In the present studies it was found that EDTA almost eliminated $C^{14}O_2$ formation from radiopalmitate when no Fe^{+++} was added (Tables IV and V). At the same time, ascorbate oxidation was greatly retarded in keeping with the report of Jäger (14). The addition of a slight excess of Fe^{+++} restored much of the capacity for $C^{14}O_2$ formation, but in no case has it been possible to obtain as much $C^{14}O_2$ in the presence of EDTA as in its absence. Of the various chelating agents tested, cysteine was the most effective in lowering the oxygen uptake without lowering the formation of radiocarbon dioxide. Thus, as little as 0.06 μmoles cysteine reduced the O_2 uptake to 19% of that of the control, while the amount of palmitate oxidized was almost normal. Folic acid, aminopterin, and A-methopterin acted similarly, but the amounts needed were much greater and the reduction of oxygen uptake was not as great as was found with the cysteine. In contrast, 8-hydroxyquinoline and EDTA were efficient inhibitors of both palmitate oxidation and oxygen uptake. An extensive series of experiments has been done to determine whether palmitate oxidation can be carried out in the presence of the proper chelating agent and with a catalytic amount of ascorbic acid. Although a large reduction in the amount of ascorbic

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TABLE V

Effect of Various Chelating Agents on Palmitate-1-C^{14} Oxidation

Each flask contained 0.1 *M* phosphate buffer (pH 7.4), 54.7 μmolar palmitate-1-C^{14}, 53.3 mmolar ascorbic acid, and a total volume of 3 ml. The incubations were for 1 hr. in O_2.

Chelating agent	Conc.	Oxygen uptake	$C^{14}O_2$ formation
	μM	*per cent of control*	*per cent of control*
None	—	100	100
8-Hydroxyquinoline	18.7	—	86.1
	37.4	73.2	61.7
	56.1	78.4	52.7
	74.8	53.1	28.4
	130.9	52.0	9.9
	187.0	52.9	5.1
Thiourea	18.7	20.5	63.1
	37.4	18.5	60.2
	56.1	17.9	69.0
	74.8	20.0	63.3
	130.9	18.3	59.8
	187.0	18.4	66.4
Cysteine	18.7	19.5	83.8
	37.4	19.7	94.2
	56.1	19.1	93.7
	74.8	20.8	100.2
	130.9	18.6	103.1
	187.0	19.4	110.1
EDTA	0.4	77.1	78.0
	8.0	21.4	8.2
	40.0	24.2	15.4
	400.0	20.1	1.6
	800.0	19.5	1.0
Folic acid	50.0	130.6	93.8
	100.0	75.6	109.0
	150.0	66.2	99.1
	200.0	55.2	97.5
	350.0	36.8	79.3
	500.0	34.9	102.2
Aminopterin	50.0	110.4	97.2
	100.0	87.3	106.6
	150.0	78.6	111.9
	200.0	77.9	71.5
	350.0	74.9	103.7
	500.0	59.0	107.3
A-methopterin	500.0	34.6	119.4

acid necessary has been achieved, recovery of all of the ascorbic acid has not been successful to date.

As shown by the results in Table IV, the reaction proceeded well at all but the lowest pH tested. At the highest pH the destruction of the ascorbate was so rapid and extensive that the pH of the mixture dropped rapidly even when the amount of buffer was increased. Weissberger and LuValle (11) in studies on the copper-ion catalysis of ascorbate oxidation have pointed out the difficulties inherent in such systems where complex and chelate formation with the buffers can occur.

Hydrogen peroxide has been suggested as a product of ascorbic acid oxidation (11). In view of the reaction of H_2O_2 with fatty acids as reported by Dakin (2, 3) and by Smedley-MacLean and Pearce (5), its action was tested in the present series of experiments by using it to replace ascorbic acid. Under no circumstance was more than 2.3% of the carboxyl-labeled palmitate oxidized (Table VI). When present with ascorbic acid, hydrogen peroxide caused a decrease in $C^{14}O_2$ formation. Thus, H_2O_2 which might arise during the oxidation of ascorbic acid was not the agent responsible for long-chain fatty acid oxidation. This conclusion was also supported by the fact that although in the presence of cysteine, total ascorbate oxidation was greatly reduced and possible H_2O_2 formation thereby also decreased, no reduction in $C^{14}O_2$ production resulted.

In addition to H_2O_2, a number of other compounds were tested as substitutes for ascorbic acid (Table VI). Many of these had an enediol group such as in dihydroxymaleic acid and catechol, but only those related to ascorbic acid itself were effective in producing appreciable radiocarbon dioxide from radiopalmitate. Although ferric ions caused a much more rapid oxidation of these compounds, little gain in $C^{14}O_2$ formation was observed. These results suggest that the type of enediol found in ascorbic acid where one hydrogen is much more dissociated than the other is necessary for activity as a catalyst. The need for such an "unbalanced" enediol is now under investigation. The three hexose reductones tested were effective catalysts, but their action may have been somewhat modified by the fact that the presence of an amine group in the same molecule may have influenced the chelation of the ferric ions. Weissberger and LuValle (11) have shown that it is probably the ascorbate ion which undergoes Cu^{++}-catalyzed oxidation. Whether or not the Fe^{+++}-catalyzed oxidation of long-chain fatty acids is also mediated through the ascorbate ion has not been conclusively demonstrated to date.

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TABLE VI

Palmitate-1-C^{14} Oxidation in the Presence of Compounds Other Than Ascorbic Acid

All flasks contained 0.1 *M* phosphate buffer, 54.7 μmolar palmitate-1-C^{14}, and a total volume of 3 ml. When present, the Fe^{+++} concentration was 0.042 μ*M*. In addition to being used separately, many of the above compounds were also tested in combination with ferric ions and other supplements as well as at concentrations other than those given above; however, no enhanced oxidation of the fatty acid was observed.

Compound	Concentration *mM*	Per cent C^{14} recovered as $C^{14}O_2$ in one hour
Adrenaline	78.9	0.41
Catechol	75.9	0.54
Phloroglucinol	80.0	0.03
Glutathione	95.9	—
Riboflavine	0.97	—
Alloxan	225.0[b]	—
Ninhydrin	193.0	—
Dihydroxymaleic acid	100.0	1.09
H_2O_2	581.0	2.17
$H_2O_2 + Fe^{+++}$	581.0	2.38
Isoascorbic acid	200.0	15.7
Dehydroascorbic acid	202.0	—
Morpholino-hexose-reductone + Fe^{+++}[a]		6.02
Piperidino-hexose-reductone + Fe^{+++}[a]		8.21
Trimethylamino-hexose-reductone + Fe^{+++}[a]		11.5

[a] These compounds were used at concentrations of 17.04, 16.88, and 13.7 mg. per flask for the morpholino-, piperidino-, and dimethylamino- derivatives, respectively.

These latter compounds were supplied by Dr. John E. Hodge of the Northern Regional Research Laboratory, Peoria, Illinois. For details regarding these reductones, consult Ref. (15).

As shown by the data in Table VII, the oxidation of long-chain fatty acids includes at least the first three carbons. Less $C^{14}O_2$ was formed from the 2- and 3-positions of stearic acid than from the carboxyl carbon. Hydroxylamine and bisulfite greatly diminished $C^{14}O_2$ formation from stearic acid labeled in the 2- and 3-positions but only moderately decreased that derived from the carboxyl-labeled stearic acid. This indicates that a carbonyl compound is an intermediate in the reaction as far as loss of carbons-2 and -3 are concerned. When such experiments were conducted in air, similar results were obtained, but less $C^{14}O_2$ was formed in all cases. Intermediates such as long-chain methyl ketones and short-

TABLE VII

The Oxidation of Stearic Acids Labeled in the 1-, 2- and 3-Positions. Total Radioactivity in carbon dioxide

Each flask contained 53.3 mmolar ascorbic acid, 0.16 μmoles of one of the fatty acids, 31 μmolar $FeCl_3$, and 0.1 *M* phosphate buffer. Incubation time was 1 hr. in 100% O_2. Total activity per flask was 24,600 counts/min.

Substrate	Control	24.7 mmolar hydroxylamine	24.7 mmolar bisulfite
Stearate-1-C^{14}	9460	7440	5400
Stearate-2-C^{14}	5680	858	2280
Stearate-3-C^{14}	2450	324	594

chain fatty acids have not as yet been isolated. Any proposed mechanism for the oxidation of fatty acids by the ascorbate–oxygen system must carry with it an explanation of the fact that only the long-chain acids are attacked. If the ascorbate oxidation initiates a free-radical oxidation of the fatty acid, then it would appear likely that the micellular character of the long-chain acids and their soaps would augment the extent of the reaction because the molecules in each micelle would be in close proximity to each other. This may in effect bear a resemblance to the mechanism proposed by Harkins (19) for soap-catalyzed emulsion polymerization.

Of interest is the fact that less $C^{14}O_2$ was derived from oleic acid-1-C^{14} and elaidic acid-1-C^{14} than from the usually more stable saturated fatty acids. Thus, in six experiments the conversion of oleic acid-1-C^{14}, elaidic acid-1-C^{14}, and stearic acid-1-C^{14} to $C^{14}O_2$ ranged from 7.9 to 19.4, 1.9 to 7.8, and 26.1 to 50.8%, respectively. In these experiments the flasks contained 31 μmolar $FeCl_3$, 54.7 μmolar carboxyl-labeled fatty acid, 53.3 μmolar ascorbic acid, and 0.1 *M* phosphate buffer. Bernheim *et al.* (16, 17) and Kuck (18) have reported on the catalytic effect of ascorbic acid on the oxidation of unsaturated acids. These authors used the thiobarbituric acid color reaction as the measure of oxidation and reported that oleic, in contrast to linoleic and linolenic acids, gave a negative result. Their results further showed that the formation of a maximum color was dependent upon the presence of a double bond near the terminal end of the fatty acid molecule. From the present studies it is evident that both oleic and elaidic acids are oxidized in the presence of ascorbic acid and oxygen with the rupture of carbon-to-carbon bonds. Since the studies of Bernheim *et al.* (16) clearly demonstrated rupture of the distal double bond of linolenic acid, it seems reasonable to assume that the bond is also broken in the case of the 9,10-position but that no

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fragment is formed which produces a color with the thiobarbituric acid. The lower yield of radiocarbon dioxide from the monoethylenic acids in the present studies can be attributed to the fact that the double bond affords a readily oxidizable site and diverts the attack from the carboxyl end of the chain. Any molecules which are disrupted at the double bond would not be expected to undergo decarboxylation because, if for no other reason, the chain length is inadequate, for even lauric acid was poorly oxidized (1). However, the double bond may also change the character of the carbon chain to decrease its effectiveness in the ascorbate–oxygen system. The fact that elaidic acid was little oxidized and yet is more like a saturated acid than is oleic acid would be in keeping with the latter explanation. Positional isomers of these acids would yield worth-while data in this regard.

As pointed out previously (1), in a number of respects the oxidation of the long-chain fatty acids by means of ascorbic acid and oxygen resembles that reported by Humphreys *et al.* (8) to occur with a microsomal fraction of peanut cotyledons. The addition of ascorbic acid to the microsomal fraction does not increase fatty acid oxidation.[7] Since even in the absence of the microsomal system the ascorbic acid should have given rise to some $C^{14}O_2$ formation, it is possible that the use of ammonium salts in the microsomal fraction experiments (8) negated fatty acid oxidation by the ascorbate–O_2 system. Another possibility is that the microsome preparation inhibits the ascorbic acid–O_2 system. In support of this is the finding in the present studies that liver homogenate almost completely inhibited the ascorbate–O_2 system. It is well known that in the presence of liver tissue ascorbic acid oxidation is greatly reduced presumably because of sulfhydryl compounds such as glutathione (20). Since cysteine itself lowers ascorbate oxidation but not $C^{14}O_2$ formation from palmitic acid-1-C^{14}, some other materials such as polyunsaturated fatty acids in liver may be responsible for the inhibition of ascorbate-catalyzed long-chain fatty acid oxidation. It would be of interest to determine whether or not a bound form of ascorbic acid or some related enediol functions in the peanut microsomal system.

Summary

Under conditions normally used in physiological experiments, carboxyl-labeled long-chain saturated fatty acids were oxidized to $C^{14}O_2$ in the presence of ascorbic acid and oxygen. The reaction was greatly

[7] Personal communication from Dr. P. K. Stumpf.

increased in the presence of ferrous and ferric ions. Ethylenediaminetetraacetic acid inhibited both ascorbate oxidation and $C^{14}O_2$ formation. Cysteine and folic acid, on the other hand, decreased ascorbate oxidation but not $C^{14}O_2$ formation.

Approximately half as much $C^{14}O_2$ was formed from the 2- and 3-carbons of stearic acid as from the carboxyl carbon. Hydroxylamine inhibited $C^{14}O_2$ formation from stearic acid-2-C^{14} but not from the carboxyl-labeled material.

Carboxyl-labeled oleic and elaidic acids were also oxidized but to a lesser extent than stearic acid, while octanoic acid-1-C^{14} formed little $C^{14}O_2$.

References

1. Geyer, R. P., Marshall, L., Ryan, M., and Westhaver, S., *Arch. Biochem. Biophys.* **56**, 549 (1955).
2. Dakin, H. D., *J. Biol. Chem.* **4**, 77 (1908).
3. Dakin, H. D., *J. Biol. Chem.* **4**, 91 (1908).
4. Clutterbuck, P. W., and Raper, H. S., *Biochem. J.* **19**, 385 (1925).
5. Smedley-MacLean, I., and Pearce, M. S. B., *Biochem. J.* **28**, 486 (1934).
6. Paquot, C., and Goursac, F. de, *Bull. soc. chim. France* **1950**, 172.
7. Mahler, H. R., *Federation Proc.* **12**, 694 (1953).
8. Humphreys, T. E., Newcomb, E. H., Bokman, A. H., and Stumpf, P. K., *J. Biol. Chem.* **210**, 941 (1954).
9. Bergstrom, S., Pääbo, K., and Rottenberg, M., *Acta Chem. Scand.* **6**, 1127 (1952).
10. Bessey, O. A., *J. Assoc. Offic. Agr. Chemists* **27**, 537 (1944).
11. Weissberger, A., and LuValle, J. E., *J. Am. Chem. Soc.* **66**, 700 (1944).
12. Udenfriend, S., Clark, C. T., Axelrod, J., and Brodie, B. B., *J. Biol. Chem.* **208**, 731 (1954).
13. Umbreit, W. W., Burris, R. M., and Stauffer, J. F., "Manometric Techniques and Tissue Metabolism, 2nd ed. Burgess Publishing Co., Minneapolis, Minn., 1949.
14. Jäger, H., *Pharmazie* **3**, 536 (1948).
15. Hodge, J. E., and Rist, C. E., *J. Am. Chem. Soc.* **75**, 316 (1953).
16. Bernheim, F., Bernheim, M. L. C., and Wilbur, K. M., *J. Biol. Chem.* **174**, 257 (1948).
17. Kenaston, C. B., Wilbur, K. M., Ottolenghi, A., and Bernheim, F., *J. Am. Oil Chemists' Soc.* **32**, 33 (1955).
18. Kuck, S. K. D., *Arch. Biochem.* **26**, 351 (1950).
19. Harkins, W. D., "The Physical Chemistry of Surface Films," pp. 332–54. Reinhold Publishing Co., New York, 1952.
20. Hopkins, F. G., and Morgan, E. J., *Biochem. J.* **30**, 1446 (1936).

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Indust. Eng. Chem. Anal. Ed., 17, 217-221. 2859

Polarographic Determination of Vitamin C in Fruits and Vegetables

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A polarographic method for determining ascorbic acid in fruits and vegetables affords a reliable means of determining vitamin C in quantities ranging from 4 to 85 micrograms per ml. of solution with an accuracy of 3.3 to 4.3%, depending upon the supporting electrolyte used. Variable results were obtained on some extracts—e.g., dehydrated beets. The half-wave potential and the diffusion current, respectively, were found to be independent of, and proportional to, the ascorbic acid concentration in the four supporting electrolytes studied. Diffusion current constants, half-wave potentials, and diffusion coefficients were determined in four supporting electrolytes. Results of vitamin C analyses on fruits and vegetables by four different methods—polarographic, visual titration, photometric, and Roe's—were reported and discussed. The polarographic method showed good agreement with the visual titration and photometric methods on certain fruit extracts. By making use of the diffusion current constant polarographic determination of vitamin C in certain materials is possible without the necessity of calibrating the instrument.

MOST of the methods that have been proposed for the estimation of vitamin C have been based upon the oxidation of ascorbic acid to dehydroascorbic acid by a specific oxidant such as 2,6-dichlorophenolindophenol. Roe (*11*) has developed a method which is based on an entirely different reaction of ascorbic acid—namely, its reaction with 2,4-dinitrophenylhydrazine.

The use of a dye as a specific oxidant has certain limitations. The indophenol dye is not ideal because of its relatively high oxidation potential; materials other than ascorbic acid may be oxidized. Its reaction with ascorbic acid is not instantaneous and therefore the time of reading the end point and the rate of addition of the dye are factors that may introduce errors. In the absence of the vitamin the dye will fade, and cause a drift in the galvanometer if used in connection with a photoelectric colorimeter. For highly colored extracts the visual titration method cannot be used, and some modification of the method must be resorted to, such as the one suggested by Bessey (*2*).

Potentiometric methods have been published for estimating vitamin C (*1*, *6*) but a continuous drift in potential throughout the whole course of the titration with the dye makes it very difficult to locate the end point with any degree of accuracy. Harris *et al.* (*5*) using a platinum-mercury electrode greatly improved the potentiometric method. Their electrode, however, was found to be slightly sluggish at the end point, and when working with materials that were high in content of reducing substances they used the less desirable bright platinum electrode. Lewis (*8*), in this laboratory, found the platinum mercury electrode acceptable on extracts from fresh materials but unsatisfactory for dehydrated products.

The polarographic method, described in this paper, differs from most methods in that a specific compound is not used as the oxidant. Instead, ascorbic acid is oxidized at the dropping mercury electrode. Essentially, it is based on current voltage curves and since ascorbic acid possesses striking reducing properties this method should be applicable to vitamin C determination. The determination is specific, is sensitive to small concentrations of ascorbic acid, and colored extracts do not necessarily interfere. Furthermore, an inspection of the anodic wave will definitely indicate whether or not interfering materials are present in the solution being analyzed.

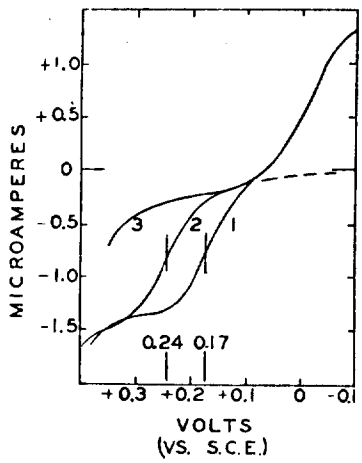

Figure 1. Shift of Half-Wave Potential with Change of pH

1, 28 micrograms of ascorbic acid per ml. at pH 3.41. 2, 28 micrograms of ascorbic acid per ml. at pH 2.17. 3, residual current, supporting electrolyte II (phosphate buffer—1.5% HPO_3). Broken line, residual current after aeration

Kodicek and Wenig (*7*) stated that dehydroascorbic acid was not reducible at the dropping mercury cathode, but a characteristic wave was obtained when the dropping mercury electrode was polarized as an anode. They suggested using a 0.066 *N* phosphate buffer (pH 7.0) in all polarographic analyses for vitamin C. Their method of analysis was suitable for orange juice but they were not successful in applying it to extracts of plant or animal tissues. Other constituents apparently hindered the electrode reaction.

Cozzi (*3*) reported the use of the polarograph in the analysis of fruit juices and concluded that it gave high results.

The present paper presents a polarographic method for determining vitamin C in plant materials which eliminates the necessity of frequently standardizing solutions, is not affected by colored extracts, and is sensitive to very low concentrations of the vitamin. Diffusion current constants and the half-wave potentials of ascorbic acid in four different supporting electrolytes are given. The results of analyses run in this laboratory by four different methods—polarographic, visual titration, photoelectric, and Roe's dinitrophenylhydrazine method (*13*)—are also reported.

PRELIMINARY INVESTIGATION

In the oxidation of organic compounds hydrogen ions are usually involved in the electrode reaction—e.g., $RH_n \rightleftharpoons$

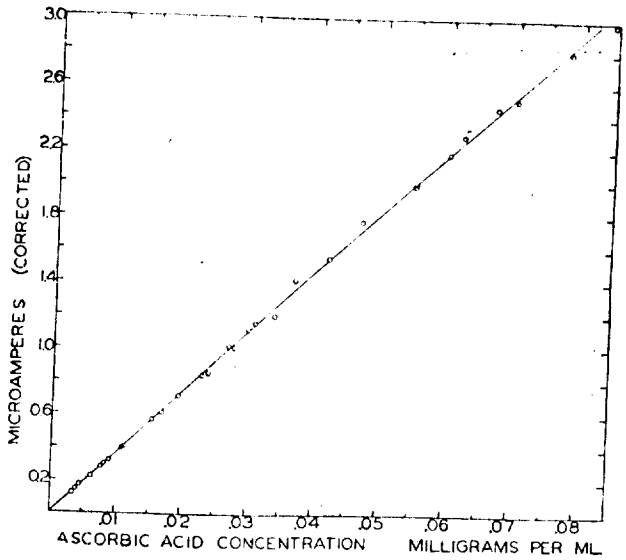

Figure 2. Calibration Curve for Ascorbic Acid in Supporting Electrolyte II

Phosphate buffer–1.5% HPO_3

$R + nH^+ + ne$. Since the hydrogen ion is a component of the electrode reaction, its concentration at the electrode surface is current-dependent. It is therefore evident that for the polarographic oxidation of ascorbic acid a strongly buffered solution is required to keep the concentration of the hydrogen ions at the electrode fairly constant, and equal to the concentration of the hydrogen ions in the whole solution.

The number of buffer solutions suitable for use with the polarograph is limited by the fact that they must contain no substance that will interfere with the ascorbic acid anodic wave.

Of approximately 24 buffers investigated only six yielded a satisfactory residual current curve. After a critical study of pH, buffering capacity, and stability of ascorbic acid in the supporting electrolyte solutions, two buffers were finally selected: potassium biphthalate–sodium hydroxide buffer (pH 6.2) and a phosphate buffer (pH 8.0).

Many extracting media were investigated but all but two, metaphosphoric and oxalic acids, were discarded because they interfered with the anodic wave of ascorbic acid or the vitamin was not sufficiently stable in them. Of these two acids oxalic is probably preferable since it can be stored indefinitely and the data indicated that it had a greater stabilizing effect on ascorbic acid and at higher pH's than those solutions containing metaphosphate.

Since the half-wave potential of ascorbic acid shifts to more positive values with increasing acidity, the pH of the supporting electrolytes must be kept within certain limits. If the pH values are too low the anodic wave of ascorbic acid is shifted toward such high potentials that the limiting current cannot develop (or is of such short duration that it is difficult to determine) because of the interference of the residual current (curve 2, Figure 1). Thus for supporting electrolytes I and II the pH must not be much below 4.6 and 3.4, respectively. On the other hand, if the pH values are much greater than this the vitamin may become unstable.

Electrolytes I and II were used in practically all the analyses performed in this laboratory. Electrolytes III and IV were used only occasionally.

In order to obtain pH values of 4.6 and 3.4, respectively, for supporting electrolytes I and II 0.25% oxalic acid and 1.5% metaphosphoric acid were used. The supporting electrolytes consisted of a 1 to 1 mixture of either acid with the corresponding buffer. For example, supporting electrolyte I consisted of one volume of potassium biphthalate buffer (pH 6.2) and one volume of 0.25% oxalic acid. Electrolyte II consisted of one volume of the phosphate buffer (pH 8.0) and one volume of 1.5% metaphosphoric acid. Both solutions had excellent buffering capacities, and pure solutions of ascorbic acid or extracts from natural products were stable in them for several hours at room temperature. Electrolyte III consisted of a 1 to 1 solution of biphthalate buffer and a solution consisting of 0.25 gram of oxalic acid dissolved in 100 ml. of 50% alcohol, while electrolyte IV contained biphthalate buffer and 3% metaphosphate in a 1 to 1 ratio.

METHOD OF ANALYSIS

Reagent. Potassium biphthalate buffer (pH 6.2), 500 ml. of 0.1 *M* potassium acid phthalate plus 470 ml. of 0.1 *N* sodium hydroxide, made up to 1 liter.

Phosphate buffer (pH 8.0), 50 ml. of 0.066 *M* potassium dihydrogen phosphate plus 950 ml. of 0.066 *M* disodium hydrogen phosphate.

Oxalic acid solution, 0.25%; metaphosphoric acid solution, 1.5%; and 0.25 gram of oxalic acid dissolved in 100 ml. of 50% alcohol.

Apparatus. The current-voltage curves were obtained by means of a Leeds & Northrup Electro-Chemograph. All the polarograms were obtained by using an external saturated calomel electrode as cathode, connected to the electrolysis cell through an agar bridge saturated with potassium nitrate.

All experiments were carried out in a water bath at 25.0° ± 0.2° C. The amount of mercury flowing from the dropping electrode per second, designated by m, was determined while dropping in air (*10*). The m and t values of the dropping capillary used were, respectively, 2.33 mg. of mercury per second, and 3.0 seconds when the capillary tip dipped into the supporting electrolyte and no potential was applied to the mercury drop. The drop time, of course, varies with the nature of the supporting electrolyte and the potential of the dropping electrode, but it was found much more expedient, at the start of an analysis, to adjust the pressure on the mercury, so that $t = 3.0$ seconds in all the supporting electrolytes used. In all cases the drop time was also measured at the approximate potential at which the diffusion current was measured. Thus for supporting electrolyte I (biphthalate–oxalic acid) $t = 2.85$ seconds, for electrolyte II (phosphate–1.5% HPO_3) $t = 2.67$ seconds, for electrolyte III (biphthalate–oxalic acid–alcohol) $t = 2.81$ seconds, and for electrolyte IV (biphthalate–3% HPO_3) $t = 2.68$ seconds, respectively, when a potential of 0.19, 0.26, 0.14, and 0.30 volt was applied to the mercury drop. The value of $m^{2/3}t^{1/6}$ for the capillary used was 2.08.

Procedure. A given weight of the material to be analyzed is whipped in a Waring Blendor with a known volume of metaphosphoric or oxalic acid for 2 to 5 minutes (*4*). The extract is then filtered through a dry fluted filter paper. A 2- or 5-ml. aliquot of the filtrate is withdrawn, an equal volume of the buffer

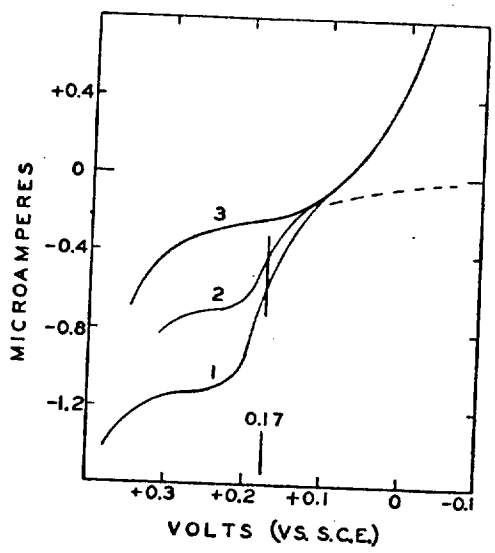

Figure 3. Current-Voltage Curves of Ascorbic Acid in Supporting Electrolyte II at 25° C.

1, 23 micrograms of ascorbic acid per ml. 2, 11.7 micrograms of ascorbic acid per ml. 3, residual current. Broken line, residual current after aeration. Range of recorder, 10 microamperes

solution is added, and the well-mixed solution is polarographed between −0.1 and +0.3 volt.

The diffusion current is then measured on the polarogram, corrected for the residual current of the supporting electrolyte, and the corresponding concentration of vitamin C is read off the calibration graph, Figure 2.

If the concentration of ascorbic acid in the aliquot used is too great to give a satisfactory limiting current, the sample may be diluted with the supporting electrolyte being used or a lower sensitivity of the instrument may be employed. In analyzing solutions one merely adds a 1- to 5-ml. aliquot to an equal volume of the supporting electrolyte, and polarographs the solution.

ANALYTICAL RESULTS

The instrument was calibrated by polarographing solutions containing known amounts of crystalline ascorbic acid, shown to have a purity of 98% by an iodine titration. The residual current was determined for each supporting electrolyte by polarographing the solution between −0.1 and +0.3 volt at each range of the recorder—e.g., 40, 28, 10, 5, 4, 3, and 2 microamperes. This corresponds to a range of $^1/_{20}$ to full sensitivity of the galvanometer. The corrected diffusion currents were then obtained by measuring the distance between the limiting current and the residual current.

All the residual current curves were similar to the one plotted in Figures 1 and 3, in that they showed a pronounced cathodic current between −0.1 and +0.1 volt. Although cupric ions will give a cathodic current within this voltage range, the wave noted was due to oxygen. Consequently it can readily be removed by aerating the solutions with either nitrogen or carbon dioxide. The residual current curve, after aeration, is indicated by the broken line in Figures 1 and 3.

Oxygen was not removed from the solutions analyzed because it did not interfere with the ascorbic acid determinations and by eliminating the aeration procedure considerable time was saved.

Table I illustrates the reproducibility of the results in a number of repeated determinations on extracts from natural products and on several pure solutions containing varying amounts of ascorbic acid. The maximum coefficient of variation for the determinations listed on natural products is ±1.6%.

The half-wave potentials, determined experimentally, the diffusion coefficients, and diffusion current constants are listed in Table II. The diffusion current constants for supporting electrolytes I, II, III, and IV, respectively, are constant to ±2.2, ±2.2, ±2.0, and ±3.9%.

Theoretically, the shift of the half-wave potential with changing pH values should be 0.059 volt per pH unit. This shift was experimentally determined in two supporting electrolytes (phosphate buffer–1.5% metaphosphate) and phthalate buffer–0.25% oxalic acid) and was found in each case to be 0.056 volt per pH unit, which is in very good agreement with the theoretical (Figure 1).

The value of the half-wave potential was found to be independent of the ascorbic acid concentration in all the supporting electrolytes studied (Figure 3).

Many naturally occurring products have been successfully analyzed in this laboratory by the polarographic method and a few of these results are reported in Tables III and IV. Fresh materials such as orange, lemon, grapefruit, tangerine, cauliflower, tomato, pepper, spinach, lettuce, apple, and potato, and plant tissues such as tomato, mustard, turnip, and beet tops, have been analyzed and found to yield satisfactory anodic waves. Dehydrated potatoes and carrots likewise have been successfully analyzed, and with special precautions the vitamin C content of dehydrated beets and onions can be determined.

Several polarograms obtained from fruit and vegetable extracts are shown in Figure 4. From 5 to 25 grams of the material were extracted with 0.25% oxalic acid, except in the case of II and I, following the procedure previously described, and a 5-ml. aliquot was polarographed. The anodic waves obtained from the extracts do not always coincide with the residual current curve in the range −0.1 to 0 volt, the former usually lying below the latter. Thus a correction must be applied to obtain the true diffusion current. Under such conditions, or when the limiting current is not well defined, it has been found advantageous to determine the true diffusion current by taking the difference in current at two predetermined potential points. By this method it is only necessary to select two reference potential points to be used in calibration and in subsequent analyses of solutions. With this particular electrolyte (I) the potentials used were 0 and 0.2 volt. The corrected diffusion current is then taken as the difference in current at the two potentials, minus the difference in current at these two potentials when the ascorbic acid concentration is known to be zero.

Many of these materials were analyzed by four different methods—polarographic, visual titration, photometric, and Roe's methods (Tables III and IV).

DISCUSSION

The calibration curves show that ascorbic acid can be determined with an accuracy of 3.3% in supporting electrolyte I when the ascorbic acid concentration lies between 13 and 85 micrograms per ml. In the concentration range 5 to 13 micrograms per ml. the accuracy is 4.0%. In electrolyte II the accuracy is 4.3% over a range in concentration of 3.5 to 85 micrograms per

Table I. Reproducibility of Results

(Micrograms of ascorbic acid per ml. of solution)

Material		Material	
Tomato	61.6	Spinach	60.0
	60.1		60.0
	61.6		62.2
	60.8		58.0
Mean	61.0		60.0
Average deviation	0.6		1.0
Coefficient of variation, %	1.0		1.6
Tomato	94.0	Cauliflower	180.0
	94.0		182.0
	94.0		180.0
Mean	94.0		180.6
Average deviation	0.0		0.9
Coefficient of variation, %	0.0		0.5
Orange	40.0	Pepper	137.6
	40.0		132.0
	40.8		130.0
	39.6		133.2
Mean	40.1		133.2
Average deviation	0.4		2.2
Coefficient of variation, %	1.0		1.6

Pure Solution of Ascorbic Acid

Contained	Found
300	300
300	300
321	325
38	38
30	30
30	30
30	30
22	21.8
11.7	11.7

Table II. Half-Wave Potentials (Volts vs. Saturated Calomel Electrode at 25° C.), Diffusion Coefficients, and Diffusion Current Constants

Supporting Electrolyte	Half-Wave Potential	Diffusion Coefficient	Diffusion Current Constant	
		Sq. cm. sec.$^{-1}$	i_d/C	$i_d/Cm^{2/3}t^{1/6}(\theta)$
Biphthalate buffer–0.25% oxalic acid (I)	0.10	5.7×10^{-6}	6.25	3.00
Phosphate buffer–1.5% HPO_3 (II)	0.17	6.2×10^{-6}	6.34	3.04
Biphthalate buffer–0.25% oxalic acid in 50% alcohol (III)	0.06	3.6×10^{-6}	4.88	2.34
Biphthalate buffer–3% HPO_3 (IV)	0.24	5.3×10^{-6}	6.02	2.88

Table III. Analyses of Tomatoes Extracted with 3% HPO_3

(Milligrams of ascorbic acid per gram of fresh weight)

Sample	Polarographic Method	Visual Titration Method[a]
633-5-5-3	0.452	0.516
1956-1-L	0.398	0.411
1554-2	0.479	0.565
633-5-S-2	0.390	0.447
1956-1-S	0.380	0.407
633-5-1-2	0.361	0.398

[a] Analyzed by J. W. Porter.

ml. With electrolyte III the accuracy is 4.0% (concentration range, 5 to 95 micrograms per ml.), and in electrolyte IV the accuracy is 4.3% over a range in concentration of 8 to 85 micrograms of ascorbic acid per ml.

The data in Table IV illustrate conclusively that the polarographic method checks very well with the visual and photometric methods on several fruit and vegetable extracts.

Theoretically, the polarographic method might be expected to give more accurate results than any method which uses the indophenol dye. For obvious reasons the values obtained by the visual titration method might be expected to be slightly higher than those obtained by the polarographic method. Theoretically the polarographic method is more specific because each compound is characterized by its half-wave potential. When several electrooxidizable substances are present in the solution each one will produce its own characteristic wave if the half-wave potentials are far enough apart. Thus, compounds such as glutathione or cysteine should not interfere in certain acid supporting electrolytes.

The data in Table III may indicate that the polarographic method is more specific than the visual titration method in these particular extracts.

The tomatoes listed in this table consisted of the red- and green-fruited species, *Lycopersicon pimpinellifolium* and *Lycopersicon peruvianum*. Several of these small tomatoes were whipped in a Waring Blendor with 3% metaphosphate and filtered, and aliquots of the filtrate were analyzed by the two methods. A few of these extracts were slightly pink in color. Sample 1554-2 gave the most highly colored filtrate and both methods showed it to contain the greatest concentration of ascorbic acid. The variation between the two analyses, however, was greatest for this sample, which would indicate that slightly colored solutions tend to give high values by the visual titration method.

In every case (Table III) whether the filtrate was colored or not, the visual titration values were from 3 to 18% higher than the polarographic values. This may be due to the fact that (*a*) a small excess of dye must be added to indicate the completion of the reaction, (*b*) the pink color in some of the extracts would tend to obscure the end point, and (*c*) the dye may have reacted with reducing materials other than ascorbic acid. All these would tend to increase the titration value.

Another sample of tomatoes (*Lycopersicon pimpinellifolium*) was extracted with oxalic acid (sample 1, Table IV). This extract was colorless but again the visual titration value, as well as the photometric value, was considerably higher than the value obtained by the polarograph. The tomato samples, 2 to 9, consisted of oxalic acid extracts of F_2 and F_3 selections of crosses of *Lycopersicon esculentum*, *L. hirsutum*, and *L. pimpinellifolium* species. Here, the polarographic values check the visual titration values within 2 to 4% and in two cases the former values were slightly higher than the visual titration values.

In the case of cauliflower (Table IV) the polarographic value was considerably higher than that obtained by the visual titration method.

In all the analyses Roe's method (*12*) gave rather high results. This was to be expected, since his method determines total vitamin C content (ascorbic plus dehydroascorbic acid), and the difference between these values and those obtained by the polarographic method was attributed to the presence of dehydroascorbic acid. However, several of these values, notably those for fresh potato and lettuce, appeared to be too high. For this reason Roe's method was included in the analyses of certain citrus fruits, where dehydroascorbic acid, if present at all, would be in very low concentration. Curiously, Roe's method, even on these products, gave very high values; in one instance (tangerine) the value was 300% greater than the value obtained by the polarographic or visual titration methods. To check this further these extracts were treated with hydrogen sulfide and subsequently analyzed by the polarographic and visual methods (Table V). Results indicated there was very little, if any, dehydroascorbic acid present in the citrus fruit extracts studied.

Additional work dealing with these four methods of analysis will be reported in a later paper.

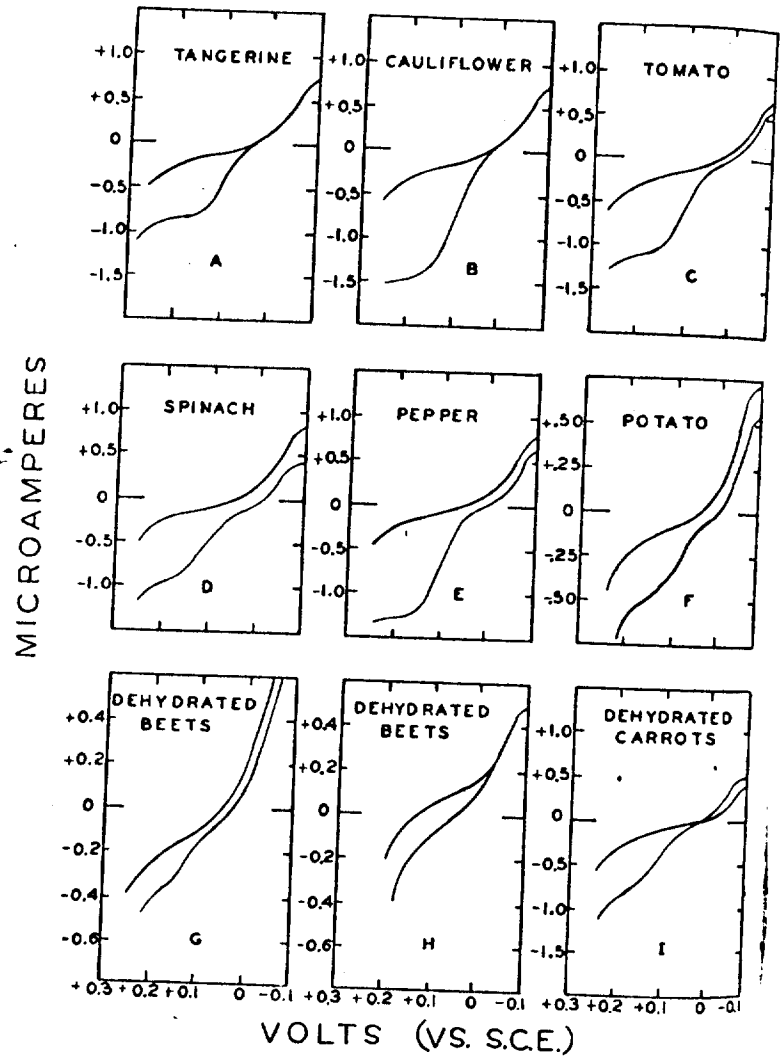

Figure 4. Typical Polarograms of Fruit and Vegetable Extracts

H and I polarographed in supporting electrolyte III; all others in supporting electrolyte I

Table IV. Ascorbic Acid in Fruit and Vegetable Extracts

Material	Ascorbic Acid, Micrograms per Milliliter of Extract: Polarographic	Visual titration[a]	Photometric[b]	Roe[b]
Ascorbic acid, 300 micrograms per ml., in 1.5% HPO_3	300	302	306	300
Ascorbic acid, 321 micrograms per ml. in 0.25% oxalic acid	325	315	312	315
Orange	40.1	41.3	...	...
Orange	27.0	.....	27.0	
Grapefruit	42.0	40.1	...	73.0
Lemon	51.1	50.4	...	72.8
Tangerine	33.0	35.0	...	108.4
Tangerine	31.0	.....	...	65.0
Tangerine	41.0	.....	41.0	...
Cauliflower	180.0	161.0	...	227.2
Apple	6.4	6.0	...	10.7
Tomato				
1	80.0	92.0	95.0	102.0
2	0.168	0.176	...	...
3	0.200	0.204	...	...
4	0.138	0.138	...	...
5	0.170	0.174	...	...
6	0.126	0.123	...	...
7	0.207	0.214	...	...
8	0.344	0.343	...	...
9	0.182	0.178	...	...
Pepper	132.5	.....	...	140.0
Lettuce	9.2	.....	...	18.2
Lettuce	4.0	.....	2.4	...
Fresh potato	8.4	.....	...	27.8
Dehydrated carrots	9.6	.....	...	10.4
Dehydrated potatoes				
1	15.0	22.5	...	27.0
2	4.0	.....	6.6	...
3	0.	.....	3.0	...
4	5.0	.....	12.5	6.0
5	13.0	.....	...	19.0
Dehydrated onions	30.0[c]	.....	25.8	...
	28.0[c]	.....	20.5	...

[a] Analyzed by J. W. Porter.
[b] Analyzed by W. R. Lewis.
[c] Poor anodic wave indicating a fairly high concentration of interfering material.

The polarographic method possesses several advantages over other published methods for vitamin C analysis. After the initial calibration no standardization of solutions is required. If oxalic acid is used as the extracting agent all solutions listed in the procedure can be stored several months. No pretreatment of the extract is required and colored extracts do not necessarily interfere, since the method is concerned with a specific and characteristic property of the reaction. Suspended matter which will affect the light absorption in the photoelectric method ordinarily does not interfere. However, with some materials—e.g., dehydrated potatoes—the colloidal content of the extract may be sufficiently high to interfere with the analysis. In some instances this can be alleviated by using the alcoholic supporting electrolyte.

In any analysis an inspection of the anodic wave will quickly and definitely indicate whether or not interfering substances are present in the extract being polarographed. This is not true of the visual, photometric, or Roe's methods, for with these one obtains a given value and has no indication of the presence or absence of interfering materials.

Another advantage of the polarographic method is that any number of analyses can be run on one and the same aliquot.

The method is accurate over a range in ascorbic acid concentration of 4 to 85 micrograms per ml. However, since there is a dilution factor of $^1/_2$ (one volume of extract plus one volume of buffer) the extracts being analyzed should possess 8 to 170 micrograms of ascorbic acid per milliliter of solution.

In the analysis of materials having a high content of interfering substances the method becomes less reliable and may fail completely. However, even though interfering substances may prevent the formation of a well-defined limiting current (Figure 4, H), reproducible results are obtained by taking the difference in current at two predetermined potential points.

Since the diffusion current constants for four supporting electrolytes have now been determined, an ascorbic acid analysis of certain materials could be made from a single polarogram. Thus the necessity of preparing standard solutions of ascorbic acid and calibrating the instrument can be eliminated. The diffusion current constant has previously been referred to as i_d/C, but according to Lingane (*9*) a more fundamental constant is $i_d/Cm^{2/3}t^{1/6}$. Thus, $C = i_d/Km^{2/3}t^{1/6}$, where K is the diffusion current constant expressed as $i_d/Cm^{2/3}t^{1/6}$. From this equation it is evident that m and t must be known for the capillary that is used. The determination of these two values is very simple and obviously requires much less time than the calibration of the instrument. Lingane (*9*) states "that with m values in the usual range from about 1 to 3 mg. per second, and drop time between 2 and 4 seconds, the accuracy of this relation is as good as the accuracy with which one can measure a diffusion current".

This procedure could certainly be used on citrus juices, but for materials such as potatoes, dehydrated products, etc., the slower procedure of recording the current at two selected potentials must be employed in order to correct for the displacement of the anodic wave above or below the residual current curve due to the presence of interfering substances.

The diffusion current constants are also of theoretical interest because they can be used to calculate the corresponding diffusion coefficients. Thus a measure of the apparent size of the ascorbic acid molecule in the different supporting electrolytes can be obtained. The diffusion coefficients are calculated by the application of the Ilkovič equation, $i_d = 605\ nD^{1/2}Cm^{2/3}t^{1/6}$, in which n is the number of electrons involved, 2 in this case, D the diffusion coefficient, C the concentration of ascorbic acid in millimoles per liter, m the milligrams of mercury flowing from the capillary tip per second, and t the time in seconds required for the formation of one drop of mercury. Thus $D = (K/1210)^2$ when K is expressed as $i_d/Cm^{2/3}t^{1/6}$ (Table II).

Since the diffusion current constant of ascorbic acid was largest in the phosphate–1.5% metaphosphate supporting electrolyte, it follows that the apparent size of the ascorbic acid molecule must be smaller in this medium than in any of the other aqueous supporting electrolytes investigated. Similarly, the apparent size of the ascorbic acid molecule must be larger in electrolyte IV than in either I or II. In general, the diffusion coefficients are much smaller in alcoholic solutions than in water solutions. It is, therefore, impossible to make such comparisons, based on diffusion coefficients, between supporting electrolyte III and the other three aqueous supporting electrolytes.

Table V. Comparison of Ascorbic Acid Contents of Fruit and Vegetable Extracts before and after H_2S Treatment

(Micrograms per ml. of extract)

Material	Before H_2S Treatment: Polarographic	Visual	Roe	After H_2S Treatment: Polarographic	Visual
Orange	40.1	41.3	...	41	39
Grapefruit	42.0	40.1	73	45	39.6
Lemon	51.1	50.4	72.8	55	50.4
Tangerine	33	35	108	40	35
Cauliflower	180	161	227	208	178

LITERATURE CITED

(1) Becker, E., and diGleria, J., *Z. Vitaminforsch.*, 6, 86 (1937).
(2) Bessey, O. A., *J. Biol. Chem.*, 126, 771 (1938).
(3) Cozzi, D., *Ann. chim. applicata*, 29, 434 (1939).
(4) Davis, W. B., Ind. Eng. Chem., 34, 217 (1942).
(5) Harris, L. J., Mapson, L. W., and Wang, Y. L., *Biochem. J.*, 36, 183 (1942).
(6) Kirk, M. M., and Tressler, D. K., Ind. Eng. Chem., Anal. Ed., 11, 322 (1939).
(7) Kodicek, E., and Wenig, K., *Nature*, 142, 35 (1938).
(8) Lewis, W. R., unpublished data, 1943.
(9) Lingane, J. J., Ind. Eng. Chem., Anal. Ed., 15, 583 (1943).
(10) Müller, O. H., *J. Chem. Education*, 18, 176 (1941).
(11) Roe, J. H., and Kuether, C. A., *J. Biol. Chem.*, 147, 399 (1942).
(12) Roe, J. H., and Oesterling, M. J., *Ibid.*, 152, 511 (1944).

Journal Paper 169, Purdue University Agricultural Experiment Station.

Nutrition Metabol. 12 76-86 1970 2894

Nutr. Metabol. *12:* 76-86 (1970)

The Effect of Ascorbic Acid on Cholesterolemia in Healthy Subjects with Seasonal Deficit of Vitamin C

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Abstract. The effect of vitamin C on cholesterolemia in a selected group of persons above 40 years of age with a seasonal deficit of ascorbic acid and with an initial level of serum cholesterol in the upper permitted limit, or hypercholesterolemia, was studied. A nutritional situation in the examined area can be characterized by a high consumption of animal fats and sucrose, all-the-year-round deficit of vitamin A, B_2 and iron and an evident seasonal deficit of vitamin C. Administration of 300 mg of ascorbic acid daily for 47 days significantly decreased the cholesterolemia. The effect of ascorbic acid was most pronounced in persons with hypercholesterolemia. In the control group not receiving ascorbic acid, the cholesterol levels showed no significant changes throughout the examined period. In the discussion it is assumed that mechanism of hypocholesterolemic effect of ascorbic acid is based on increasing catabolism of cholesterol by vitamin C.

Among the relatively numerous references found about the effect of ascorbic acid on cholesterolemia in men there is no agreement concerning the influence of vitamin C on the level of cholesterol in blood. In normocholesterolemic persons vitamin C does not affect serum cholesterol level [TJAPINA, 1952; BUKOVSKAJA, 1957; ANDERSON *et al.*, 1958; HRUBÁ and MAŠEK, 1962; BRONTE-STEWARD *et al.*, 1963]. In persons with increased levels of serum cholesterol, some papers indicate in a part of the examined subjects a hypocholesterolemic effect of the ascorbic acid (TJAPINA, 1952; SEDOV, 1956; BUKOVSKAJA, 1957; GRABENKO, 1958; FEDOROVA, 1960; SOKOLOFF *et al.*, 1966], however, some authors maintain that the vitamin C is without any effect [KRIVORUCHENKO, 1963, 1965; SAMUEL and SALCHI, 1964]. The most of the papers, trying to prove the hypocholesterolemic effect of ascorbic acid, are criticised on the ground that this effect has been attained under simultaneous effect of further factors with hypo-

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cholesterolemic influence, as e.g. protective diet, curative regime, etc. Another shortcoming of the all previously cited papers is the fact, that the state of vitamin C saturation has not been studied in the patients subjected to investigations.

Data obtained by epidemiological study of two population sets in Eastern Slovakia showed a statistically highly significant negative correlation between some lipid metabolism parameters, especially the level of cholesterolemia and that of vitamin C in blood [KAJABA, 1964; KAJABA and BUČKO, 1968]. These data indicated that an increase of vitamin C in blood is accompanied with a decrease of average cholesterolemia in population groups. Similarly, experimental studies performed on guinea-pigs revealed the close relationship between vitamin C and cholesterol metabolism (GINTER *et al.*, 1965, 1967].

The presented study is based on the assumption [GINTER, 1968], that eventual hypocholesterolemic effect of the ascorbic acid could be most clearly evident at an intentional selection of a group of persons with higher levels of serum cholesterol and at the same time with biochemically proved low vitamin C saturation. The objective of the study is to test this assumption by a model epidemiological experiment on selected individuals.

Material and Methods

In the preceeding years we have performed a year-round study of food-stuffs consumption by 18 selected typically agricultural families in the study area Voderady in the Southwest part of Slovakia with an objective of obtaining a picture about the method and seasonal variability of nutrition within the locality under study. Since this is an area, where a further broadly based observation of nutritional state of inhabitants did not reveal any important changes of the style of living and nutritional habits, the obtained data on caloric and biological value of consumption can be accepted as material reliably illustrating the situation in nutrition of the given region also in the period of our experiment.

For dietetic research, the inventory method [BUDLOVSKY, 1960] and data obtained about food-stuffs were calculated from commonly used biological indexes using Czechoslovak Tables of Nutritional Value of Food-stuffs [KRONDLOVÁ-ŠKOPKOVÁ and ŠMRHA, 1965]. The food-stuffs consumption is presented both in absolute values of calculations in relation to nutritional factors, both in comparison with the found consumption of the currently accepted Czechoslovak recommended doses [MAŠEK *et al.*, 1962], in the form of percentage maintenance of a dose, as well as the structure of consumption of basic nutrients and their triple relationship.

In the clinical and biochemical research of the so-called healthy population in the Voderady area (the village of Voderady, Cífer, Slovenská Nová Ves and Pác), age

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group above 40 years, one of the authors was examining by the end of 1967 apart from other indexes the level of cholesterol and vitamin C in blood by a relatively large group of persons. From the whole group of 256 persons under investigation (181 women, 175 men) 50 persons were selected with low levels of vitamin C in blood (about 0.6 mg%) and cholesterolemia at the upper limit of the standard or beyond it (232–312 mg%).

The selected subjects were divided into two groups, experimental and a control one. Persons in the control group (villages Cífer and Pác) were unaware of taking part in an experiment; only at the end of the experiment, were blood samples taken for biochemical examination. The control group persons (villages Voderady, Slovenská Nová Ves, Pác) had blood samples for determinations of cholesterol and vitamin C content in blood taken on January 23, 1968. Each person received a container with 141 tablets (100 mg of vitamin C in each tablet) of Celaskon Spofa, and were instructed to take, 3 times daily, 1 tablet of the 'vitamin preparation'. The daily dose of vitamin C was calculated for exactly 47 days, so that a real intake of tablets could be checked at the end of the experiment. They all received instructions about not changing their nutritional habits.

After 7 weeks of vitamin C administration (March 12, 1968), blood samples were taken from each member of the experimental group and the number of tablets, not taken, were checked. Persons, having at that time more than 30 tablets (about 20% of the total dose of vitamin C) were excluded from further experiments. Following the blood sampling, a saturation test was performed: after intravenous administration of 100 mg of ascorbic acid, the elimination of vitamin C in 3-h urine was examined. In the control group, a similar examination was carried out a day after the taking of blood samples. After excluding the individuals which either did not use the required number of tablets or developed a more serious disease during the experiment, the experimental group consisted of 24 persons (13 men and 11 women) and there were 18 persons in controls (10 men and 8 women), in an age group of 42 to 65 years.

The analytical methods employed: vitamin C in total blood [BESSEY, in GYÖRGYI, 1950], vitamin C in urine [ROE and KUETHER, 1943], cholesterol in blood serum [PEARSON *et al.*, 1953]. The variation coefficient obtained by analysis of 10 parallel samples was shown to be for the method of vitamin C in blood determination $\pm 2{,}6\%$, for the method of cholesterolemia determination $\pm 0.5\%$.

The results were statistically evaluated following the calculation SE by Student's t-test and the pair test, evaluating differences in the studied parameters by individual persons.

Results and Discussion

Tables I and II present the results of a year-round study of alimentation of selected typically agricultural families in the area under investigation, both of these are expressed in an average all-year-round measuring, both quarterly in an effort to record seasonal differences in the nutrition. Consumption of basic nutrients is relatively satisfactory, proteins reaching in total estimates about 10% less value than is the recommended rate, nevertheless, animal proteins are in-

Table I. Caloric and nutrient intake in the rural population (peasants) of the investigated area Voderady in the course of one year, as compared with recommended allowances

Seasons	Total calories		Total proteins		Animal proteins		Plant proteins		Fats		Carbohydrates				Ratio		
	abs. g	%[1]	abs. g	%[1]	abs. g	%[1]	abs. g	%[1]	abs. g	%[1]	total abs. g	%[1]	sugar abs. g	%[2]	P:F:C	Pa:P pl	Fa:F pl
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
I. trimester	3,010	100	86	86	45	89	41	82	105	105	427	100	89	21	11.6:31.6:56.8	52.2:47.8	69.3:30.7
II. trimester	2,982	99	86	86	47	94	39	78	108	108	417	98	95	23	11.5:32.6:55.9	54.6:45.4	70.0:30.0
III. trimester	2,949	98	87	87	46	92	41	82	101	101	427	100	87	20	11.6:30.6:57.8	52.9:47.1	72.2:27.8
IV. trimester	3,241	108	94	94	51	102	43	86	124	124	439	103	92	21	11.6:34.2:54.2	54.1:45.9	62.7:37.3
Daily average per year	3,045	102	88	88	47	94	41	82	109	109	428	101	91	21	11.5:32.3:56.2	53.5:46.5	68.4:31.6

[1] Percent of recommended Czechoslowak allowances
[2] % of carbohydrate
P proteins, *P–a* proteins animal, *P–p* proteins plant, *F* fats, *C* carbohydrates

Table II. Vitamin and mineral intake in the rural population (peasants) of the investigated area Voderady in the course of one year, as compared with recommended allowances

Seasons	Ca		Fe		P	Vitamin A		Vitamin B_1		Vitamin B_2		Vitamin PP		Vitamin C	
	abs. mg	%[1]	abs. mg	%[1]	abs.[2] mg	abs. J.U.	%[1]	abs. mg	%[1]	abs. mg	%[1]	abs. mg	%[1]	abs. mg	%[1]
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
I. trimester	819	102	11.75	84	1,456	2,222	44	1,624	108	1,112	56	16.62	111	40.2	54
II. trimester	876	110	11.25	80	1,493	2,326	46	1,488	99	1,062	53	15.31	102	37.1	49
III. trimester	859	107	11.61	83	1,488	3,859	77	1,487	99	1,134	57	16.30	109	86.4	115
IV. trimester	813	102	13.12	94	1,545	3,402	68	1,557	104	1,270	64	19.44	130	53.4	71
Daily average per year	842	105	11.93	85	1,496	2,952	59	1,539	103	1,114	57	16.92	113	54.3	72

[1] Per cent of recommended Czechoslowak allowances
[2] Per cent of recommended allowances according to SHERMAN

cluded in this to a smaller degree than proteins of plant origin. Carbohydrates according to their balanced value of consumption throughout the year kept at the level of the recommended supply. This does not apply to structure of their consumption, where the ratio of sugar participating in carbohydrate calories attains higher values similarly as was found during our previous findings in Eastern Slovakia [KAJABA and BUČKO, 1968a]. The nutrition of the inhabitants of the studied area is with an exception of summer months characterized moreover by an excessive consumption of animal fats.

Consumption of protective substances is characterized by a year-round deficiency of vitamin A, B_2 and iron. The intake of vitamin C shows typical seasonal variations, depending on access to these sources. This is seen by insufficient maintenance of the recommended supply during the critical nutrient period in the winter and spring months. From the point of view of the objective of our study it is important to state, that in the experimental period the consumption of vitamin C over the given area was attaining only about 50% of the recommended values.

Table III lists the cholesterolemia data in the experimental and control group prior to administration of vitamin C, recorded from November 28th to December 8th, 1967 and after the end of the experiment March 12th–13th, 1968. The initial cholesterol level in blood serum was in both groups practically the same. The administration of vitamin C resulted in the experimental group in a slight, but sta-

Table III. The influence of vitamin C on cholesterolemia (mg%). In the table means ± SE are given

Group	December 1967	March 1968	Statistical significance	
			Student's t-test	t-test of pair sample
Control group	251±3	263±6	P>0.1	P<0.1
Vitamin C treated group	255±2	238±8	P<0.05	P<0.02
Statistical significance				
Student's t-test (control × treated group)	P>0.5	P<0.05		
t-test of pair sample	–	P<0.01		

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tistically significant decrease of cholesterolemia. This decrease was evident both by comparing the final and initial cholesterol levels by the experimental group and when comparing the level of cholesterolemia between the control and experimental group at the end of the experiment. When evaluating differences using the method of the pair t-test, even statistically more persuasive results were observed. On the contrary in the control group, when comparing the data from December and March a trend towards a modest increase of cholesterolemia was observed, however the increase was not statistically significant.

Determination of the level of cholesterolemia in the experimental group immediately prior to starting with vitamin C administration in January 1968, revealed that there was no difference in comparison with initial values determined in December 1967 (average values in December 255 ± 2, in January 258 ± 8 mg%). A decrease in the cholesterol level occurred under the effect of vitamin C only at a later period. The decrease of cholesterolemia caused by administration of ascorbic acid was also significant, when data from the end of the experiment were evaluated (March 1968) by the pair t-test ($P < 0.02$) in comparison with data recorded in January 1968.

The hypocholesterolemic effect of vitamin C was most pronounced in persons with serum cholesterol values over 240 mg% (table IV). In this group the cholesterolemia level was decreased due to the effect of the vitamin C supply with a considerable significance, on the average by 34 mg%, the observed decrease being exceptionally great in some people.

Table V shows a concentration of vitamin C in total blood in control and experimental subjects at the beginning and at the end of the experiment. In December 1967, there were no differences in levels of vitamin C of control and experimental groups. At the end of the experiment in March 1968, this differences was statistically evident in favour of the experimental group receiving vitamin C. This difference is influenced by the fact, that in the course of winter months from December to March, the vitamin C level in the blood of the control group decreased significantly ($P < 0.02$, evaluated by the pair t-test), while in the experimental group, the administration of vitamin C prevented this decrease.

A very surprising fact is, that daily intake of 300 mg of vitamin C for 47 days did not result in a more pronounced increase of ascorbin-

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Table IV. The influence of vitamin C on serum cholesterol levels (mg%) in persons with hypercholesterolemia

Person examined	January 1968	March 1968	Difference
K.M. ♀	340	260	—80
K.M. ♀	320	260	—60
R.A. ♀	313	321	+ 8
Č.A. ♂	305	275	—30
M.J. ♂	305	248	—57
P.J. ♂	291	199	—92
K.A. ♂	291	260	—31
O.J. ♂	291	283	— 8
B.L. ♂	269	283	+14
M.H. ♀	255	217	—38
F.F. ♂	254	220	—34
Š.A. ♂	247	231	—16
P.J. ♂	243	239	— 4
Mean ± SE	286±9	253±9	—33±9
Statistical significance:			P<0.01

Table V. Blood vitamin C levels (mg%) in control and vitamin C treated group

Group	December 1967	March 1968
Control group	0.56±0.07	0.40±0.09
Vitamin C treated group	0.62±0.05	0.67±0.06
Statistical significance	P>0.5	P<0.02

emia in persons belonging to the experimental group. A saturation test after the intravenous administration of ascorbic acid however has shown, that saturation of tissues of persons included in the experimental group, is considerably higher (figure 1). The obtained data in spite of a considerable individual variability demonstrate, that elimination of vitamin C in urine was in the experimental group higher by nearly ten times. The results obtained agree well with the authors of the

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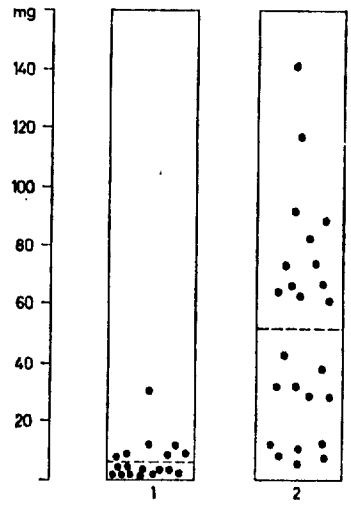

Fig. 1. Vitamin C excretion (mg) in 3 h urine after intravenous injection of 100 mg ascorbic acid. *1* Control group, *2* vitamin C treated group. The difference is statistically highly significant ($P<0.001$).

the saturation test [PLENERT and HEINE, 1967], who report that in saturated persons the elimination of vitamin C in 3-h urine should be about 50% of the administered dose. The values under 15% are considered as defective. Taken from this standpoint, the saturation in the control group persons was extremely low, since from 18 observed persons, only in one case a value higher than 15% was found; this is an indication of unsatisfactory saturation of the Czechoslovak population by vitamin C in winter [BUDLOVSKÝ, 1966; KAJABA, 1966; HEJDA *et al.*, 1965; HEJDA, 1967].

The data obtained demonstrate the causal relationship between saturation of an organism with vitamin C and cholesterolemia. In recent years various authors pointed out the seasonal fluctuation of cholesterolemia in men [THOMAS *et al.*, 1961; TOCHOWICZ *et al.*, 1962; THORP, 1963]: the highest concentrations of serum cholesterol could be observed in the period of minimal intake of vitamin C (winter, spring), lowest in summer and autumn. A similar fluctuation of cholesterolemia was observed in children, too [KAJABA and BUČKO, 1968], with an inverse trend of changes of vitamin C levels in blood (maximum in summer, minimum in winter months). Statistical anal-

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ysis of this material has shown the existence of a significant negative correlation between ascorbinemia and cholesterolemia. Therefore it is probable, that a cause of seasonal fluctuations of cholesterolemia is – not excluding other factors – also the different intake of vitamin C in winter, or summer, respectively.

Further evidence on the participation of vitamin C in cholesterol metabolism was obtained by experimental animals. In guinea-pigs with chronic hypovitaminosis C a significant increase of cholesterol concentration in tissues occurs, especially in liver [GINTER *et al.*, 1967] and an increase of beta-lipoprotein level in blood serum [GINTER *et al.*, 1965]. In guinea-pigs fed on an atherogenic diet, the chronic deficit of ascorbic acid results in an increased accumulation of cholesterol in a whole range of tissues, including aorta walls [GINTER *et al.*, 1969]. Indirect evidence obtained by a study of cholesterol metabolism in hypovitaminic guinea pigs indicates that a shortage of vitamin C results in a decrease in cholesterol catabolism. Therefore it is likely that the hypocholesterolemic effect of the ascorbic acid is based on an increase of cholesterol catabolism, which is finally manifested by a decrease of cholesterolemia.

The fact, that various authors [KRIVORUCHENKO, 1963, 1965; SAMUEL and SALCHI, 1964] were not successful in proving the hypocholesterolemic effect of ascorbic acid, is most likely to be explained by a high saturation of the examined persons with vitamin C, due to which any further supply of ascorbic acid remained ineffective. The evident hypocholesterolemic effect of ascorbic acid, found in the presented study, is undoubtedly influenced by the fact that persons under investigation were showing a clear-cut seasonal vitamin C hyposaturation. We do not consider as impossible that a strictly controlled intake of higher doses of ascorbic acid will under similar conditions result in an even more extensive decrease of cholesterolemia, as that described in this study.

References

ANDERSON, J.; GRANDE, F. and KEYS, A.: Dietary ascorbic acid and serum cholesterol. Fed. Proc. *17:* 468–470 (1958).

BRONTE-STEWART, B.; ROBERTS, B. and WELLS, V. M.: Serum cholesterol in vitamin C deficiency in man. Brit. J. Nutr. *17:* 61–68 (1963).

2894

BUDLOVSKÝ, J.: Stav výživy obyvatelstva na Slovensku (Vyd. SAV, Bratislava 1960).

BUDLOVSKÝ, J.: Die Versorgung der Bevölkerung der Slowakei mit Vitamin C im Winter und Frühjahr. Nahrung *10:* 665–676 (1966).

BUKOVSKAJA, A. V.: Primenenije askorbinovoj kisloty u bolnych aterosklerozom. Sovetsk. Med. *21:* 77–82 (1957).

FEDOROVA, E. P.: Dlitelnoje lečenije askorbinovoj kislotoj bolnych aterosklerozom koronarnych sosudov. Sovetsk. Med. *24:* 56–60 (1960).

GINTER, E.; BOBEK, P. and GERBELOVÁ, M.: The influence of scorbut and prolonged low intake of vitamin C on serum lipoproteins in guinea pigs. Nutr. Dieta *7:* 103–107 (1965).

GINTER, E.; BOBEK, P.; KOPEC, Z.; OVEČKA, M. and ČEREY, K.: Metabolic disorders in guinea pigs with chronic vitamin C hyposaturation. Z. Versuchstierk. *9:* 228–235 (1967).

GINTER, E.: Vplyv vitamínu C na metabolizmus cholesterolu a aterogenézu. Čs. Fysiol. *17:* 423–436 (1968).

GINTER, E.; BOBEK, P.; BABALA, J. and BARBIERIKOVÁ, E.: The effect of ascorbic acid on the lipid metabolism of guinea pigs fed an atherogenic diet. Cor Vasa *11:* 65–73 (1969).

GRABENKO, I. K.: K voprosu o lečenii bolnych aterosklerozom, pp. 105–108 (Trudy 14. Vsesoj. sjezda terapevtov, Moskva 1958).

GYÖRGYI, P.: Vitamin methods. C-vitamin determination according to O. A. BESSEY (Academic Press, New York 1950).

HEJDA, S.; OŠANCOVÁ, K. AND CIBOCHOVÁ E.: Spotřeba vitamínu C v naší populaci v dlouhodobém sledování. Čs. Gastroent. Výž. *19:* 438–442 (1965).

HEJDA, S.: Výživa a nutriční stav našich starých lidí (St. zdravot. nakl., Praha 1967).

HRUBÁ, F. und MAŠEK, J.: Einige Aspekte der Wirkung hoher Dosen von L-Ascorbinsäure auf den gesunden Menschen. Nahrung *6:* 507–517 (1962).

KAJABA, I.: Výživa tukmi v spádovej oblasti VSŽ vzhľadom na kritériá nutričné, biochemické, klinické-caliperometrické. Čs. Gastroent. Výž. *18:* 365–373 (1964).

KAJABA, I.: Die Häufigkeit der Obesität und einige Parameter des Fettstoffwechsels bei der Landbevölkerung in der Ostslowakei. III. CIMR 1966, Bratislava, ČSSR Acta Tome III, p. 251.

KAJABA, I. and BUČKO, A.: Health and nutritional status of children in an industrialised and agricultural area of eastern Slovakia. III. Investigations of the lipid metabolism. Rev. czechoslov. Med. *14:* 180–191 (1968).

KAJABA, I. and BUČKO, A.: I. Dietary pattern and health status. Rev. czechoslov. Med. *14:* 118–126 (1968a).

KRIVORUČENKO, I. V.: O lečenii askorbinovoj kislotoj bolnych aterosklerozom. Terap. Arch. *35:* 48–53 (1963).

KRIVORUČENKO, I. V.: Obmen lipidov i ateroskleroz, pp. 194–196 (Izd. Nauka, Moskva–Leningrad 1965).

KRONDLOVÁ-ŠKOPKOVÁ, M. and ŠMRHA, O.: Tabulky výživných hodnot potravin (St. zdravot. nakl., Praha 1965).

MAŠEK, J.; KŘIKAVA, L.; OŠANCOVÁ, K.; HEJDA, S. and HÁTLE, J.: Nové československé dávky živin. Čas. Lék. čes. *101:* 806–811 (1962).

PEARSON, J.; STERN, S. and MCGAVACK, T. H.: A rapid accurate method for the determination of total cholesterol in serum. Anal. Chem. *25:* 813–817 (1953).

PLENERT, W. und HEINE, W.: Normalwerte. Untersuchungsergebnisse beim gesunden Menschen unter besonderer Berücksichtigung des Kindesalters (VEB Verlag Volk und Gesundheit, Berlin 1967).

2894

ROE, J. H. and KUETHER, C. A.: Determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivate of dehydroascorbic acid. J. biol. Chem. *147:* 339–406 (1943).

SAMUEL, P. and SALCHI, O. B.: Effect of vitamin C on serum cholesterol in patients with hypercholesterolemia and arteriosclerosis. Circulation *29:* 24–25 (1964).

SEDOV, K. R.: Profilaktika i lečenije ateroskleroza askorbinovoj kislotoj. Terap. Arch. *28:* 58–65 (1956).

SOKOLOFF, B.; HORI, M.; SAELHOF, C.; McCONNEL, B. and IMAI, T.: Effect of ascorbic acid on certain blood fat metabolism factors in animals and man. J. Nutr. *91:* 107–118 (1967).

THOMAS, C. B.; HOLLJES, H. W. D. and EISENBERG, F. F.: Observation on seasonal variations in total serum cholesterol level among healthy young prisoners. Ann. intern. Med. *54:* 413–430 (1961).

THORP, J. M.: The control of lipid metabolism, p. 161 (Grant, London 1963).

TJAPINA, L. A.: Vlijanije askorbinovoj kisloty na cholesterinemiju pri gipertoničeskoj bolezni i ateroskleroze. Gipertoničeskaja bolezn. Trudy AMN SSSR *2:* 108–113 (1952).

TOCHOWICZ, L.; CIBA, T.; KOCEMBA, J. and SZOPIŃSKA-CIBA, L.: Sezonowa zmiennoćś poziomu cholesterolu w surovicy. Polski Tyg. lek. *17:* 587–592 (1962).

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Am J Clin Nutr 24 (10): 1238-1245. Illus. 1971 2897

Lowered cholesterol catabolism in guinea pigs with chronic ascorbic acid deficiency[1]

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There is much disagreement regarding the effects of acute ascorbic acid deficiency on cholesterol metabolism in the guinea pig (1–6). The contradictions in results may be explained to a certain extent by the dynamic character of avitaminosis C development, during which the character of metabolic disturbances is changed according to development of scurvy. When following the dynamics of the changes in adrenal cholesterol levels during development of avitaminosis C in guinea pigs, we have found (7) that distinct cholesterol accumulation can be observed at the beginning. During further stages, the cholesterol level reached its normal value and only in the last stage of scurvy, which was accompanied by a decrease in body weight, did the cholesterol concentration in adrenals decrease to below control values. Results attained in animals with acute scurvy may be, however, affected by secondary nonspecific effects, such as food refusal followed by a sudden drop in body weight, hemorrhaging, total metabolic disorganization, et cetera. Some of these changes (e.g., hemorrhage) cannot be simulated by pair-feeding. Moreover, acute experimental scurvy does not reproduce the latent ascorbic acid deficiency observed in humans.

The aim of our experiments was to approach the contemporary state in human nutrition and to use a metabolically better defined state than acute scurvy. All these reasons have led us to elaborate a model of chronic hypovitaminosis C in guinea pigs (8). When using this model, we have found that in male guinea pigs with chronic ascorbic acid deficiency a significant accumulation of cholesterol occurs in the liver (9, 10). When guinea pigs were fed an atherogenic diet supplemented with 0.3% cholesterol, the effect of ascorbic acid deficiency was reflected in an increased accumulation of cholesterol in a number of other tissues, including the aorta wall (11). A significant negative correlation was confirmed as existing between the tissue cholesterol concentration and saturation of tissues with ascorbic acid, i.e., with decreasing saturation of tissues with the vitamin, the cholesterol accumulation in the relevant tissue was increasing (12). The increased cholesterol accumulation in the tissues of hypovitaminotic guinea pigs can be explained neither by increased synthesis of endogenous cholesterol from acetate-1-^{14}C (7, 13) nor by increased absorption of exogenous cholesterol (14). Speed of these processes is either unchanged or even decreased in guinea pigs with hypovitaminosis C. The aim of the experiments reported here was to determine whether an increased cholesterol accumulation in the tissues of guinea pigs with an ascorbic acid deficiency is caused by retarded cholesterol catabolism.

Materials and methods

Growing male guinea pigs were used in all experiments; they were fed a scorbutigenic diet ad libitum (10). All experiments were arranged as follows: the guinea pigs were divided into a control and a hypovitaminotic group. The control guinea pigs were administered 10 mg ascorbic acid/animal per 24 hr perorally. Hypovitaminosis C was evoked in the other group by 14-day administration of a scorbutigenic diet without any addition of ascorbic acid, and after that, by oral administration of a maintenance dose of 0.5 mg ascorbic acid/animal per 24 hr. Ascorbic acid in a 20% sucrose solution was administered by peroral probe three times weekly thereafter. Such a nutritional regimen was followed by a sudden decrease in tissue vitamin C levels of hypovitaminotic animals compared with the ascorbic acid concentrations

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The American Journal of Clinical Nutrition 24: October 1971, pp. 1238–1245. Printed in U.S.A.

observed in the tissues of acute scorbutic guinea pigs (9). The maintenance dose of ascorbic acid, however, prevented manifestations of distinct avitaminosis C. Hypovitaminotic guinea pigs consumed the same diet as the control group; their behavior was normal and their weight curves were similar to those of control animals that were given doses of ascorbic acid 20 times higher than the maintenance dose. All experiments were long term, i.e., the guinea pigs were in the state of chronic hypovitaminosis C for 5 to 7 months.

The first experiment, lasting 22 weeks, was carried out with 10 control and 8 hypovitaminotic guinea pigs initially weighing about 400 g. Twenty days before the end of the experiment, the guinea pigs were given intraperitoneally solutions of cholesterol-4-^{14}C (Amersham, Great Britain; specific activity 24.6 mCi/mmole) in a dose about 4.4 μCi/animal. The exact dose administered was determined by differential weighing of the injection needle with syringe before and after application of labeled cholesterol. Labeled cholesterol was dissolved in methanol with Tween 80; the methanol was evaporated and the residue was dissolved in sterile, physiological saline solution. The same application of labeled cholesterol was used also in the remaining experiments. After administration of cholesterol-4-^{14}C, the guinea pigs were placed in metabolic cages and were allowed free access to food. At 2-day intervals their stools were quantitatively collected. These were dried at 105 C to constant weight; the samples obtained from individual animals were thoroughly mixed together. For analysis, average samples were used which represented approximately 10% of the total weight of dry stool. The samples were extracted for 30 min with boiling ethanol, and then for 12 hr with ethanol in a Soxhlet apparatus. Only negligible traces of activity were found in extracted stool. Five milliliters of the extract was mixed with 5 ml 10% NaOH and hydrolyzed for 8 hr at 160 C. Neutral sterols were extracted from the hydrolysate into five 10-ml portions of petrolether; bile acids after acidification to pH 1 with concentrated sulfuric acid were extracted into five 10-ml portions of ethylether (15). Absolute activity of ^{14}C samples was determined by scintillation spectrometry in a Nuclear Chicago scintillation counter model Mark I, using external standard for quenching correction. Twenty days after the cholesterol-^{14}C application, the guinea pigs were decapitated and total activity of ^{14}C in their blood serum and thoracic aorta was determined by scintillation spectrometry, after being dissolved in a Nuclear Chicago solubilizer.

In the second experiment, 11 control and 12 hypovitaminotic guinea pigs initially weighing about 450 g were used. The experiment lasted 31 weeks. Seventy-two hours before its end, the guinea pigs were given intraperitoneally a solution of cholesterol-4-^{14}C (the same preparation as in the first experiment) in a dose of 0.5 μCi/100 g body wt. After 72 hr, the guinea pigs were decapitated. In their blood serum and liver after extraction (16), concentration of total cholesterol was determined by the Liebermann–Burchard reaction (17). The concentration of vitamin C in liver and spleen was determined after extraction in 6% trichloroacetic acid (18). The appropriate amount of liver and pooled samples of gallbladder bile were hydrolyzed for 8 hr at 160 C in a mixture of 5 ml 10% aqueous NaOH and 2.5 ml 96% ethanol. Extraction of neutral sterols and bile acids from the hydrolysate and determination of the ^{14}C activity was performed, as already mentioned in the first experiment. More than five sterol extractions are not necessary because in the next five petrolether extracts only negligible traces of activity were found. Thin-layer chromatography revealed that the fraction of bile acids did not contain any detectable amounts of cholesterol.

The third experiment was carried out with 10 control and 5 hypovitaminotic guinea pigs initially weighing about 300 g. This experiment lasted 28 weeks. At the end of the experiment, guinea pigs were given intraperitoneally a solution of cholesterol-26-^{14}C (Amersham, Great Britain; specific activity 24 mCi/mmole) in a dose of 0.6 to 0.7 μCi/100 g body wt. The guinea pigs were individually placed in the metabolic cages (diameter 29 cm), in which they had free access to food. CO_2-free air was admitted to the cages, aspirated at a rate of 400 to 600 ml/min and bubbled into two washing bottles with fritted-glass stoppers connected in a series and filled with 2.5 N water solution KOH. Expired $^{14}CO_2$ was collected in 48 hr-intervals for 10 days. The known quantity of KOH solution with absorbed $^{14}CO_2$ was acidified and $^{14}CO_2$ was absorbed into ethanolamine in an apparatus according to Saba and DiLuzio (19). The radioactivity of $^{14}CO_2$ absorbed in ethanolamine was assayed as in the first experiments by scintillation spectrometry.

Results of all experiments were statistically evaluated by Student's t test and correlations were determined by the method of the least squares on the Olivetti Programma 101 computer.

Results

Results of the first experiment, in which output of ^{14}C in stool was followed after intraperitoneal administration of cholesterol-4-^{14}C in the fraction of neutral sterols and bile acids, are shown in Fig. 1. Hypovitaminosis C had no effect on excretion of ^{14}C in the fraction of neutral sterols. The average output of ^{14}C in the fraction of bile acids slightly decreased in hypovitaminotic guinea pigs. The ratio of activity of ^{14}C-bile acids to ^{14}C-sterols was lower in the hypovitaminotic group in almost all intervals. In regard to the fact that in this experiment only pooled samples of stool were analyzed, it was not

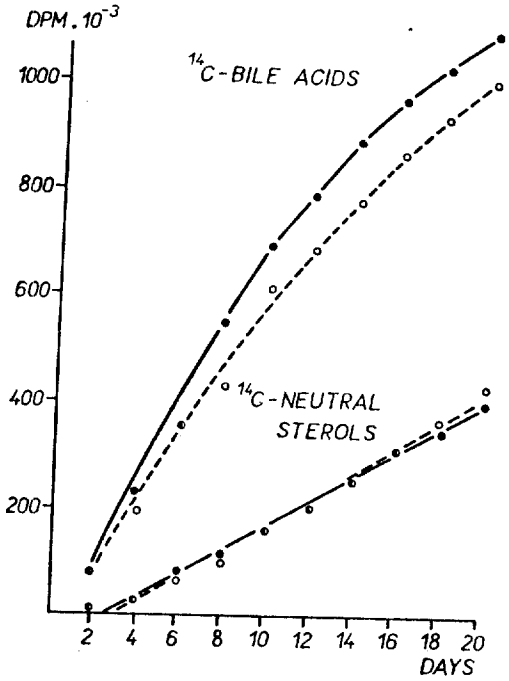

FIG. 1. Excretion of ^{14}C in stool of guinea pigs in the fraction of bile acids and neutral sterols after intraperitoneal administration of cholesterol-4-^{14}C. ●——● control. ○--○ hypovitaminosis C.

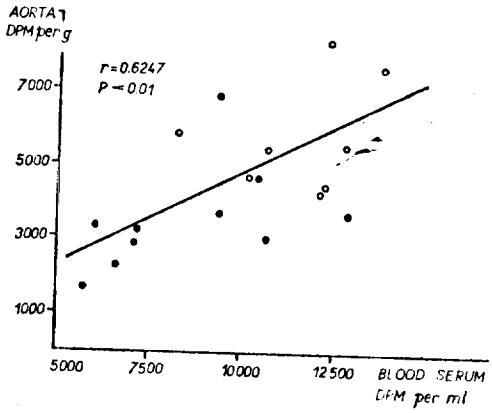

FIG. 2. Correlation between ^{14}C activity in blood serum and thoracic aorta of guinea pigs 20 days after ip administration of cholesterol-4-^{14}C. ● control. ○ hypovitaminosis C.

possible to determine the degree of statistical significance of the differences observed.

Twenty days after intraperitoneal administration of cholesterol-4-^{14}C, significantly higher ^{14}C-total activity was found in the blood serum and thoracic aorta of hypovitaminotic guinea pigs than in the control group (blood serum: control 854 ± 73 dpm, hypovitaminosis C, 1,151 ± 61 dpm/100 μl, $P < 0.01$; thoracic aorta: control 355 ± 61 dpm, hypovitaminosis C, 577 ± 52 dpm/100 mg, $P < 0.01$). Statistical analysis of the combined sets of control and hypovitaminotic guinea pigs has shown that there is a middle closed correlation between the level of ^{14}C in the blood serum and thoracic aorta ($P < 0.01$) (Fig. 2).

Table 1 lists the concentrations of ascorbic acid and total cholesterol in control and hypovitaminotic guinea pigs from the second experiment. The results prove that ascorbic acid concentrations in the liver and spleen of hypovitaminotic guinea pigs significantly decreased. Hypovitaminosis C caused a significant increase in the level of total cholesterol in liver, which is in agreement with results of our previous works (7, 9, 10).

Table 2 gives the distribution of ^{14}C in the fraction of neutral sterols and bile acids in liver and gallbladder bile 72 hr after intraperitoneal administration of cholesterol-4-^{14}C. These results prove that distribution of ^{14}C between the fraction of neutral sterols and bile acids was significantly changed. In the liver of ascorbic acid-deficient guinea pigs a not sufficiently significant tendency to higher values of activity in the fraction of sterols and significantly lower activity in the

TABLE 1

Vitamin C and total cholesterol concentration in control and vitamin C hyposaturated guinea pigs

Parameter	Tissue	Control	Hypovitaminosis C	P
Ascorbic acid mg/100 g, wet tissue	Liver	5.28 ± 0.30	0.84 ± 0.20	<0.001
	Spleen	15.41 ± 0.95	5.11 ± 0.52	<0.001
Total cholesterol mg/100 ml	Blood serum	136 ± 11	152 ± 18	NS
mg/100 g	Liver	357 + 22	661 ± 96	<0.01

Values are means ± SEM. NS = not significant.

TABLE 2
The influence of hypovitaminosis C on the distribution of ^{14}C in neutral sterols and bile acids 3 days after ip injection of cholesterol-4-^{14}C

Sample	Fraction	Control	Hypovitaminosis C	P
Liver, dpm per g wet tissue	Neutral sterols	23,970 ± 2,292	26,505 ± 2,353	NS
	Bile acids	3,397 ± 635	2,017 ± 173	<0.05
	Bile acids / Neutral sterols	0.157 ± 0.030	0.080 ± 0.007	<0.02
Gallbladder bile, dpm per g	Neutral sterols	1,273	1,367	Analyses were performed on pooled samples
	Bile acids	132,307	108,755	
	Bile acids / Neutral sterols	103.9	79.6	

Values for liver are means ± SEM.

fraction of bile acids was observed. Due to these changes, the ^{14}C-bile acids: ^{14}C-sterols decreased in liver and gallbladder bile. (It reached almost one-half of the control values in liver.) These results prove that in liver of guinea pigs with chronic vitamin C deficiency, transformation of cholesterol to bile acids is significantly retarded.

Results of the third experiment, in which a different method for following cholesterol catabolism was used, prove the above conclusion. Figure 3 represents cumulative oxidation of intraperitoneally administered cholesterol-26-^{14}C to $^{14}CO_2$ in control and hypovitaminotic guinea pigs. In agreement with the results of the first two experiments, the rate of this process is significantly decreased in ascorbic acid-deficient guinea pigs. Differences in the amount of expired $^{14}CO_2$ between the control and hypovitaminotic group are highly significant in all intervals ($P < 0.01$–0.001).

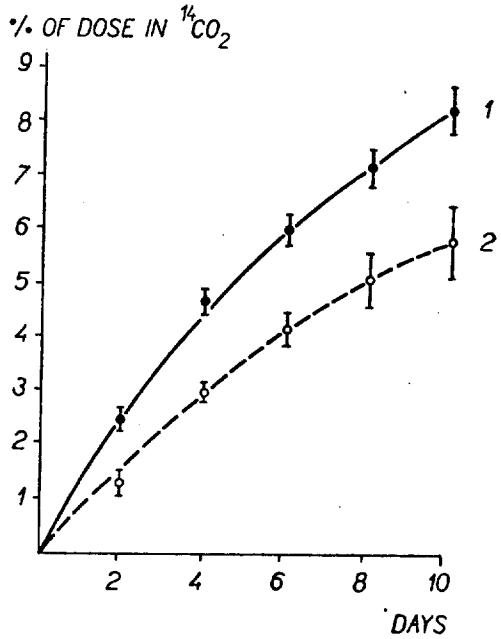

FIG. 3. Cumulative elimination of $^{14}CO_2$ in the expired air as percent of the dose of 26-^{14}C-cholesterol. Vertical bars represent ± SEM. 1 = control. 2 = hypovitaminosis C.

Discussion

Cholesterol level in blood and tissues under a defined nutrition regimen is the result of a combination of many processes. Theoretically the increased cholesterol levels, regularly observed in the tissues of hypovitaminotic guinea pigs could result from any or a combination of the following alternatives in cholesterol metabolism: 1) a redistribution of cholesterol between various tissues and blood; 2) an increase in absorption of exogenous cholesterol; 3) an increase in endogenous cholesterol synthesis; 4) a decrease in fecal excretion of cholesterol; and 5) a decrease in cholesterol transformation to bile acids.

Theoretically, we could consider also other alternatives, e.g., transformation of cholesterol to steroid hormones. However, quantitatively, these processes are negligible for the total cholesterol balance (20).

The first alternative is highly improbable because in hypovitaminotic guinea pigs an accumulation of cholesterol was observed in many organs, especially in those which contribute to a substantial degree to the body pool of cholesterol (e.g., liver, small intestine, adrenals) (11, 12). The second alternative was examined by an experiment in which distribution of intragastrically applied cholesterol-4-^{14}C was followed (14). The results obtained have shown that an increased accumulation of cholesterol in hypovitaminotic guinea pigs cannot be explained by an increased absorption of exogenous cholesterol. On the contrary, this process is slowed during chronic hypovitaminosis C. Increased levels of tissue cholesterol in hypovitaminotic guinea pigs cannot be explained either by the third alternative, as endogenous synthesis of cholesterol from acetate-1-^{14}C is not changed during chronic hypovitaminosis C, or even a slight decrease (7, 13).

As the increased accumulation of cholesterol in vitamin C-deficient guinea pigs can be explained neither by an increased absorption of exogenous cholesterol nor by an increased biosynthesis of endogenous cholesterol, it is probable that chronic vitamin C-deficiency affects the metabolism of cholesterol within the sphere of its catabolism. The first in the series of experiments, results of which are presented in this paper, has shown that the output of ^{14}C-neutral sterols in stools after intraperitoneal administration of cholesterol-4-^{14}C is not affected by hypovitaminosis C. Thus, the only explanation is in the last alternative, i.e., retarded transformation of cholesterol to bile acids.

Catabolism of cholesterol to bile acids can be followed by using two isotopic methods. When cholesterol labeled with ^{14}C in position 4 of the cyclic structure is administered, the activity that occurs in the bile acid fraction is the measure of the speed of this process. Mammals do not have an enzymatic system that is able to split the cholesterol nucleus (21). When cholesterol labeled in position 26 on the side chain is given, the isopropyl fragment from the side chain is split off during cholesterol catabolism and the ^{14}C is released in the form of carbon dioxide (22). The speed of cholesterol catabolism is measured according to the amount of ^{14}C in expired CO_2. In order to prove our hypothesis that chronic hypovitaminosis C causes retardation of cholesterol catabolism to bile acids, both above-mentioned techniques were employed. The results we attained proved this hypothesis to be correct: in guinea pigs with long-term vitamin C-deficiency, transformation of cholesterol-4-^{14}C to bile acids is slowed down and so is the oxidation of cholesterol-26-^{14}C to $^{14}CO_2$.

The mechanism of ascorbic acid participation in the transformation of cholesterol to bile acids is unknown. It seems that the basic biological role of ascorbic acid consists in its participation in hydroxylation reactions, e.g., in the synthesis of hydroxyproline from proline (23), in catabolism of aromatic amino acids (24), in hydroxylation of dopamine (25), tryptophan (26), acetanilid (27), tyramine (28), and other substances. Mechanism of the majority of the above-mentioned ascorbic acid-dependent hydroxylation reactions consists, according to Staudinger (29), in the fact that ascorbic acid forms with molecular oxygen, in an enzymatically catalyzed reaction, hydroxyl radicals that can be used for hydroxylation of various substrates. For the enzymes, catalyzing reactions of this sort in which one oxygen atom incorporates into hydroxylated substrate and the second atom is simultaneously reduced to water, the term "mixed-function oxidases" is used. A similar function to that of vitamin C can be played in these reactions by other electron donors such as nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), nicotinamide adenine dinucleotide, reduced form (NADH), dihydroxyfumaric, and tetrahydrofolic acids.

Hydroxylation reactions also play an important role in the transformation of cholesterol to bile acids (30). Björkhem et al. (31) have described the major pathway in the conversion of cholesterol to chenodeoxycholic acid in guinea pigs: cholesterol → cholest-5-ene-3β,7α-diol → 7α-hydroxycholest-4-en-3-one → 7 α-hydroxy-5β-cholestan-3-one → 5β-cholestane-3α,7α-diol → 5β-cholestane-3α,7α,26-triol → chenodeoxycholic acid. During the first reaction, cholesterol

nucleus is hydroxylated in position 7; during the second to last, hydroxylation of side chain in position 26 proceeds. As the function of ascorbic acid in hydroxylation reactions is generally accepted, we suggest as a working hypothesis that ascorbic acid has a similar function in the transformation of cholesterol into bile acids. The scheme of this hypothetic role of ascorbic acid is in Fig. 4. The direct participation of ascorbic acid in the above-mentioned processes is supported also by data on the stimulatory effect of ascorbic acid on the synthesis of bile acids in vitro in liver mitochondria from scorbutic guinea pigs (32), as well as by our finding of substantially increased oxidation of cholesterol-26-^{14}C to $^{14}CO_2$ shortly after resaturation of ascorbic acid-deficient guinea pigs with a large intraperitoneally administered dose of ascorbic acid (unpublished observations). In this connection also, the data on the stimulatory effect of ascorbic acid on oxidation of 7-dehydrocholesterol in the mouse liver microsomal system (33) and on transformation of deoxycholic acid to cholic acid in nonbiological systems (34) are very interesting. There are other authors who emphasize the important role of Fe^{2+} ions during reactions of this nature (28, 33–36).

The question of the potential role of latent hypovitaminosis C in the pathogenesis of atherosclerosis is interesting. According to Willis (37), within a short period of time scorbutic guinea pigs show atheromatous changes in the aorta without being fed a cholesterol atherogenic diet. Gore et al. (38, 39) examined a great number of scorbutic guinea pigs, but did not find any similar changes. We have not found any significant morphological changes in the aorta and coronary arteries of scorbutic guinea pigs that were given an atherogenic cholesterol diet during the entire period of development of acute avitaminosis C (11). There are more data on protective effects of massive doses of ascorbic acid on experimental cholesterol atheromatosis (40–42). Unfortunately, these experiments were carried out with the animals (rabbits and rats) synthesizing ascorbic acid. Metabolic response of these animal species to the large amounts of exogenous vitamin C differs from that of the organisms

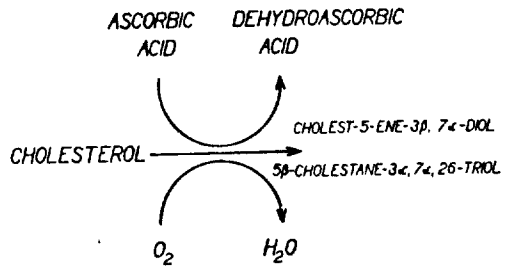

FIG. 4. Hypothetical role of ascorbic acid in hydroxylation of cholesterol during its transformation to bile acids.

that are not able to synthesize ascorbic acid (43), and the results attained must be very carefully extrapolated to the human organism. In guinea pigs fed atherogenic cholesterol diets, large amounts of the vitamin did not prevent the appearance of morphological changes in the vascular system, but pathomorphological changes in vessels of guinea pigs with chronic ascorbic acid deficiency were more distinct (12). In our opinion, the fact that chronic ascorbic acid deficiency with decreasing cholesterol catabolism causes an increasing accumulation of cholesterol in tissues creates a metabolic situation that increases the risk that pathomorphological changes in vessels will occur. There is a prospect that knowledge of the function of ascorbic acid in catabolism of cholesterol, obtained on the model of latent chronic hypovitaminosis C in guinea pigs, will make it possible to extrapolate also to a human organism, as when we succeeded in decreasing the level of serum cholesterol in hypercholesteremic persons with seasonal vitamin C deficiency through resaturation with massive doses of ascorbic acid (44).

Summary

Chronic ascorbic acid deficiency was provoked in male guinea pigs by 14-day administration of a scorbutigenic diet followed by peroral administration of a maintenance dose of vitamin C (0.5 mg/animal per day). A control group was fed the same scorbutigenic diet supplemented perorally by 10 mg vitamin C/animal per day. The experiments lasted 5 to 7 months. The course of weight

curves was not affected significantly by hypovitaminosis C.

Hypovitaminosis C caused a substantial decrease in vitamin C levels in tissues of guinea pigs and accumulation of total cholesterol in the liver. Hypovitaminosis C did not affect fecal excretion of ^{14}C in the fraction of neutral sterols in guinea pigs injected intraperitoneally with cholesterol-4-^{14}C, and it slightly decreased fecal excretion of ^{14}C bile acids (during 20 days following administration of labeled cholesterol). Twenty days after application of cholesterol-4-^{14}C more ^{14}C was found in the blood serum and thoracic aorta of hypovitaminotic guinea pigs than in the control group. Three days after intraperitoneal injection of cholesterol-4-^{14}C, less ^{14}C in the fraction of bile acids was found in liver and gallbladder bile of hypovitaminotic guinea pigs than in the control group. Chronic hypovitaminosis C decreased significantly the oxidation of intraperitoneally injected cholesterol-26-^{14}C to $^{14}CO_2$ during 10 days following administration of labeled cholesterol.

The results prove that in guinea pigs with chronic ascorbic acid deficiency, the catabolism of cholesterol to bile acids is significantly decreased. A working hypothesis is postulated that ascorbic acid is required for cholesterol hydroxylation during its transformation to bile acids.

References

1. Mouriquand, G., and A. Leulier. Sur la teneur en cholestérine de quelques organes des cobayes soumis au régime scorbutigene. *Compt. Rend. Soc. Biol.* 93: 1314, 1925.
2. Banerjee, S., and H. D. Singh. Cholesterol metabolism in scorbutic guinea pigs. *J. Biol. Chem.* 233: 336, 1958.
3. Bolker, H. I., S. Fishman, R. D. H. Heard, V. J. O'Donnell, J. L. Webb and G. C. Willis. The incorporation of acetate-1-^{14}C into cholesterol and fatty acids by surviving tissues of normal and scorbutic guinea pigs. *J. Exptl. Med.* 103: 199, 1956.
4. Banerjee, S., and P. K. Ghosh. Metabolism of acetate in scorbutic guinea pigs. *Am. J. Physiol.* 199: 1064, 1960.
5. Oesterling, M. J., and C. N. H. Long. Adrenal cholesterol in scorbutic guinea pigs. *Science* 113: 241, 1951.
6. Baldvin, R. A., H. E. Longenecker and C. G. King. *Arch. Biochem.* 5: 137, 1944. Cited from Oesterling and Long (5).
7. Ginter, E., L. Bilisics and J. Červeň. Cholesterol metabolism under conditions of acute and chronic vitamin C deficiency in guinea pigs. *Physiol. Bohemoslov.* 14: 466, 1965.
8. Ginter, E., P. Bobek and M. Ovečka. Model of chronic hypovitaminosis C in guinea-pigs. *Intern. J. Vitamin Res.* 38: 104, 1968.
9. Ginter, E., P. Bobek, Z. Kopec, M. Ovečka and K. Čerey. Metabolic disorders in guinea pigs with chronic vitamin C hyposaturation. *Z Versuchstierk.* 9: 228, 1967.
10. Ginter, E., R. Ondreička, P. Bobek and V. Šimko. The influence of chronic vitamin C deficiency on fatty acid composition of blood serum, liver triglycerides and cholesterol esters in guinea pigs. *J. Nutr.* 99: 261, 1969.
11. Ginter, E., P. Bobek, J. Babala and E. Barbieriková. The effect of ascorbic acid on the lipid metabolism of guinea pigs fed an atherogenic diet. *Cor Vasa* 11: 65, 1969.
12. Ginter, E., J. Babala and J. Červeň. The effect of chronic hypovitaminosis C on the metabolism of cholesterol and atherogenesis in guinea pigs. *J. Atherosclerosis Res.* 10: 341, 1969.
13. Ginter, E., and R. Nemec. Metabolism of 1-^{14}C-acetate in guinea pigs with chronic vitamin C hyposaturation. *J. Atherosclerosis Res.* 10: 273, 1969.
14. Ginter, E., J. Červeň and L. Mikuš. The effect of chronic hypovitaminosis C on the absorption of cholesterol-4-^{14}C in guinea pigs. *Physiol. Bohemoslov.* 18: 459, 1969.
15. Siperstein, M. D., M. E. Jayko, J. L. Chaikoff and W. G. Dauben. Nature of the metabolic products of ^{14}C cholesterol excreted in bile and feces. *Proc. Soc. Exptl. Biol. Med.* 81: 720, 1952.
16. Folch, J., M. Lees and G. H. Sloane-Stanley. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226: 497, 1957.
17. Cook, R. P. *Cholesterol.* New York: Academic, 1958, p. 487.
18. Roe, J. H., and C. A. Kuether. Determination of ascorbic acid in whole blood and urine through the 2:4-dinitrophenyl-hydrazine derivate of dehydroascorbic acid. *J. Biol. Chem.* 147: 399, 1943.
19. Saba, T. M., and N. R. DiLuzio. Method for collection and determination of $^{14}CO_2$ for in vitro metabolic studies. *J. Lipid Res.* 7: 566, 1966.
20. Chevallier, F. Dynamics of cholesterol in rats, studied by the isotopic equilibrium methods. *Adv. Lipid Res.* 5: 209, 1967.
21. Chaikoff, I. L., M. D. Siperstein, W. G. Dauben, H. L. Bradlow, J. F. Eastham, G. M. Tomkins, J. R. Meier, R. W. Chen, S. Hotta and P. A. Srere. C^{14}-cholesterol. II. Oxidation of carbons 4 and 26 to carbon dioxide by the intact rat. *J. Biol. Chem.* 194: 413, 1952.
22. Siperstein, M. D., and I. L. Chaikoff. Con-

2897

version of cholesterol to bile acids. *Federation Proc.* 14: 767, 1955.

23. ROBERTSON, W. VAN B. The biochemical role of ascorbic acid in connective tissue. *Ann. N.Y. Acad. Sci.* 92: 159, 1961.
24. LA DU B. N., AND V. G. ZANNONI. The role of ascorbic acid in tyrosine metabolism. *Ann. N.Y. Acad. Sci.* 92: 175, 1961.
25. LEVIN, E. Y., B. LEVENBERG AND S. KAUFMAN. The enzymatic conversion of 3,4-dihydroxyphenyl-ethylamine to norepinephrine. *J. Biol. Chem.* 235: 2080, 1960.
26. COOPER, J. R. The role of ascorbic acid in the oxidation of tryptophan to 5-hydroxytryptophan. *Ann. N.Y. Acad. Sci.* 92: 208, 1961.
27. DEGKWITZ, E., AND HJ. STAUDINGER. Untersuchungen zur Hydroxylierung von Acetanilid mit Lebermikrosomen normaler und skorbutischer Meerschweinchen. *Z. Physiol. Chem.* 342: 63, 1965.
28. UDENFRIEND, S., C. T. CLARK, J. AXELROD AND B. B. BRODIE. Ascorbic acid in aromatic hydroxylation. I. A model system for aromatic hydroxylation. *J. Biol. Chem.* 208: 731, 1954.
29. SCHNEIDER, W., AND HJ. STAUDINGER. Zum Wirkungsmechanismus von Vitamin C. *Klin. Wochschr.* 42: 879, 1964.
30. VAN BELLE, H. *Cholesterol, Bile Acids and Atherosclerosis.* Amsterdam: North-Holland, 1965, p. 73.
31. BJÖRKHEM, I., H. DANIELSSON AND K. EINARSSON. On the conversion of cholesterol to 5β-cholestane-3α,7α-diol in guinea pig liver homogenates. *European J. Biochem.* 2: 294, 1967.
32. GUCHAIT, R., B. C. GUHA AND N. C. GANGULI. Metabolic studies on scorbutic guinea pigs. III. Catabolism of /4-^{14}C/ cholesterol in vivo and in vitro. *Biochem. J.* 86: 193, 1963.
33. KANDUTSCH, A. A. Oxidation of 7-dehydrocholesterol by a mouse liver microsomal system dependent on reduced pyridine nucleotides. *J. Lipid Res.* 7: 603, 1966.
34. MATKOVICS, B., P. PÉNZES AND GY. GÖNDÖS. The in vitro transformation of deoxycholic acid into cholic acid with ferroascorbate system. *Steroids* 5: 451, 1965.
35. BRODIE, B. B., J. AXELROD, P. A. SHORE AND S. UDENFRIEND. Ascorbic acid in aromatic hydroxylation. II. Products formed by reaction of substrates with ascorbic acid, ferrous ion, and oxygen. *J. Biol. Chem.* 208: 741, 1954.
36. BRESLOW, R., AND L. N. LUKENS. On the mechanism of action of an ascorbic acid-dependent nonenzymatic hydroxylating system. *J. Biol. Chem.* 235: 292, 1960.
37. WILLIS, G. C. The reversibility of atherosclerosis. *Can. Med. Assoc. J.* 77: 106, 1957.
38. GORE, I., Y. TANAKA, T. FUJINAMI AND M. L. GOODMAN. Aortic acid mucopolysaccharides and collagen in scorbutic guinea pigs. *J. Nutr.* 87: 311, 1965.
39. GORE, I., T. FUJINAMI AND T. SHIRAHAMA. Endothelial changes produced by ascorbic acid deficiency in guinea pigs. *Arch. Pathol.* 80: 371, 1965.
40. MYASNIKOV, A. L. Influence of some factors on development of experimental cholesterol atherosclerosis. *Circulation* 17: 99, 1958.
41. SOKOLOFF, B., M. HORI, C. SAELHOF, B. MCCONNELL AND T. IMAI. Effect of ascorbic acid on certain blood fat metabolism factors in animals and man. *J. Nutr.* 91: 107, 1967.
42. ZAITSEV, V. F., L. A. MYASNIKOV, L. V. KASATKINA, N. M. LOBOVA AND T. I. SUKASOVA. The effect of ascorbic acid on experimental atherosclerosis. *Cor Vasa* 6: 19, 1964.
43. GINTER, E., J. BABALA AND E. POLÓNYOVÁ. Vitamin C und Metabolismus der Lipoide bei mit atherogener Diät gefütterten Kaninchen. *Biológia (Bratislava)* 25: 579, 1970.
44. GINTER, E., I. KAJABA AND O. NIZNER. The effect of ascorbic acid on cholesterolemia in healthy subjects with seasonal deficit of vitamin C. *Nutr. Metabol.* 12: 76, 1970.

Nutr 101 (2): 197-204 Illus. 1971

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Metabolism of L-Ascorbic Acid-1-^{14}C in Guinea Pigs with Alimentary Cholesterol Atheromatosis

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ABSTRACT A diet containing 0.3% cholesterol fed to guinea pigs for 18 weeks caused an accumulation of cholesterol in blood serum, liver and adrenals, atheromatous changes in coronary arteries and a marked increase in the weight of liver and spleen, in comparison with controls fed the same diet without cholesterol. Ascorbic acid intake was the same in these two groups (10 mg per animal per day, given through a stomach tube). Ascorbic and dehydroascorbic acid concentration in liver and adrenals was not changed in the cholesterol-fed animals, but the total quantity of ascorbic acid in their liver and spleen was substantially higher. Urinary excretion of ascorbic, dehydroascorbic and diketogulonic acids was decreased in this group while oxidation of intraperitoneally administered ascorbic acid-1-^{14}C to $^{14}CO_2$ remained unaltered. Urinary excretion of ^{14}C was lower in the cholesterol-fed group. Specific activity of ascorbic acid in the tissues of cholesterol-fed guinea pigs was found to be significantly decreased at 96 hours after administration of labeled ascorbic acid compared to that at 48 hours. Guinea pigs with experimental atheromatosis had a significantly larger body pool and a moderately, though significantly, prolonged biological half-life of ascorbic acid. In guinea pigs with dietary cholesterol atheromatosis, an increased ascorbic acid retention exists. J. Nutr. *101:* 197-204, 1971.

A long-term chronic ascorbic acid deficiency in guinea pigs results in cholesterol accumulation in the liver (1). If animals are fed an atherogenic diet with added cholesterol, the effects of ascorbic acid deficiency are even more pronounced: in comparison with guinea pigs well saturated with ascorbic acid, the cholesterol level is significantly raised in several tissues including the aorta wall (2, 3). The mechanism of this phenomenon is not quite clear but it is probably related to the lowered rate of cholesterol catabolism in ascorbic acid deficient guinea pigs (4, 5). If ascorbic acid is involved in catabolic reactions in which cholesterol is converted into bile acids, then the question arises whether exogenous cholesterol feeding affects ascorbic acid metabolism. The present study is an attempt to answer this question by a long-term study of ascorbic acid-1-^{14}C metabolism in guinea pigs with a high level of ascorbic acid saturation fed an atherogenic diet containing cholesterol.

MATERIAL AND METHODS

Male guinea pigs, initial weight 400 to 600 g, were fed ad libitum the modified Lunde's scorbutogenic diet (6). Ten milligrams ascorbic acid was administered orally to each animal per day. Thus, the exact ascorbic acid intake was known. An experimental group was fed the same diet supplemented with 0.3% cholesterol. They received the same ascorbic acid supplement as the controls. This cholesterol supplemented diet has been shown to cause a significant accumulation of cholesterol in tissues and, within 4 months, to induce atheromatous changes in the vascular system, particularly in the coronary arteries of guinea pigs (7).

After 18 weeks on this diet, the controls and the cholesterol-fed guinea pigs were given intraperitoneally solution of 5 mg ascorbic acid-1-^{14}C (produced in the USSR) in sterile saline. The radiochemical purity

Received for publication June 22, 1970.

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and specific activity of the ascorbic acid-1-^{14}C was determined by paper radiochromatography (Whatman No. 1, solvent system butanol–acetic acid–water, 4:1:5). The radiochemical purity was 91% and specific activity 2.63 μCi/mg. The solutions were prepared from crystalline ascorbic acid-1-^{14}C just before application. This quantity (5 mg) was supplemented with 5 mg nonlabeled ascorbic acid given orally to make up the usual daily intake of 10 mg of ascorbic acid. The exact quantity of administered ascorbic acid-1-^{14}C was ascertained by differential weighing of a syringe and needle before and after injection. After receiving the labeled ascorbic acid the animals were immediately placed in glass metabolic cages where they had free access to food. The $^{14}CO_2$ exhaled in 12-hour periods was collected in two washing bottles with fritted-glass stoppers connected in series and filled with 2.5 N water solution of KOH. Urine stabilized with 0.5 ml of concentrated acetic acid was also collected in the same intervals. Controls and cholesterol-fed animals were divided into subgroups (always eight animals per subgroup) and killed by decapitation at 48 or 96 hours after labeled ascorbic acid administration.

The concentration of total cholesterol and of ascorbic acid (9) in blood and several tissues was determined by Liebermann-Buchard's reaction (8). The concentration of ascorbic acid in blood and tissues was also determined (9). The concentration of ascorbic, dehydroascorbic and diketogulonic acids in the liver, adrenals and urine following isolation of bis-hydrazone of diketogulonic acid by thin-layer chromatography on silica gel was assayed (10). The absolute activity of $^{14}CO_2$ was determined according to Saba and Di Luzio (11) by scintillation spectrometry in toluene-based scintillation solution in a scintillation counter,[2] using an external standard for quenching correction. Total ^{14}C activity in urine and in six tissues dissolved in NCS (Nuclear Chicago Solubilizer) was assayed in the same instrument. Liver and myocardium with a part of aorta were examined histologically.

The evaluation of the results by means of Student's t test and the calculation of the biological half-life of ascorbic acid were performed by a computer.[3] Altogether, data from 32 animals (16 controls and 16 cholesterol-fed guinea pigs) were processed in this way.

RESULTS AND DISCUSSION

There was no substantial difference between the weight gain curves of controls and cholesterol-fed guinea pigs. Weight increase in the latter group was somewhat lower but this difference was not significant (table 1). The atherogenic diet caused a highly significant weight increase of the liver and spleen (as much as threefold that in controls), and a decrease in adrenal weight (table 1).

The intake of a cholesterol diet caused a striking accumulation of cholesterol in liver (up to ten times the values in the controls). The cholesterol level in cholesterol-fed animals increased significantly also in blood serum and adrenals, whereas it remained unaltered in their intestine and brain (table 2).

A histological investigation of the myocardium of guinea pigs fed an atherogenic diet revealed atheromatous changes in the

[2] Mark I, Nuclear Chicago Corp., Chicago, Ill.
[3] Olivetti Programma 101.

TABLE 1

Body and organ weights of control and cholesterol-fed guinea pigs

Organ	Control group	Cholesterol-fed group	Statistical significance
Body weight, g (beginning)	453 ± 17 [1]	480 ± 17	—
Body weight, g (end of experiment)	645 ± 19	634 ± 19	—
Liver, g	20.85 ± 0.89	57.37 ± 2.97	$P < 0.001$
Spleen, g	0.85 ± 0.04	2.53 ± 0.40	$P < 0.001$
Kidneys, g	3.96 ± 0.11	3.71 ± 0.08	—
Adrenals, mg	438 ± 36	339 ± 21	$P < 0.05$

[1] Mean ± SEM, 16 guinea pigs per group.

coronary arteries. Myocardial cells showed an enhanced oxyphylia and pyknosis of nuclei, with scanty mononuclei and histiocytes around the vessels. Edema of the wall and signs of endothelial proliferation in the aorta were evident in some of these animals. Steatosis of the liver was observed in all these guinea pigs, and monocellular necroses with surrounding leukocytic reaction was observed in the majority. Foam-cell formation was also noted in the arteries of porto-biliary spaces and occasionally clusters of thrombocytes and leukocytes in the rami of the portal vein were present. Immature blood-forming elements could also be observed in the vessels as well as sinusoids.

A decrease in ascorbic acid concentration in the spleen of cholesterol-fed guinea pigs was of high statistical significance (table 3), which is in agreement with our earlier findings (12), while its level was not changed significantly in the other organs and blood. On the other hand, the total quantity of ascorbic acid increased substantially in the liver and spleen of animals fed an atherogenic diet but decreased moderately in their adrenals (table 3). These changes are evidently closely related to substantial weight increases in the liver and spleen and to a significant weight decrease in the adrenals of cholesterol-fed animals.

Sokoloff et al. (13), without giving any numerical data, state that "dehydroascorbic acid is present in considerable amounts in the blood and tissues of animals and humans with atherosclerosis and/or disturbed fat metabolism." Our results do not support this claim. Concentrations of ascorbic, dehydroascorbic and diketogulonic acids in the liver and adrenals were not significantly altered in cholesterol-fed guinea pigs (except for a decline of diketogulonic acid in adrenals). The total amount of ascorbic and dehydroascorbic acids in the liver of cholesterol-fed guinea pigs was significantly elevated (table 4) which was in agreement with the results presented in table 3. On the other hand, urinary excretion of ascorbic, dehydroascorbic and diketogulon-

TABLE 2

Total cholesterol concentration in blood serum and tissues of control and cholesterol-fed guinea pigs

Tissue	Control group	Cholesterol-fed group	Statistical significance
	mg/100 g tissue		
Liver	355 ± 18 [1]	3740 ± 170	$P < 0.001$
Adrenals	5649 ± 575	8133 ± 534	$P < 0.01$
Brain	1051 ± 54	1068 ± 31	—
Small intestine	271 ± 9	280 ± 12	—
	mg/100 ml		
Blood serum	126 ± 6	256 ± 22	$P < 0.001$

[1] Mean ± SEM, 16 guinea pigs per group.

TABLE 3

Ascorbic acid concentration and total content in tissues of control and cholesterol-fed guinea pigs

Tissue	Control	Cholesterol-fed	Control	Cholesterol-fed
	mg/100 g tissue		*mg/organ*	
Liver	8.12 ± 0.39 [1]	8.10 ± 0.35	1.681 ± 0.094	4.648 ± 0.326 [a]
Spleen	29.28 ± 0.77	23.62 ± 0.95 [a]	0.247 ± 0.011	0.576 ± 0.080 [a]
Kidneys	5.08 ± 0.30	5.19 ± 0.21	0.198 ± 0.011	0.192 ± 0.008
Adrenals	57.33 ± 1.09	57.56 ± 1.77	0.253 ± 0.023	0.196 ± 0.014 [b]
Small intestine	13.08 ± 0.55	12.02 ± 0.40		
Brain	13.88 ± 0.48	13.80 ± 0.62		
	mg/100 ml			
Total blood	0.56 ± 0.04	0.65 ± 0.06		

[1] Mean ± SEM, 16 guinea pigs per group; [a] difference from controls significant at $P < 0.001$ level; [b] difference significant at $P < 0.05$ level.

ic acids was significantly lower in cholesterol-fed guinea pigs (table 4).

The lowered urinary excretion of ascorbic acid and its oxidation products is evidently related to a lower ^{14}C urinary excretion in guinea pigs fed an atherogenic diet (table 5). An open question is, what part of the total radioactivity found in urine belongs to oxalic acid which is the principal catabolic product derived from the first two carbons of ascorbic acid (14–17). Pilot analyses led us to think that cholesterol-fed guinea pigs excrete larger amounts of ^{14}C-oxalic acid in urine than the controls.

The major part of ascorbic acid-1-^{14}C was catabolized by oxidation to $^{14}CO_2$, which is in agreement with findings by other authors (14–16, 18, 19) who studied the catabolism of labeled ascorbic acid in guinea pigs. Immediately following injection of ascorbic acid-1-^{14}C a high rate of $^{14}CO_2$ expiration occurred, falling to a constant rate after 24 hours (3 to 5% of administered dose per 12 hours). This indicates that the labeled ascorbic acid was in equilibrium with the nonradioactive ascorbic acid already present in the tissues.

The dynamics of $^{14}CO_2$ release, schematized in figure 2, agreed with data reported by other authors (14, 15, 20, 21). Oxidation of ascorbic acid-1-^{14}C to $^{14}CO_2$, except for two cases (which are to be discussed later) was not affected by the atherogenic diet. The amount of $^{14}CO_2$ expired during 12 hour intervals is practically the same in the controls and in the cholesterol-fed animals (see table 5). The activity of the enzymatic system catalyzing ascorbic acid

TABLE 4

Ascorbic acid (AA), dehydroascorbic acid (DAA) and diketogulonic acid (DGA) in tissues and urine of control and cholesterol-fed guinea pigs

Sample	Fraction	Control	Cholesterol-fed	Control	Cholesterol-fed
		mg/100 g tissue		*μg/organ*	
Liver	AA	6.57 ± 1.23 [1]	5.57 ± 0.83 [1]	1301 ± 223	3359 ± 669 [b]
	DAA	0.46 ± 0.16	0.43 ± 0.09	96 ± 35	259 ± 60 [c]
	DGA	0.02 ± 0.01	0.06 ± 0.04	5 ± 2	33 ± 22
Adrenals	AA	45.63 ± 7.58	43.87 ± 5.27	209 ± 49	154 ± 24
	DAA	1.33 ± 0.37	2.47 ± 0.75	7 ± 2	8 ± 2
	DGA	0.64 ± 0.12	0.02 ± 0.01 [a]	2 ± 0.3	0.1 ± 0.03 [a]
		μg/12 hr sample			
Urine	AA	31.5 ± 3.6	19.8 ± 2.2 [b]		
	DAA	3.7 ± 0.7	1.2 ± 0.3 [b]		
	DGA	1.7 ± 0.4	0.6 ± 0.2 [c]		

[1] Mean ± SEM, 16 guinea pigs per group; [a] difference from controls significant at $P < 0.001$ level; [b] difference significant at $P < 0.01$ level; [c] difference significant at $P < 0.05$ level.

TABLE 5

Expired $^{14}CO_2$ and ^{14}C activity in urine of control and cholesterol-fed guinea pigs after injection of L-ascorbic acid-1-^{14}C

Interval after injection	Expired $^{14}CO_2$		^{14}C in urine	
	Control	Cholesterol-fed	Control	Cholesterol-fed
hr	*% of dose*		*% of dose*	
0–12	10.18 ± 0.89 [1]	10.49 ± 0.63	4.30 ± 0.68	3.85 ± 0.30
12–24	6.08 ± 0.52	6.12 ± 0.27	2.19 ± 0.19	1.59 ± 0.13 [a]
24–36	4.84 ± 0.33	5.19 ± 0.20	1.77 ± 0.18	1.13 ± 0.12 [a]
36–48	4.61 ± 0.38	4.87 ± 0.39	1.77 ± 0.25	0.88 ± 0.16 [b]
48–60	2.94 ± 0.17 [2]	3.74 ± 0.24 [a]	1.45 ± 0.15	0.88 ± 0.10 [a]
60–72	3.35 ± 0.16	3.79 ± 0.23	0.83 ± 0.12	0.82 ± 0.08
72–84	2.95 ± 0.50	2.95 ± 0.39	0.95 ± 0.06	0.73 ± 0.08 [a]
84–96	3.30 ± 0.22	3.56 ± 0.41	0.66 ± 0.24	0.75 ± 0.10

[1] Mean ± SEM, 14 guinea pigs per group (intervals 0–48 hr).
[2] Control group eight guinea pigs, cholesterol-fed group seven guinea pigs (intervals 48–96 hr.).
[a] Difference from controls significant at $P < 0.05$ level. [b] Difference from controls significant at $P < 0.01$ level.

decarboxylation in the liver is evidently very stable, because even severe interventions into the organism of guinea pigs, e.g., wounding (20, 21) and toxin stress (18, 19), also failed to affect ascorbic acid oxidation to CO_2 to a distinct degree.

The data listed in table 5 permit calculation of the percentage of ^{14}C present in the body of the animals at different time intervals after ascorbic acid-1-^{14}C administration, as fecal waste in this case is negligible (14). Retention of ^{14}C activity, calculated as the difference between that administered as ascorbic acid-1-^{14}C and the total excreted (respiratory plus urinary), is plotted on a logarithmic scale against time in figure 1. The descending pattern of the curves shows the biological half-life of ascorbic acid in guinea pigs with experimental atheromatosis to be moderately prolonged.

In accordance with the decrease of urinary ^{14}C excretion in cholesterol-fed guinea pigs, its retention in total liver and spleen was significantly increased at 48 and 96 hours after administration of ascorbic acid-1-^{14}C. This finding is related to a considerable increase in the weight of these organs in animals receiving an atherogenic diet. Several authors (14, 16, 18, 22) have shown that following ascorbic or dehydroascorbic acid-1-^{14}C administration to guinea pigs, no more than 5%, and probably less, is present in the tissues as radioactivity other

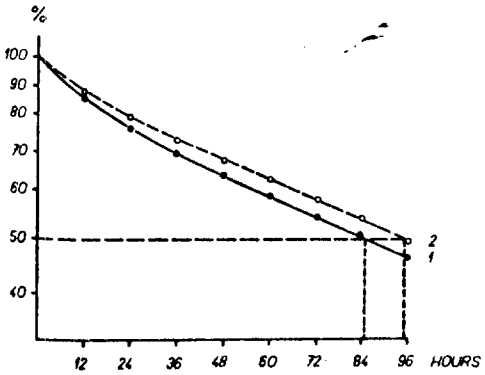


Fig. 1 ^{14}C radioactivity retained by guinea pigs as percentage of the administered dose of ascorbic acid-1-^{14}C: 1, control group (mean of eight animals); 2, cholesterol-fed group (mean of seven animals). The difference of ascorbic acid half-lives is statistically significant ($P < 0.05$).

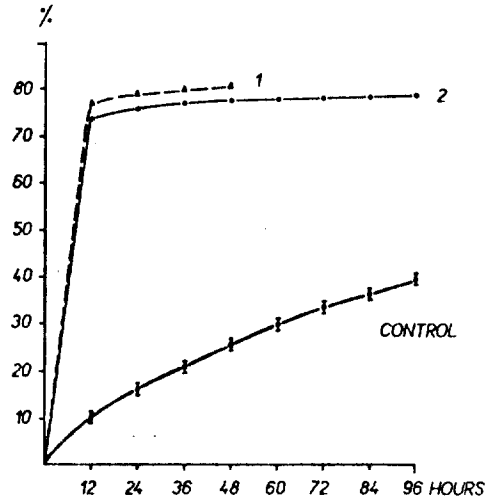


Fig. 2 ^{14}C in respiratory CO_2 as percentage of intraperitoneally injected ascorbic acid-1-^{14}C activity: control, means of eight normal guinea pigs. Vertical bars represent ± SEM; 1 and 2, two cholesterol-fed guinea pigs with abnormal ascorbic acid-1-^{14}C catabolism.

than ascorbic acid radioactivity. Since the amount of ^{14}C in the guinea pig tissues is equivalent to the amount of ascorbic acid remaining, it can be used to evaluate the specific activity of tissue ascorbic acid. Specific ascorbic acid activity was calculated as the ratio of ^{14}C quantity in tissue to ascorbic acid concentration as determined chemically (table 3). Table 6 presents values of specific activity expressed in this form for five tissues, at 48 and 96 hours after ascorbic acid-1-^{14}C administration. Data for the 48-hour interval show that the values for specific activities in all the five organs followed a very similar pattern, which suggests a homogenous ascorbic acid-1-^{14}C distribution through the body pool of ascorbic acid. Differences between the control and the cholesterol-fed groups are very small. At the 96-hour interval the specific activity of ascorbic acid in the majority of animals fed an atherogenic diet is significantly lower than in the controls.

A comparison of the specific activities of ascorbic acid found 48 and 96 hours after administration of ascorbic acid-1-^{14}C showed that this activity was decreased at 96 hours to approximately half of its 48-hour value in all these organs, i.e., the body

pool of ascorbic acid at this time was diluted by a factor of two. During this time guinea pigs received orally 2 × 10 mg of ascorbic acid. The total amount of ascorbic acid in the body of guinea pigs weighing about 600 g was only slightly over 20 mg (cf. body pool data in table 7), which agrees well with the presumed twofold dilution of the body pool of ascorbic acid.

These data enable the basic parameters of ascorbic acid metabolism to be calculated. Figure 1 indicates that the logarithm of the percentage of ^{14}C remaining in a body is a linear function of time starting 36 hours after labeled ascorbic acid injection. This type of curve is invariably obtained after injection of ascorbic acid-1-^{14}C into guinea pigs (19). For each animal killed 96 hours after labeled ascorbic acid was administered, an equation was calculated that expresses the dependence of the logarithm of activity remaining in the body on the time for the interval 36 to 96 hours. These equations were used for calculations of the value of biological half-life for each animal (i.e., time from injection of ascorbic acid-1-^{14}C at which 50% of ^{14}C has been excreted, table 7). The values found are about 4 days, which is in a good agreement with the results by other authors (15, 18–20, 24). Biological half-life of ascorbic acid in cholesterol-fed guinea pigs is moderately, though significantly, prolonged. As a result, the turnover time, too, is prolonged: half-life $\times \frac{1}{0.693}$.

Body pool of ascorbic acid was calculated from the following relation:

$$\text{Body pool} = \frac{\text{dpm remaining in the body}}{\text{specific activity of ascorbic acid}}$$

The specific activity of ascorbic acid was calculated using the mean values of five organs (table 6). In the controls the value of body pool was about 35 mg per kilogram, in the cholesterol-fed group it was significantly higher. The values obtained agree well with those reported by other authors (18, 24). The slight differences in the pool values may be explained by the fact that the ascorbic acid pool depends on the nutrition history of the guinea pigs. When a body pool is expressed in terms of body weight, the value of 21.25 ± 0.83 mg

TABLE 6

Specific activity of ascorbic acid in the tissues of control and cholesterol-fed guinea pigs 48 and 96 hours after injection of L-ascorbic acid-1-^{14}C

Tissue	48 Hours after injection		96 Hours after injection	
	Control [1]	Cholesterol-fed [2]	Control [1]	Cholesterol-fed [2]
	dpm/μg ascorbic acid			
Liver	1023 ± 115	953 ± 61	579 ± 37	408 ± 32 [b]
Spleen	915 ± 97	939 ± 90	575 ± 39	383 ± 20 [b]
Kidneys	1005 ± 145	1005 ± 97	560 ± 36	508 ± 29
Adrenals	972 ± 53	1042 ± 97	740 ± 61	483 ± 30 [b]
Small intestine	953 ± 87	947 ± 14	577 ± 45	449 ± 27 [a]
	dpm/100 mg tissue			
Thoracic aorta	2166 ± 369	2340 ± 340	1267 ± 167	1259 ± 245

[1] Mean ± SEM, eight guinea pigs per group.
[2] Mean ± SEM, seven guinea pigs per group. [a] difference from controls significant at $P < 0.05$ level; [b] difference from controls significant at $P < 0.001$ level.

TABLE 7

Biological half-life, body pool, turnover time and turnover rate of L-ascorbic acid in control and cholesterol-fed guinea pigs

Parameter	Control group [1]	Cholesterol-fed group [2]	Statistical significance
Half-life, hr	85 ± 2.7	95 ± 3.8	$P < 0.05$
Body pool, mg/kg	35.5 ± 1.4	43.4 ± 0.9	$P < 0.001$
Turnover time, days	5.1 ± 0.2	5.7 ± 0.2	$P < 0.05$
Turnover rate, mg/kg/day	7.0 ± 0.2	7.7 ± 0.4	—

[1] Mean ± SEM, eight guinea pigs per group.
[2] Mean ± SEM, seven guinea pigs per group.

of ascorbic acid per body is obtained for the controls, and 24.52 ± 1.12 per body for the cholesterol-fed animals. This significant difference (approximately 3.3 mg) is in good quantitative agreement with the increase of the total amount of ascorbic acid in the liver and spleen of cholesterol-fed guinea pigs, as obtained by chemical analysis (table 3).

The turnover rate of ascorbic acid is given by the ratio body pool/turnover time. Its value in normal guinea pigs amounts to about 7 mg per kilogram per 24 hours (table 7). The mean value of turnover rate was somewhat higher in cholesterol-fed guinea pigs, but the difference is not significant. Hellman and Burns (23) report a turnover rate of 9.3 mg per kilogram per 24 hours, but this is valid for guinea pigs with a higher body pool of ascorbic acid.

The results obtained show that in guinea pigs with experimental cholesterol atheromatosis increased ascorbic acid retention exists. A significant increase in the body pool of ascorbic acid occurs in cholesterol-fed guinea pigs. The principal reason for this phenomenon is a significant increase in liver weight, related to a substantial rise in the ascorbic acid content in this organ which amounts to approximately three times the normal value. Guinea pigs fed a cholesterol diet tend to maintain ascorbic acid levels of the liver within a normal range despite the fact that liver weight increases enormously. This is supported by enhanced ascorbic acid retention manifesting itself by a significantly lower excretion of ascorbic acid and its metabolites in urine. A prolongation of the biological half-life of ascorbic acid-1-^{14}C in cholesterol-fed animals is conditioned by this factor (i.e., lower urinary excretion of ^{14}C). Ascorbic acid-1-^{14}C oxidation to $^{14}CO_2$ is not, except two cases, affected by experimental atheromatosis.

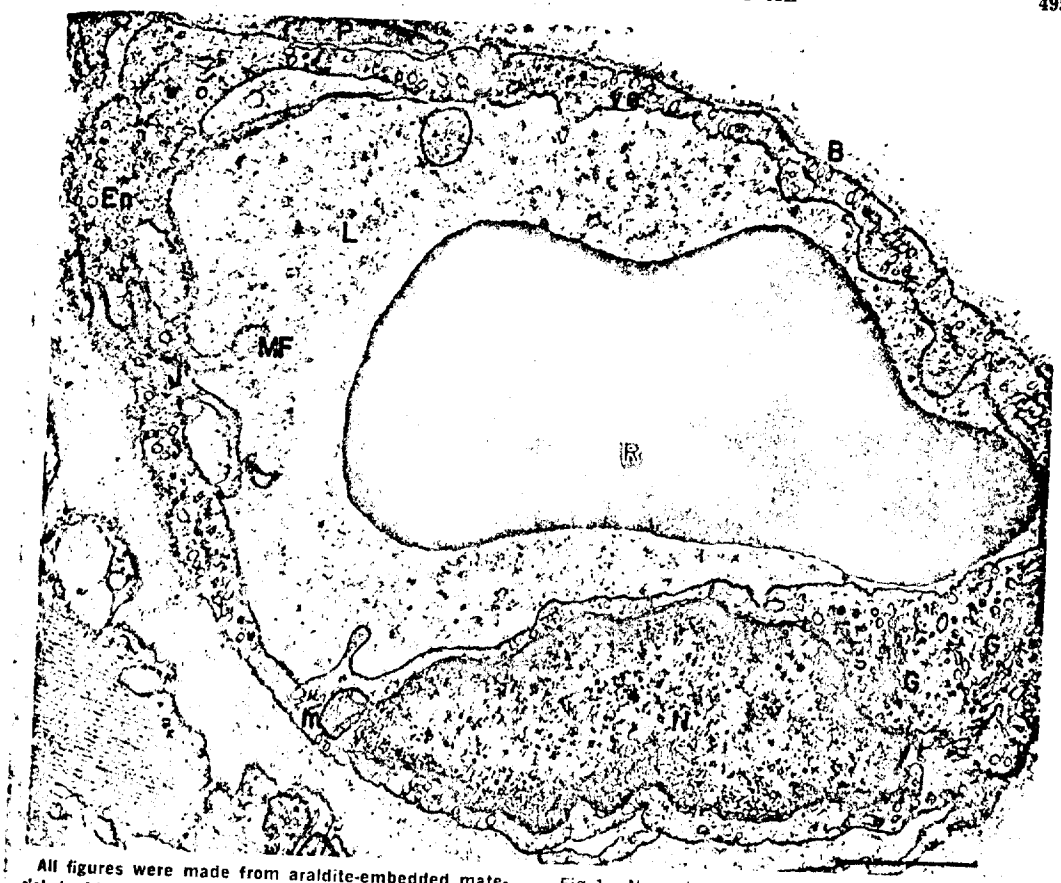
The dose of exogenous ascorbic acid employed in this experiment (10 mg per animal per 24 hours) exceeded the value of the turnover rate and enabled the ascorbic acid level in the liver of cholesterol-fed guinea pigs to remain within a normal range despite the fact that the weight of this organ increased nearly threefold as a result of an atherogenic diet. Our previous studies (12) have shown that a lowered supply of exogenous ascorbic acid resulted in a decreased vitamin C level in the liver of cholesterol-fed guinea pigs, and even in symptoms of a manifest deficiency (hemorrhages in joints of the extremities). The modified ascorbic acid metabolism in cholesterol-fed guinea pigs is primarily due to the effects of the atherogenic cholesterol diet on their liver and not their vascular system. An altered ascorbic acid metabolism was also observed in our short-term experiments (12), although no morphological changes were yet occurring in vessels. Ascorbic acid-1-^{14}C deposition in the thoracic aorta was not altered in this experiment even in guinea pigs with a markedly developed atheromatosis (table 6). Whether the described changes in ascorbic acid-1-^{14}C metabolism in cholesterol-fed guinea pigs are brought about by nonspecific hepatotoxic action of cholesterol, or whether they are related to a definite specific function of ascorbic acid in cholesterol metabolism, remains an open question.

Anomalous catabolism of ascorbic acid was observed in two cholesterol-fed guinea pigs. A very high fraction of the dose (over 70%) was converted to respiratory $^{14}CO_2$ during the first 12 hours after injection of ascorbic acid-1-^{14}C (fig. 2). Excretion of ^{14}C in urine and its retention by tissues in these two animals was but a fraction of the values found in the controls and the other cholesterol-fed animals. The concentration of ascorbic acid, its oxidation products and cholesterol in tissues determined chemically proved not to be different from those in the other cholesterol-fed guinea pigs. This anomaly is not attributable to impurities in ascorbic acid-1-^{14}C, for purity of a sample has been checked by paper radiochromatography and no such anomaly was observed in other animals which were given an identical solution of labeled ascorbic acid practically at the same time. A similar anomaly in ascorbic acid 1-^{14}C catabolism was observed by other authors as well, particularly in scorbutic guinea pigs (19, 20). Salomon (19) reports that "it occurs more frequently in animals that may be thought to be less resistant to physiological stress." There is every probability that an atherogenic cholesterol diet may produce such a situation in guinea pigs. In cholesterol-fed rats, which are far more re-

sistant to the effects of a high cholesterol diet than guinea pigs, we have observed a lower resistance towards heat stress (24).

LITERATURE CITED

1. Ginter, E., P. Bobek, Z. Kopec, M. Ovečka and K. Čerey 1967 Metabolic disorders in guinea pigs with chronic vitamin C hyposaturation. Z. Versuchstierk. *9:* 228.
2. Ginter, E., P. Bobek, J. Babala and E. Barbieriková 1969 The effect of ascorbic acid on the lipid metabolism of guinea-pigs fed an atherogenic diet. Cor Vasa *11:* 65.
3. Ginter, E., J. Babala and J. Červeň 1969 The effect of chronic hypovitaminosis C on the metabolism of cholesterol and atherogenesis in guinea pigs. J. Atheroscler. Res. *10:* 341.
4. Guchhait, R., B. C. Guha and N. C. Ganguli 1963 Metabolic studies on scorbutic guinea pigs. III. Catabolism of (4-^{14}C) cholesterol in vivo and in vitro. Biochem. J. *86:* 193.
5. Ginter, E., and J. Červeň 1969 Metabolism of cholesterol-4-^{14}C in guinea pigs with chronic vitamin C hyposaturation. Abstracts of papers, 8th International Congress of Nutrition. Prague, G 10.
6. Ginter, E., R. Ondreička, P. Bobek and V. Šimko 1969 The influence of chronic vitamin C deficiency on fatty acid composition of blood serum, liver triglycerides and cholesterol esters in guinea pigs. J. Nutr. *99:* 261.
7. Babala, J., and E. Ginter 1968 Coronary changes experimentally produced in guinea-pigs by hypercholesterolemia. Nutr. Dieta *10:* 133.
8. Richterich, R. 1965 Klinische Chemie. Theorie und Praxis. S. Karger, Basel (Switzerland), New York.
9. Roe, J. H., and C. A. Kuether 1943 Determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenyl-hydrazine derivate of dehydroascorbic acid. J. Biol. Chem. *147:* 399.
10. Zloch, Z., and E. Ginter 1970 Thin-layer chromatography determination of L-ascorbic, L-dehydroascorbic and 2,3-diketo-L-gulonic acids in animal tissues, blood and urine. Z. Klin. Chem. Klin. Biochem. *8:* 302.
11. Saba, T. M., and N. R. Di Luzio 1966 Method for collection and determination of ^{14}CO for in vitro metabolic studies. J. Lipid Res. *7:* 566.
12. Ginter, E. 1970 Effect of dietary cholesterol on vitamin C metabolism in laboratory animals. Acta Med. Acad. Sci. Hung. *27:* 23.
13. Sokoloff, B., M. Hori, C. C. Saelhof, T. Wrzolek and T. Imai 1966 Aging, atherosclerosis and ascorbic acid metabolism. J. Amer. Geriat. Soc. *14:* 1239.
14. Burns, J. J., H. B. Burch and C. G. King 1951 The metabolism of 1-^{14}C-L-ascorbic acid in guinea pigs. J. Biol. Chem. *191:* 501.
15. Burns, J. J., P. G. Dayton and S. Schulenberg 1956 Further observations on the metabolism of L-ascorbic acid in guinea pigs. J. Biol. Chem. *218:* 15.
16. Dayton, P. G., F. Eisenberg, Jr. and J. J. Burns 1959 Metabolism of ^{14}C-labelled ascorbic, dehydroascorbic and diketogulonic acids in guinea pigs. Arch. Biochem. Biophys. *8:* 111.
17. Banay, M., and E. Dimant 1962 On the metabolism of L-ascorbic acid in the scorbutic guinea-pig. Biochim. Biophys. Acta *59:* 313.
18. Salomon, L. L. 1957 Ascorbic acid catabolism in guinea pigs. J. Biol. Chem. *228:* 163.
19. Salomon, L. L. 1962 Catabolism of L-ascorbic acid in guinea pigs. J. Nutr. *76:* 493.
20. von Schuching, S., T. Enns and A. F. Abt 1960 Connective tissue studies. IV. L-Ascorbic-1-^{14}C acid excretion in intact and wounded guinea pigs on varying vitamin C intakes. Amer. J. Physiol. *199:* 423.
21. Abt, A. F., and S. von Schuching 1961 Catabolism of L-ascorbic-1-^{14}C acid as a measure of its utilization in the intact and wounded guinea pig on scorbutic, maintenance, and saturation diets. Ann. N.Y. Acad. Sci. *92:* 148.
22. Hammarström, L. 1966 Autoradiographic studies on the distribution of ^{14}C-labelled ascorbic acid and dehydroascorbic acid. Acta Physiol. Scand. *70:* Suppl. 289, 1-84.
23. Hellman, L., and J. J. Burns 1958 Metabolism of L-ascorbic acid-1-^{14}C in man. J. Biol. Chem. *230:* 923.
24. Bobek, P., and E. Ginter 1966 Metabolism of lipids in rats exposed to heat under conditions of a normal and a high fat-high cholesterol diet. J. Nutr. *89:* 373.



All figures were made from araldite-embedded material doubly stained with uranium and lead. The linear marker at one side of the lower margin, indicates the length of 1μ at the magnification of the electron micrograph. Key to abbreviations: R indicates red blood cell; N, nucleus; G, Golgi complex; M, mitochondrion; J, intercellular junction; MF, marginal fold; En, endothelial cell; Er, endoplasmic reticulum; Ve, vesicle; B, basement membrane; L, lumen; Th, thrombocyte; Gap, junctional separation; Se, swollen endothelial cell process; Cs, cell defect; and H, histiocyte.

Fig 1.—Normal myocardial blood capillary consisting of a lining endothelium and an outer basement membrane. The endothelium is extremely thin except for the portion containing the nucleus, the Golgi complex, and most of the mitochondria. Pinocytotic vesicles are abundant in the cytoplasm. Note the narrow, frequently sinuous cell junctions and the marginal folds which extend from some (slightly enlarged from × 31,000).

digitated, separated by a narrow intercellular space measuring about 100 A. Not infrequently, a marginal flap or fold extends some distance from the edge of one cell into the lumen or along the surface of its neighbor. More or less well developed desmosomes may be seen towards the luminal aspect of some of the junctions.

In scorbutic guinea pigs, two types of sharply focal lesions were seen associated with capillary hemorrhage. These, more frequent, and severe after three and five weeks of deficiency than after two, consisted of junctional separation (Fig 2-6) and cellular disruption (Fig 7 and 8). Numerically, the former was seen three to four times as often as the latter, but both resulted in discontinuities which exceeded the normal junctional space by a 100-fold or more.

The intercellular gap in junctional separation was associated with attenuation or even disappearance of the basement membrane underlying the adjacent endothelial cells; similarly, there was depletion of the underlying collagen. Erythrocytes and/or tracer particles were sometimes

Table 1 - Urinary sugar excretion prior, during and after daily addition of 1500 mg ascorbic acid, in mg glucose per 100 ml.

Test subject	Period prior to administration $\bar{X} \pm S_{\bar{X}}$	Period of ascorbic acid administration $\bar{X} \pm S_{\bar{X}}$	Period after administration $\bar{X} \pm S_{\bar{X}}$
1	2.21 ± 1.36	6.83 ± 3.16	4.21 ± 2.64
2	5.09 ± 1.07	2.08 ± 0.87	2.36 ± 0.74
3	4.60 ± 0.74	3.77 ± 1.13	2.60 ± 1.04
4	3.61 ± 1.17	3.16 ± 1.18	4.15 ± 0.89
5	3.42 ± 1.27	4.44 ± 1.33	5.73 ± 1.26
6	3.54 ± 1.98	6.42 ± 1.51	2.99 ± 1.11

In summary, the following could thus be established: the renal glucose excretion of a healthy person is not affected by the daily addition of 1500 mg ascorbic acid to the customary diet.

Bibliography

1. Glatzel, H., M. Ruberg-Schweer: Med. Klin. 61 (1966), 1249.

2. Renschler, H.E., H. Weicker, H. von Baeyer: Dtsch. med. Wschr. 90 (1965), 2349.

ZUM THEMA

Med Klin 61: 1879, 25 Nov 66

„Zur Frage der Glukosurie nach hohen Askorbinsäuredosen"

Von H. Glatzel und M. Rüberg-Schweer

[H. Glatzel und M. Rüberg-Schweer: Med. Klin. 61 (1966), 1249]

In unserem Beitrag „Zur Frage der Glukosurie nach hohen Askorbinsäuredosen" [1] haben wir vor kurzem über Untersuchungen berichtet, bei denen sich ergab, daß nach Zugabe von täglich 1500 mg Askorbinsäure zu Normalkost die Glukoseausscheidung im Urin nicht, wie von tschechischen Autoren angegeben, zunimmt, sondern (gegenüber der Vor- und Nachperiode ohne Vitamin-C-Zulage) sogar abnimmt. Die Glukosebestimmung im Urin war mit Glukoseoxydase (Farbtest von Boehringer) durchgeführt worden.

Da diese Methode bei so geringen Zuckermengen nicht ganz zuverlässig zu sein schien, haben wir jetzt bei 6 weiteren Versuchspersonen die Zuckerkonzentration im Urin jeweils an 7 aufeinanderfolgenden Tagen vor, während und nach Zugabe von 1500 mg Askorbinsäure mit dem empfindlicheren *UV-Test* bestimmt (Boehringer Mannheim, enzymatische Bestimmung der D-Glukose mit Hexokinase/-Glukose-6-Phosphat-Dehydrogenase, UV-Test mit TPN). Zugesetzte Glukose (10 mg je 100 ml) konnte mit dieser Methode in wiederholten Ansätzen zu 80 bis 90 % wiedergefunden werden. Störend scheint sich die Gegenwart von reichlich Uratkristallen im Urin auszuwirken. Jedenfalls fanden sich in uratreichen trüben Urinen auffallend kleine Glukosemengen.

Die Glukosekonzentration des Urins lag in dieser Versuchsreihe zwischen 2 und 7 mg je 100 ml (*Tabelle 1*). *Renschler et al.* [2] fanden am häufigsten 8 bis 9 mg je 100 ml. Die Unterschiede zwischen Vorperiode und Belastungsperiode bzw. zwischen Belastungsperiode und Nachperiode sind mit *einer* Ausnahme (Versuchsperson 2, Vorperiode/Belastungsperiode, $p < 0{,}05$) statistisch nicht signifikant.

Bei 3 Versuchspersonen haben wir an 13 Versuchstagen auch die Glukosegesamtausscheidung je Tag bestimmt. Bei 2 von ihnen (bei 1 signifikant) war die Glukoseausscheidung in 24 Stunden während der Vitaminbelastungsperiode kleiner, bei der 3. Versuchsperson war sie größer als während der Nachperiode.

Zusammenfassend läßt sich somit feststellen: *Die renale Glukoseausscheidung des Gesunden wird durch Zugabe von täglich 1500 mg Askorbinsäure zu landesüblicher Kost nicht beeinflußt.*

Versuchsperson	a) Vorperiode $\bar{x} \pm s_{\bar{x}}$	b) Askorbinsäureperiode $\bar{x} \pm s_{\bar{x}}$	c) Nachperiode $\bar{x} \pm s_{\bar{x}}$
1	2,21 ± 1,36	6,83 ± 3,16	4,21 ± 2,64
2	5,09 ± 1,07	2,08 ± 0,87	2,36 ± 0,74
3	4,60 ± 0,74	3,77 ± 1,13	2,60 ± 1,04
4	3,61 ± 1,17	3,16 ± 1,18	4,15 ± 0,89
5	3,42 ± 1,27	4,44 ± 1,33	5,73 ± 1,26
6	3,54 ± 1,98	6,42 ± 1,51	2,99 ± 1,11

Tabelle 1. Harnzuckerausscheidung vor, während und nach Zulage von täglich 1500 mg Askorbinsäure (mg Glukose/100 ml).

Literatur

1. *Glatzel, H., M. Rüberg-Schweer:* Med. Klin. 61 (1966), 1249.
2. *Renschler, H. E., H. Weicker, H. von Baeyer:* Dtsch. med. Wschr. 90 (1965), 2349.

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ORAL SURGERY 28: 480-3, 1969

Periodontics

The effects of ascorbic acid supplements in guinea pigs administered thyroid extract

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In a previous study[1] it was found that thyroid extract had a very profound effect in guinea pigs maintained on a normal diet. It was suggested at that time that the dental and periodontal effects bore a striking resemblance to those observed in ascorbic acid deficiency.

Utilizing the histologic tooth structures of the incisor of the guinea pig as a bioassay of the ascorbic acid nutritional state in addition to other criteria, such as blood alkaline phosphatase and collagen formation (to be published), we carried out studies to test the hypothesis that hyperthyroidism, effected by the feeding of thyroid extract, increases the utilization or demand for ascorbic acid and to determine whether hyperthyroidism influences the commonly recognized effects of ascorbic acid.

MATERIALS AND METHODS

Guinea pigs, weighing 250 to 300 grams each, were divided into groups, as follows:

1. Control animals fed the basal diet supplemented with ascorbic acid. Twenty-four animals were divided into eight groups of three and were fed 0.0, 0.25, 0.5, 0.75, 2.5, 5.0, 10.0, and 20 mg. ascorbic acid daily.
2. A second set of twenty-four animals was similarly divided and similarly treated, except that, in addition, each animal received 15 mg. thyroid extract daily.
3. Six animals were fed the basal diet, supplemented only by thyroid extract.

The scorbutigenic (basal) diet was composed of oats, 40 per cent; wheat

bran, 15.0 per cent; alfalfa leaf, 8.0 per cent; whole milk, 20.0 per cent; casein purified, 10.0 per cent; corn oil, 5.0 per cent; sodium chloride (reagent), 0.5 per cent; calcium carbonate, 1.0 per cent; and magnesium sulfate, 0.5 per cent.

A bioassay method of evaluating ascorbic acid was used. In the incisor teeth of guinea pigs maintained on a normal diet, dentine is continually deposited at the formative end to compensate for the continual wear of the tooth at the incisal edge. At the formative end, the circularly arranged tall columnar cells forming the odontoblastic layer become differentiated from a mass of connective tissue of the embryonic type. A matrix of collagenous material appears between the basal surfaces of the odontoblasts and the epithelial cells of the enamel organ with which they were previously in contact. The odontoblasts move incisally at approximately the rate of tooth eruption. At the same time, they retreat toward the center of the pulp, depositing additional dentine matrix behind them. This matrix becomes calcified a short distance from the ends of the odontoblasts, separated by an intervening layer of uncalcified predentine. Each odontoblast leaves in dentine a cell process prior to calcification. The odontoblasts become more and more crowded as they migrate incisally and centrally and gradually degenerate, forming dentine of an inferior quality.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Finally, near the incisal end, the odontoblasts become incorporated in the calcified pulp tissue which seals off the pulp chamber. The complete life cycle of the odontoblast, from its first appearance at the formative end of the tooth to its degeneration at the incisal end, normally consumes a period of 5 weeks.

Several methods of evaluating the state of ascorbic acid deficiency in guinea pigs, utilizing the incisor as a biologic indicator, have evolved. The one used in this study was one we previously reported,[2] which utilized a cross section of the mandibular incisor cut at the notch of the mandible. The height of the odontoblast used and the type and degree of dentine production are the key indicators. Sections were stained with hematoxylin and eosin as well as with a special connective tissue stain utilized in this laboratory.

FINDINGS

Pulpal changes in the incisor teeth of guinea pigs placed on a scorbutigenic diet and also in those supplemented with ascorbic acid were found to be similar to changes previously described. In animals on a scorbutigenic diet, dentine production ceased and the odontoblastic layer disappeared. Any remaining odontoblasts were no longer columnar in shape but, rather, small oval cells with degenerative nuclei. Large engorged blood vessels were routinely noted. In animals given supplements of 0.25 mg. of ascorbic acid, dentine production was irregular, with cells at times incorporated into the structure. As the supplement was increased, regular dentine with a regular odontoblastic layer was produced (Fig. 1). Of interest was confirmation of a previous finding (1951) that the height of the odontoblasts increased in proportion to the amount of ascorbic acid in the daily supplement.

Administration of thyroid extract to animals on a non-ascorbic acid supplemented basal diet caused changes similar to those found in ascorbic acid deficiency (Fig. 2). In those animals receiving a daily supplement of 0.5 mg. ascorbic acid the effect of thyroid was, for the most part, to produce a condition comparable to that observed in animals receiving a subminimal amount of ascorbic acid (Fig. 3). Although in some instances some spiked projections of osteodentine were produced, the entire osteoblastic layer disappeared. The tooth structure of an animal on a basal diet supplemented by thyroid extract and 5 mg. of ascorbic acid daily is shown in Fig. 4. Note that although the odontoblasts tend to be columnar, they are normally arranged.

In the animals fed thyroid along with deficient levels of ascorbic acid supplementation the changes noted, as interpreted from histologic examination, ranged from maximal changes in the odontoblasts at the zero or lower levels to complete normalcy at high levels of ascorbic acid administration. The observations suggest that the feeding of thyroid extract significantly raises the requirement for ascorbic acid. In animals on the scorbutigenic diet or the diet supplemented with low levels of ascorbic acid, the changes varied from complete scorbutus to a slight degree of spike formation of osteodentine and small, poorly formed spindle-shaped osteoblasts. As the ascorbic acid supplement increased, dentine production and the osteoblastic layer became regular and their height increased.

Unpublished studies reveal that the critical supplement of ascorbic acid required for phosphatase production by guinea pig osteoblasts is between 0.225 and 0.25 mg. daily and rises to 0.35 mg. daily in response to thyroid feeding. It has also been noted that, with minimum levels of ascorbic acid, the thyroid-supplemented animals can produce only about 50 per cent of the amount of collagen produced by animals fed the unsupplemented diet.

DISCUSSION AND SUMMARY

From the foregoing findings, it is concluded that increased thyroid feeding to guinea pigs increases the daily ascorbic acid requirements. It may be possible to state that it raises the tissue utilization of ascorbic acid as it relates to pulp tissue. This may be true of other tissues of the body. It is also concluded that the use of supplements of ascorbic acid will satisfy this increased need of the odontoblastic layer of the pulp during the hyperthyroid state. Thus, the effects of thyroid as seen in the pulp of the guinea pig incisor are negated by increasing the daily intake of ascorbic acid.

REFERENCES

1. Goldman, H. M.: Experimental Hyperthyroidism in Guinea Pigs, Am. J. Orthodontics & Oral Surg. 29: 665, 1943.
2. Goldman, H. M., and Gould, B. S.: Histologic Bioassay of 3-Methyl-1-Ascorbic Acid, J. Nutrition 43: 193, 1951.

Arch. Intern. Med. 63(3): 531-546 1939 3049

ASCORBIC ACID IN BLOOD AND URINE AFTER ORAL ADMINISTRATION OF A TEST DOSE OF VITAMIN C

A SATURATION TEST

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Numerous methods have been proposed for the determination of vitamin C deficiency and for the estimation of the degree of saturation of the bodily tissues with vitamin C. The majority of these methods involve measuring the amount of ascorbic acid excreted in the urine in twenty-four hours (1) while the subject is taking his usual diet and (2) after the administration of a test dose of vitamin C. These procedures necessitate the complete cooperation of the patient in order to avoid loss of part of the specimen, and there is difficulty in preserving the urine against oxidation, with resultant loss of vitamin C. For these reasons the methods are not applicable to ambulatory patients. The best single test of vitamin C nutrition is considered by many to be the determination of the amount of reduced ascorbic acid in the blood during fasting. It gives an indication of the degree of tissue saturation but not a complete one, as there may be fluctuations with daily variation in the quantity of vitamin C in the diet. The aim of this study was to find a method of determining the state of vitamin C nutrition which would be applicable to ambulatory patients, which would require only a few hours to perform and which would avoid some of the difficulties that have been enumerated.

We studied 22 persons (25 tests being performed): 3 normal adults, 6 persons with psychoneuroses, 8 with chronic diseases which were not etiologically related to vitamin deficiency, 2 with pellagra and 3 with evidence of multiple vitamin deficiency. A careful history in regard to diet was elicited from each person, attention being especially directed to the intake of vitamin C. In 3 cases in which a marked deficiency of ascorbic acid was found at the original examination, a second test was carried out subsequent to a period of treatment with

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vitamin C. No patient with fever or an acute infectious process was included in this study.

METHOD

A specimen of blood and one of urine were collected from the patient in the fasting state. Afterward 600 mg. of ascorbic acid [1] was administered by mouth. Specimens of blood and urine were obtained at the end of one, three and six hours. Each specimen of urine included the entire amount voided after the time of the previous collection. Similarly, all urine passed from the sixth to the twelfth and from the twelfth to the twenty-fourth hour after the test dose was saved for examination. In a few instances a twenty-four hour specimen was collected the day prior to the administration of the test dose of ascorbic acid for comparison of findings. The urine was kept in dark, tightly stoppered bottles on ice and was acidified with glacial acetic acid (approximately 10 per cent by volume), these procedures having been shown to retard the oxidation of vitamin C. Day specimens were analyzed at once in most instances, always within two hours. One cubic centimeter of acidified urine, diluted with 10 cc. of distilled water, was titrated against a solution of 2,6-dichlorophenolindophenol of such a concentration that 1 cc. of the dye was equivalent to 0.02 mg. of ascorbic acid. The quantity of urine in each of the specimens was measured, and the amount of ascorbic acid present was calculated. When the urine was highly colored, it was often necessary to dilute it with more than 10 cc. of distilled water in order to obtain a definite end point. When a large amount of vitamin C was present in the specimen, dilution was first made, after which 1 cc. of the diluted specimen was used in titration with the dye.

As a result of special observations we found that there was only a slight diminution in the amount of vitamin C in the urine if analysis was delayed for one or two hours when it had been collected as described, but after twenty-four hours the decrease at times amounted to 20 per cent or more. Similar findings have been reported by Abbasy, Harris, Ray and Marrack [2] and by Wright.[3]

The amount of reduced ascorbic acid in the blood plasma was determined by the method of Farmer and Abt.[4] Ten cubic centimeters of blood was collected in a tube containing oxalate and centrifuged immediately. Two cubic centimeters of plasma was transferred to another tube, to which was added 4 cc. of distilled water and 4 cc. of a 5 per cent solution of metaphosphoric acid. After further centrifugation, 2 cc. of the supernatant fluid was titrated against a solution of 2,6-dichlorophenolindophenol of such a concentration that 1 cc. of the dye was equivalent to 0.01 mg. of ascorbic acid. The solutions of the dye (2,6-dichlorophenolindophenol) were prepared from tablets, each of which was equal to 1 mg. of ascorbic acid. These were checked against freshly prepared solutions of ascorbic acid of known concentration and found to be accurate within the limits of experimental error of this method.

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1. The ascorbic acid used was supplied by Hoffmann-LaRoche, Inc., Nutley, N. J.

2. Abbasy, M. A.; Harris, L. J.; Ray, S. N., and Marrack, J. R.: Diagnosis of Vitamin C Subnutrition by Urinalysis, Lancet **2**:1399, 1935.

3. Wright, I.: The Present Status of the Clinical Use of Cevitamic Acid, Am. J. M. Sc. **192**:719, 1936.

4. Farmer, C. J., and Abt, A. F.: Determination of Reduced Ascorbic Acid in Small Amounts of Blood, Proc. Soc. Exper. Biol. & Med. **34**:146, 1936.

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A microburet reading to 0.01 cc. was used in all titrations, the dye being titrated into the fluid being tested. The latter was placed in a white evaporating dish and a blue light used to facilitate determination of the end point, which was considered to be the first pink color lasting thirty seconds. All titrations were completed within two minutes. A distilled water blank value was determined, this value being subtracted from the final buret reading. Each titration was done in duplicate by each of two observers, and the average of these values was used in calculating the content of ascorbic acid in the specimen.

TABLE 1.—*Vitamin C Content of the Blood and Urine*

Test No.	Case No.	Diagnosis	Dietary History of Vitamin C	Control, 24 Hour Output in Urine, Mg.	Blood (Fasting), Mg. per 100 Cc.	Vitamin C Content After Administration of 600 Mg. of Ascorbic Acid: Blood, Highest Concentration, Mg. per 100 Cc.	6 Hour Output in Urine, Mg.	Percentage of 24 Hour Output in Urine	24 Hour Output in Urine, Mg.	Percentage of Test Dose Excreted
1	1	Hypovitaminosis.....	Very poor	.....	0.05	0.31	0.95	88	1.08	0.2
2	2	Hypovitaminosis.....	Very poor	.....	0.05	0.12	0.62	55	1.12	0.2
3	3	Hypovitaminosis.....	Very poor	.....	0.06	0.20	0.49	17	2.86	0.5
4	4	Pellagra.....	Very poor	1.820	0.09	0.50	2.60	77	3.38	0.6
5	5	Rheumatic heart disease	Very poor	.....	0.07	0.24	0.71	20	3.54	0.6
6	6	Epilepsy.....	Poor ?	.....	0.21	0.86	3.07	75	4.06	0.7
7	7	Syphilitic heart disease	Very poor	.....	0.17	0.64	0.33	7	4.45	0.7
8	8	Psychoneurosis.....	Poor	.....	0.04	0.84	2.24	40	5.61	0.9
9	9	Psychoneurosis.....	Poor	10.670	0.19	0.47	2.80	41	6.87	1.1
10	10	Psychoneurosis.....	Fair	.....	0.19	1.05	3.43	43	7.87	1.3
11	11	Normal.....	Fair ?	9.003	0.17	0.76	3.11	35	8.87	1.5
12	12	Psychoneurosis.....	Poor	6.150	0.21	1.15	3.97	41	9.55	1.6
13	13	Hookworm.....	Poor	9.210	0.52	1.71	3.46	36	9.63	1.6
14	14	Arteriosclerosis; allergy	1,050 mg. in 21 days; poor previously	.....	0.34	1.55	4.99	35	14.12	2.3
15	15	Mild pellagra.....	Fair	.....	0.74	2.72	26.93	..	....	...
16	16	Psychoneurosis.....	Fair	.....	1.22	1.74	49.50	..	....	...
17	2	Hypovitaminosis; after treatment	3,900 mg. in 13 days	46.640	0.98	2.18	50.19	70	70.84	12.0
18	17	Psychoneurosis.....	Fair	.....	0.43	2.32	70.49	85	82.70	13.7
19	18	Diabetes.....	Good	.....	0.80	2.48	81.57	74	109.53	18.0
20	19	Chronic bacillary dysentery	Very good	.....	0.82	2.99	48.59	41	116.31	19.0
21	20	Normal.....	Good	12.190	0.65	2.00	103.05	75	137.09	23.0
22	4	Pellagra; after treatment	4,500 mg. in 15 days	.....	1.61	2.48	136.05	75	179.70	30.0
23	21	Rheumatoid arthritis..	Excellent	.....	1.74	2.16	209.02	91	228.34	38.0
24	22	Normal.....	Excellent	17.800	1.38	2.97	109.73	45	240.40	40.0
25	3	Hypovitaminosis; after treatment	3,500 mg. in 9 days	.....	1.98	2.60	180.16	62	291.15	48.5

RESULTS

A number of pertinent findings made in the 25 tests which were performed are given in table 1. The results are listed in ascending order according to the amount of ascorbic acid excreted in the urine in twenty-four hours after the administration of the test dose. It may be seen that, in general, the better the dietary history in regard to vitamin C, the greater the output in the urine after the test dose. The amount of ascorbic acid excreted in six hours was calculated as well as the output in twenty-four hours, and the results were found to be

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closely parallel. This observation will be discussed in detail later. The amount of reduced ascorbic acid in the blood during fasting likewise showed a close correlation with the previous dietary intake and also with the quantity of vitamin C excreted in the urine. The concentration of ascorbic acid in the blood reached higher levels after the administration of the test dose when the amount present during fasting was high. Little information regarding saturation of the tissues with vitamin C was obtained from examining the urine for twenty-four hours prior to the giving of a test dose of the vitamin except in 2 instances, 1 patient (case 4) showing extreme deficiency and another (case 2, test 17) a normal condition.

On the basis of the following considerations, the first 14 tests have been considered as indicative of vitamin C deficiency and the last 11 tests as indicative of a fairly normal state of nutrition. Abbasy, Harris, Ray and Marrack [2] have shown that for the normal person the minimal daily excretion of vitamin C in the urine in twenty-four hours is 13 mg. and that after the administration of a test dose of 700 mg. there is an excretion of 40 to 50 mg. in the subsequent twenty-four hours. The first 14 tests, that is, those designated as indicating deficiency, fail to meet these criteria; while the last 9 tests, considered as indicating a normal condition, satisfy these requirements. Two patients (cases 15 and 16) were given only six hour tests but were placed in the group of normal patients for reasons which will be discussed later. Van Eekelen [5] has stated that when vitamin C is being administered daily in amounts varying from 250 to 400 mg., as saturation of the tissues is reached there is a definite rise in the urinary output of vitamin C (above 30 mg.). Even with a single large dose of ascorbic acid, a urinary excretion of 70 mg. or more in twenty-four hours, as shown in tests 17 through 25, seems significant of near saturation.

The last column in table 1 indicates the percentage of the test dose excreted in the twenty-four hours following its administration. Hawley and Stephens [6] found that saturated subjects excreted 40 to 100 per cent of an oral test dose of 100 to 200 mg. in this period. Wright [3] stated that about 30 per cent of a test dose of 500 to 1,000 mg. should be excreted in twenty-four hours and that an output of less than 20 per cent indicates suboptimal storage of vitamin C. Youmans [7] concluded that excretion of 30 per cent or more of a test dose of 600 mg. is the lower limit of saturation. Using these criteria, the last 5, and

5. Van Eekelen, M.: On the Amount of Ascorbic Acid in Blood and Urine: Daily Human Requirements for Ascorbic Acid, Biochem. J. **30**:2291, 1936.

6. Hawley, E. E., and Stephens, D. J.: Rate of Urinary Excretion of Test Doses of Ascorbic Acid, Proc. Soc. Exper. Biol. & Med. **34**:854, 1936.

7. Youmans, J. B.; Corlette, M. B.; Akeroyd, J. H., and Frank, H.: Studies of Vitamin C Excretion and Saturation, Am. J. M. Sc. **191**:319, 1936.

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possibly the last 7, tests may be considered to indicate saturation and the preceding 2 near saturation.

Detailed Study of Urinary Excretion.—Examination of the specimen of urine obtained from the patient in the fasting state gave little and often misleading information. The content of ascorbic acid varied considerably both in the normal and in the deficient persons (table 2), and there was marked overlapping of findings. This confirms the view of a number of investigators that examination of a single specimen of urine is valueless.

TABLE 2.—*Ascorbic Acid in the Urine After a Test Dose of Ascorbic Acid* *

		Output of Ascorbic Acid, Mg.					
	Test No.	During Fasting	1 Hr.	1-3 Hr.	3-6 Hr.	6-12 Hr.	12-24 Hr.
Deficient persons	1	0.41	0.05	0.37	0.53	†	0.13
	2	0.14	0.25	0.16	0.21	0.24	0.26
	3	0.21	0.37	0	0.12	†	2.38
	4	0.46	0.30	0.78	1.52	†	0.78
	5	0.07	0.07	0.07	0.57	†	2.83
	6	2.27	0.43	1.14	1.50	†	0.99
	7	0.18	0.08	0.02	0.24	1.67	2.45
	8	0.63	0.29	0.67	1.29	1.03	2.34
	9	0.81	0.37	0.88	1.55	1.64	2.42
	10	0.41	0.33	1.02	2.08	1.11	3.33
	11	0.25	0.35	0.73	2.02	3.03	2.73
	12	0.17	0.47	1.10	2.42	3.19	2.40
	13	1.54	1.27	1.31	0.88	1.92	4.25
	14	0.18	0.32	1.27	3.40	6.61	2.52
Normal persons	15	0.07	0.22	12.29	14.42	‡	‡
	16	9.63	5.57	16.80	27.12	‡	‡
	17	‡	2.14	20.64	27.40	12.84	7.81
	18	0.32	0.36	11.47	58.65	7.47	4.75
	19	0.06	0.79	16.49	64.31	20.17	7.76
	20	0.36	1.36	3.21	44.03	†	67.72
	21	1.07	0.53	18.24	84.28	28.52	5.52
	22	0.44	0.74	94.90	40.41	41.80	1.84
	23	1.94	5.45	112.07	91.50	11.41	7.90
	24	0.66	0.97	54.94	53.82	116.48	14.19
	25	1.72	3.14	54.27	122.75	50.24	64.75

* The test dose consisted of 500 mg. of ascorbic acid.
† Included in the twelve to twenty-four hour specimen.
‡ No specimen was collected.

One hour after the administration of the test dose, all but 2 of the group of normal persons showed an increase in the amount of ascorbic acid in the urine, but in only 3 instances was it of significant degree (to 1 mg. or more). For the group of deficient persons there was a rise in only 4 tests, this increase amounting in each case to less than 0.3 mg. The urine which was collected between the first and the third hour after the administration of the test dose showed a marked increase in the quantity of ascorbic acid excreted by all normal persons, the amount varying from 3.2 to 112 mg. For the subjects who showed deficiency, there was no increase in the output of vitamin C in this period, or else the rise amounted to less than 1 mg. In the specimen collected between the third and the sixth hour after the test dose, the

quantity of ascorbic acid eliminated by normal persons ranged from 14.4 to 122.7 mg.; for those who were deficient the quantity ranged from 0.12 to 3.4 mg. At the end of twelve hours the excretion of vitamin C continued to be increased for the normal persons but at a slower rate, and at the end of twenty-four hours the excretion had decreased almost to the level noted before the test dose was given.

For the subjects who were normal in regard to vitamin C, the rate of excretion per hour was greatest between the first and the third hour in 6 instances, varying from 1.6 to 56 mg., and between the third and the sixth hour in 5 instances, varying from 4.8 to 40.9 mg. The amount of ascorbic acid per cubic centimeter of urine was calculated throughout our investigation but was found to be an unreliable measure of the actual excretion, owing to wide variations in dilution and concentration; hence the total output in the urine during a given period should always be determined.

In summary, when vitamin C nutrition is normal, the response to 600 mg. of ascorbic acid given orally is a marked rise in urinary excretion, beginning at the end of the first hour and reaching a peak some time between the first and the sixth hour, followed by a gradual decline, approaching the original level at the end of twenty-four hours. When the vitamin C stores of the body are deficient, there is no rise in the output of vitamin C in the urine, or an insignificant one, after the test dose.

PROPOSED SIX HOUR URINARY EXCRETION TEST FOR SATURATION

Since the greatest increase in excretion of ascorbic acid in the urine occurred during the first six hours after the administration of 600 mg. of this substance, the total output in this period was calculated and compared with the amount excreted in twenty-four hours. In chart 1 the findings are shown graphically, the solid columns representing the six hour and the total columns the twenty-four hour excretions. It may be seen that when the excretion during the twenty-four hour period was high or low the same was true regarding the six hour period. In the group of normal persons from 41 to 91 per cent (average 61 per cent) of the total amount of vitamin C excreted in twenty-four hours was excreted in the first six hours (table 1); in the group of deficient persons from 7 to 88 per cent (average 44 per cent) was excreted. Hawley and Stephens[6] reported similar findings, using a test dose of 100 to 200 mg. given by mouth. They found that saturated subjects excreted 55 per cent and depleted ones 47 per cent of the twenty-four hour output in the first six hours. In our series the actual amount of vitamin C eliminated in six hours after the administration of the test dose varied from 48.5 to 209 mg. for the normal persons and from

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0.3 to 4.9 mg. for the depleted persons. When over 20 per cent of the amount of vitamin C administered was excreted in twenty-four hours (e. g., when the tissues were nearly saturated), the six hour output was above 100 mg. In 2 instances only a six hour test was performed. One patient (case 16) excreted 49.5 mg. in this period; the other (case 15), who had pellagra, excreted 27 mg. The findings in each of these instances have been considered to represent normal nutrition. An output of 49.5 mg. is comparable to that found in several tests of normal

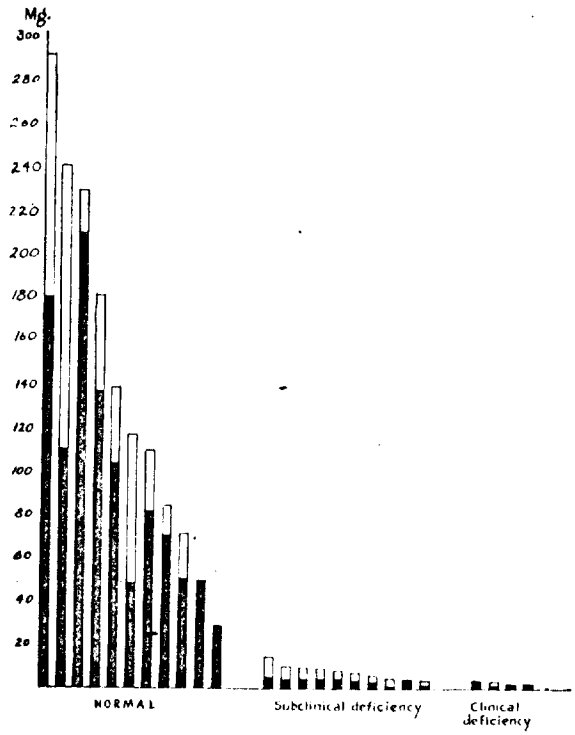

Chart 1.—Urinary excretion of vitamin C after the administration of a test dose of 600 mg. of ascorbic acid. The black columns indicate the six hour output, and the white columns indicate the twenty-four hour output.

persons. An excretion of 27 mg. may indicate slight submutrition, but examination of the blood in this case showed no evidence of deficiency.

In view of these findings, it seems that as much information can be obtained by examining the urine for the content of ascorbic acid excreted in six hours as by examining that excreted in twenty-four hours after the administration of a test dose of this substance. To test the vitamin C nutrition, 600 mg. of ascorbic acid may be given

by mouth and the urine collected for the subsequent six hours. If 50 mg. or more is excreted, the person may be considered normal in regard to the ascorbic acid content of the body; while if over 100 mg. is excreted, partial or actual saturation of the tissues is indicated. Excretion of less than 50 mg. may be associated with depletion of the vitamin C stores of the body.

Harris and Abbasy [8] have recently proposed a simplified procedure to determine the state of vitamin C nutrition, in which the output of ascorbic acid is measured between the fourth and the seventh hour after the administration of a test dose of this substance. Our findings for a comparable period, between the third and the sixth hour, showed an output for the normal persons of from 27.4 to 122.7 mg. (table 2), the amount excreted averaging 44 per cent of the total twenty-four hour excretion. By the depleted persons from 0.1 to 3.4 mg. was excreted, the output averaging 23 per cent of the twenty-four hour excretion. Examination of the urine for this short interval after the test dose, during which the maximum increase in the excretion of vitamin C occurs, gave considerable information regarding the state of vitamin C nutrition.

ASCORBIC ACID IN THE BLOOD

In the Fasting State.—In our series of 25 tests the level of reduced ascorbic acid in the blood varied from 0.04 to 1.98 mg. per hundred cubic centimeters (table 1). For the group considered normal according to findings obtained on examination of the urine, from 0.43 to 1.98 mg., with an average of 1.11 mg., of ascorbic acid was present per hundred cubic centimeters of blood; for the group considered deficient, from 0.4 to 0.52 mg., with an average of 0.17 mg., per hundred cubic centimeters. Six subjects showed less than 0.1 mg. per hundred cubic centimeters, 4 of them exhibiting clinical evidence of vitamin deficiency. Normal blood values during fasting range from 0.65 to 2 mg. per hundred cubic centimeters, according to various observers, figures above 1.3 mg. being indicative of saturation. [9] Only 1 of the group

8. Harris, L. J., and Abbasy, M. A.: A Simplified Procedure for the Vitamin C Urine Test, Lancet **2**:1429, 1937.

9. (*a*) Wright I. S.; Lilienfeld, A., and MacLenathen, E.: Determination of Vitamin C Saturation: A Five Hour Test After an Intravenous Test Dose, Arch. Int. Med. **60**:264 (Aug.) 1937. (*b*) Pijoan, M., and Klemperer, F.: Determination of Blood Ascorbic Acid, J. Clin. Investigation **16**:443, 1937. (*c*) Abt, A. F.; Farmer, C. J., and Epstein, I. M.: Normal Cevitamic Acid Determinations in Blood Plasma and Their Relationship to Capillary Resistance, J. Pediat. **8**:1, 1936. (*d*) Greenberg, L. D.; Rinehart, J. F., and Phatak, N. M.: Studies on Reduced Ascorbic Acid Content of Blood Plasma, Proc. Soc. Exper. Biol. & Med. **35**:135, 1936. (*e*) Van Eekelen.[5]

of normal persons showed less than 0.65 mg., presenting a value of 0.43 mg. This person's response to a test dose of vitamin C was normal as far as urinary excretion was concerned. The low value may have been due to a reduced intake of this vitamin for a day or two prior to the test, the diet previously having been adequate. This one finding indicates that occasionally a single determination of the ascorbic acid in the blood may lead to fallacious interpretation. Of the 4 persons with blood values above 1.3 mg. per hundred cubic centimeters, which is considered the level of saturation, all excreted 30 per cent or more of the test dose in the urine in twenty-four hours (table 1) and 109 mg. or more in six hours. The subjects showing values between 0.65 and 1.3 mg. per hundred cubic centimeters, which is considered normal, excreted 12 to 23 per cent of the test dose in the urine in twenty-four hours and 48.5 mg. or more in six hours. The patients with values of less than 0.65 mg. per hundred cubic centimeters had an output of 0.2 to 2.3 per cent of the test dose in the urine in twenty-four hours and an output of less than 5 mg. in six hours. There was 1 exception; the person previously mentioned who showed a blood level of 0.43 mg. per hundred cubic centimeters during fasting excreted 13.7 per cent of the test dose in twenty-four hours and 70 mg. in six hours. These figures show the close correlation between urinary and blood findings as indicative of normal or deficient vitamin C nutrition.

After the Administration of 600 Mg. of Ascorbic Acid as a Test Dose.—The blood was examined for the content of reduced ascorbic acid one, three and six hours after the oral administration of 600 mg. of ascorbic acid (chart 2). At the end of one hour there was a rise in the concentration of ascorbic acid; this rise persisted or reached a peak at the end of three hours. After six hours had elapsed there was usually a slight fall in the amount of vitamin C in the blood. The curves resembled those for a dextrose tolerance test, as has been suggested by Greenberg, Rinehart and Phatak.[9] These investigators obtained a maximum increase two to four and one-half hours after giving 6 ounces (177 cc.) of orange juice. Taylor, Chase and Faulkner [10] administered 1 Gm. of ascorbic acid and found that normal persons showed a rise in the concentration in the blood which reached a peak in one hour and subsided in five hours. In 1 case of scurvy the rise was delayed, reaching a maximum at three hours and remaining high for two hours longer. In our series, the group of normal persons (11 tests) showed a rise to a maximum of 1.7 to 2.9 mg. of ascorbic acid per hundred cubic centimeters of blood. In all but 1 instance a height of 2 mg. or more was attained. For the group of deficient persons (14 tests) the highest

10. Taylor, F. H. L.; Chase, D., and Faulkner, J. M.: Estimation of Reduced Ascorbic Acid in Blood Serum and Plasma, Biochem. J. **30**:1119, 1936.

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concentration varied from 1.2 to 1.7 mg. of ascorbic acid per hundred cubic centimeters, a rise above 1.1 mg. occurring in only 2 tests. In all but 3 instances the maximum concentration was reached one to three hours after the test dose was given. A delayed rise may be due to slow absorption from the gastrointestinal tract or to diminished excretion by the kidneys due to renal damage. One person who showed this type of curve had chronic bacillary dysentery, 1 had generalized arteriosclerosis with evidence of diminished renal function and a third was a normal adult in good health. In patients in whom disease of the gastrointestinal tract is interfering with absorption, vitamin C nutrition may be tested by administering ascorbic acid intravenously.

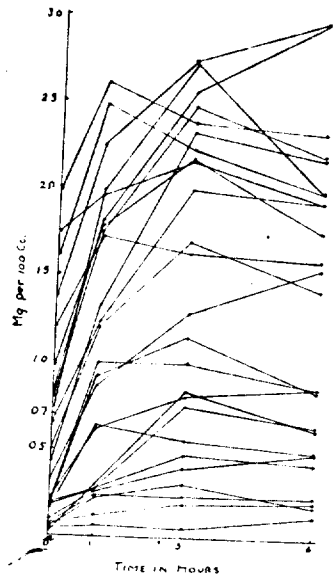

Chart 2.—The reduced ascorbic acid content of the blood after a test dose of 600 mg. of ascorbic acid.

PROPOSED TEST OF THE BLOOD FOR VITAMIN C NUTRITION

The state of saturation of the tissues may be determined by examining the blood during fasting and one and three hours after the administration of 600 mg. of ascorbic acid. An original level of reduced ascorbic acid of over 0.7 mg. per hundred cubic centimeters, reaching 2 mg. or more after the test dose, may be considered indicative of normal vitamin C nutrition. A value of over 1.3 mg. per hundred cubic centimeters for the blood during fasting indicates tissue saturation. If the amount of vitamin C in the blood during fasting is less than 0.7 mg.

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and if it fails to rise to 2 mg. or more at the end of three hours after the test dose, vitamin C deficiency is present.

THE RENAL THRESHOLD OF VITAMIN C

The findings obtained on examination of the blood and of the urine showed a close correlation. When the level of ascorbic acid in the blood during fasting was high or when a high level was reached and

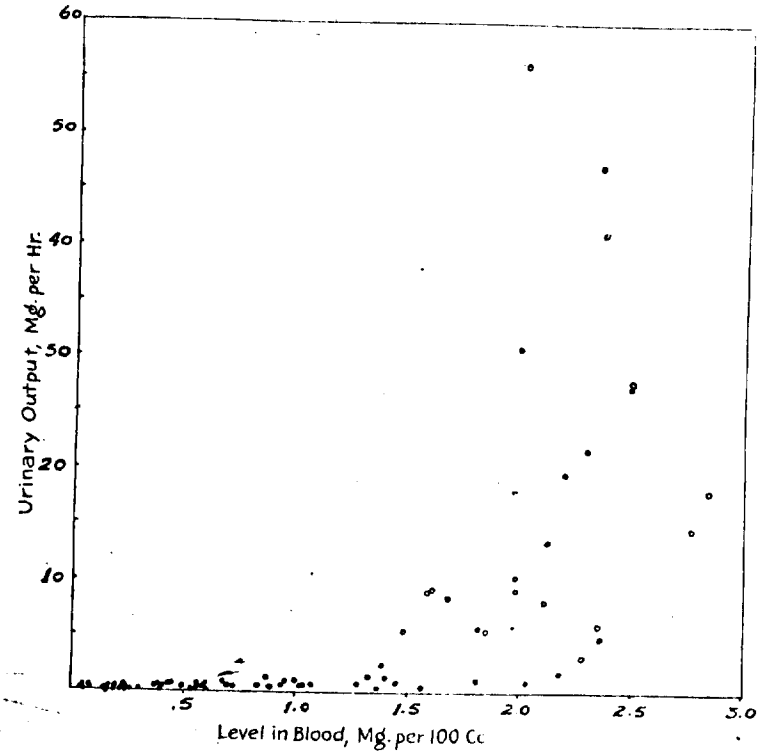

Chart 3.—The relation between the amount of ascorbic acid in the blood and the amount excreted in the urine.

maintained after the administration of a test dose, a large amount was excreted in the urine; when the quantity in the blood during fasting was low and when the rise was small, the urinary excretion was minimal. In chart 3 the amount of vitamin C excreted per hour in the urine is plotted against the concentration of ascorbic acid in the blood during the same period. The latter figure was obtained by averaging the concentration in the blood at the beginning and at the end of the particular period of urinary excretion. It can be seen that little vitamin C was

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excreted by the kidneys until the level in the blood reached approximately 1.4 mg. per hundred cubic centimeters, at which time an increase in the excretion of vitamin C occurred. Van Eekelen [5] has stated that when the level of ascorbic acid in the blood is greater than 1.3 mg. per hundred cubic centimeters, the excretion in the urine is increased. Faulkner and Taylor [11] have reported a renal threshold of vitamin C in the neighborhood of 1.4 mg. of reduced ascorbic acid per hundred cubic centimeters of blood.

STUDIES OF THREE PATIENTS WITH VITAMIN DEFICIENCY

Three patients who showed clinical signs of vitamin deficiency were studied at the time of admission to the hospital and after a period of treatment with vitamin C (table 1, tests 2, 3, 4, 17, 22 and 25). One of the patients had pellagra, with both cutaneous and gastrointestinal manifestations. Her diet had consisted of dried beans, rice, bread and coffee, with inclusion of meat and canned vegetables only once or twice weekly and citrus fruits about once a month. A second patient was originally considered as having a gastric neurosis, but it was subsequently shown that she was extremely deficient in vitamin C. For two months her diet had been extremely meager, containing small amounts of lean meat, rice and a few cooked vegetables, with no citrus fruits, tomatoes or cabbage. The third patient exhibited stomatitis, esophagitis, proctitis, vaginitis and evidence of loss of weight and dehydration. Her diet had been deficient for several months both in quantity and in quality, and she had never eaten citrus fruits or tomatoes to any extent. After the state of vitamin C nutrition had been tested, each of these patients was given 300 to 400 mg. of ascorbic acid daily, and at the end of nine to fifteen days a second test was performed.

The results of the primary and subsequent tests are shown in chart 4. At the time of the original examination, after the administration of 600 mg. of ascorbic acid, the urinary excretion was 1.1, 2.9 and 3.4 mg., respectively, of vitamin C in twenty-four hours, 0.5, 0.6 and 2.6 mg., respectively, being excreted in six hours. In other words, practically no ascorbic acid was excreted in the urine, as these small values may have been due to reduction of the dye (2,6-dichlorophenolindophenol) by other substances. The blood at this time showed during fasting 0.05, 0.06 and 0.09 mg., respectively, of ascorbic acid per hundred cubic centimeters. After the test dose the concentration rose to a peak of 0.12, 0.2 and 0.5 mg., respectively, per hundred cubic centimeters. After the period of vitamin C therapy and after the test dose the urinary excretion was 70.8, 179.7 and 291.1 mg., respectively, in twenty-four

11. Faulkner, J. M., and Taylor, F. H. L.: Observations on the Renal Threshold for Ascorbic Acid in Man, J. Clin. Investigation **17**:69, 1938.

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hours and 50.2, 136 and 180.2 mg., respectively, in six hours. The level of reduced ascorbic acid in the blood during fasting measured 0.98, 1.61 and 1.98 mg., respectively, per hundred cubic centimeters, rising after the test dose to 2.18, 2.48 and 2.6 mg., respectively, per hundred cubic centimeters. In other words, the vitamin C nutrition was normal, as judged by the examination of the urine and of the blood. From a clinical standpoint, all 3 patients were markedly improved. The gastrointestinal symptoms had cleared up in 1 case, the stomatitis, proctitis and vaginitis had disappeared in 1 case and the lesions of pellagra had nearly disappeared in the third case. The third patient was the only one to receive treatment other than vitamin C in addition to the ordinary hospital diet. She was also given large amounts of brewers' yeast daily.

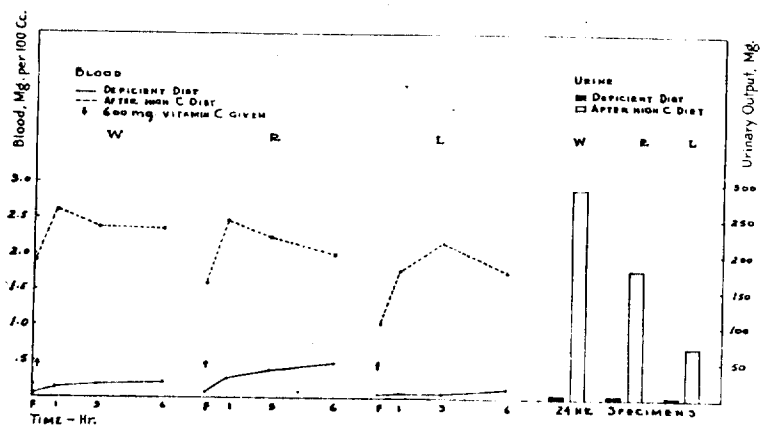

Chart 4.—The ascorbic acid content of the blood and urine in deficiency states (3 cases) when the original diet was being taken and after a high intake of vitamin C.

Of the 2 patients in this series who had pellagra, 1 showed marked deficiency of vitamin C, as already described (table 1, case 4); the other, for whom only a six hour test of blood and urine was performed, revealed findings at the low limit of normal (table 1, case 15). It seems likely that when a person exhibits deficiency of one vitamin, there may be deficiency of other vitamins as well. States of multiple vitamin deficiency have been recognized with increasing frequency in recent years. A further study of the state of vitamin C nutrition in patients with pellagra is being undertaken. It is conceivable that some of the symptoms of this disease are due to lack of substances other than vitamin B_2 (or G), the pellagra-preventive factor.

FREQUENCY OF VITAMIN C DEFICIENCY

Deficiency of ascorbic acid is a frequent finding in routine examination of patients in clinics and hospitals, as shown by Harris, Abbasy, Yudkin and Kelly.[12] In this small series of 22 patients, of whom 17 were apparently normal clinically in regard to vitamin C nutrition, 14 showed evidence of deficiency with special testing. Numerous vague complaints, such as fatigue, anorexia and indigestion, may be related to deficiency of this nutritive factor. More definite disorders, such as stomatitis, vaginitis and proctitis, may be caused by an insufficient amount of vitamin C in the tissues of the body. On the other hand, in view of the prevalence of subclinical vitamin C deficiency, caution should be used before attributing to ascorbic acid the role of an etiologic factor in any pathologic state. A depletion of the vitamin C content of the tissues may be only a concomitant finding or a result rather than a cause of the disease which is being studied.

COMMENT

The results of this study and the findings of Harris and Abbasy,[8] previously mentioned, indicate that it is not necessary to measure the excretion of ascorbic acid for twenty-four hours after giving a test dose to determine the state of vitamin C nutrition. Since this investigation was started, a short test has been reported by Wright and his co-workers,[9a] consisting of a study of urinary excretion for five hours after the administration of 1,000 mg. of ascorbic acid intravenously. The excretion of 400 mg. in this period was considered to indicate normal nutrition. Ralli, Friedman and Kaslow [13] have proposed the use of a three hour test of urinary excretion after the administration of 100 mg. of vitamin C by vein, having found that normal persons excrete 40 per cent and depleted subjects 11 per cent of the injected vitamin in three hours. The principle in each of these procedures is the same, the variants being the amount of vitamin C used as a test dose, the method of administration and the number of hours during which urine is collected. A dose of 600 mg. was chosen for our study because it was large enough to minimize the effect of reducing substances other than vitamin C in the urine and also to diminish the significance of the inherent error in the determination of the end point. Similar amounts

12. Harris, L. J.; Abbasy, M. A.; Yudkin, J., and Kelly, S.: Vitamins in Human Nutrition: Vitamin C Reserves of Subjects of Voluntary Hospital Class, Lancet **1**:1488, 1936.

13. Ralli, E. P.; Friedman, G. J., and Kaslow, M.: Excretory Test for Vitamin C Deficiency and Subnutrition, Proc. Soc. Exper. Biol. & Med. **36**:52, 1937.

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have been used in previous investigations, and an opportunity was afforded for comparison of results. The oral route is the simplest method of administration and is satisfactory in most instances, although occasionally findings may be inaccurate because of poor intestinal absorption. Van Eekelen and Heinemann [14] have suggested that intravenous administration or the use of large doses, over 300 mg., may simulate saturation. Erroneous conclusions may result if ascorbic acid is spilled into the urine, insufficient time being allowed for absorption by the tissues. Wright [9a] has stated that in order to avoid the danger of irregular intestinal absorption, ascorbic acid should be given by vein. In all examinations of the urine the presence of reducing substances other than vitamin C is a potential source of error. Van Eekelen and Heinemann [14] have indicated that this is especially true when the diet is high in protein or when diabetes is present. They have advocated removal of these reducing substances by precipitation with mercuric acetate. King,[15] however, has criticized this procedure because it introduces new sources of error. In choosing a method to determine the vitamin C nutrition and in evaluating the results, all these facts should be kept in mind. It is our opinion that for routine clinical use the administration of an oral test dose of ascorbic acid with examination of the urine excreted in a period of three or six hours is a satisfactory procedure.

Examination of the blood is free from much of the aforementioned criticism, and a saturation test dependent on estimation of the content of reduced ascorbic acid in the blood may prove to be more reliable than tests of the urine. Such a procedure has been suggested as a result of this study.

With the availability of methods which require only a few hours for their performance and which are applicable to ambulatory patients, an opportunity is afforded for studying large groups of persons, as a result of which the role of vitamin C in a number of pathologic states may be clarified.

SUMMARY

The state of vitamin C nutrition may be determined by measuring the excretion of ascorbic acid in the urine for six hours after the administration of 600 mg. of this substance. An alternative procedure consists in the determination of the amount of reduced ascorbic acid in the blood during fasting and at the end of one hour and of three

14. Van Eekelen, M., and Heinemann, M.: Critical Remarks on the Determination of Urinary Excretion of Ascorbic Acid, J. Clin. Investigation **17**:293, 1938.

15. King, C. G.: Vitamin C, Ascorbic Acid, Physiol. Rev. **16**:238, 1936.

hours after the administration of a similar test dose. Criteria of normal vitamin C nutrition, of saturation and of depletion of the tissues are enumerated for each of these tests.

There is a definite relation between the level of reduced ascorbic acid in the blood and the amount of this substance excreted by the kidneys; when the former exceeds 1.4 mg. per hundred cubic centimeters, urinary excretion increases.

Mild vitamin C deficiency is extremely prevalent, and too much importance should not be attached to depletion of the ascorbic acid content of the body in any disease.

Arch. Pathol. 1968, 85, 493-502

Capillary Hemorrhage in Ascorbic-Acid-Deficient Guinea Pigs

Ultrastructural Basis

Ira Gore, MD; Masaya Wada, MD; and Max L. Goodman, MD, Boston

An electron microscopic study of the capillaries in scorbutic guinea pigs disclosed defects which permitted the abnormal passage of erythrocytes and other blood constituents. The lesions, which were focal, consisted of endothelial junctional separations and of cytoplasmic disruption. The former was the more common, but both lesions were accompanied by attenuation and loss of the basement membrane and by depletion of pericapillary collagen.

SPONTANEOUS or readily induced hemorrhage, a manifestation of capillary fragility, is one of the cardinal clinical features of scurvy.[1] As summarized by Stolman and associates,[2] studies using conventional microscopy have suggested, variously, that the basic lesion involves the endothelium, the intercellular cement substance, or the supporting tissues of the vessel wall. The additional resolution permitted by electron microscopy, however, has questioned the existence of an intercellular cement substance,[3] and has demonstrated the inadequacy of the light microscope for demonstrating changes in the endothelium.[4] While a depletion of collagen in the scorbutic state was demonstrated by Wolbach and Howe in 1926[5] and repeatedly confirmed,[6-15] there is no direct evidence to show how this permits the occurrence of spontaneous hemorrhage. Gore and associates[16] found that depletion of subendothelial collagen in the aortas of scorbutic guinea pigs was accompanied by widening of the intercellular spaces and suggested that the same change involving capillary channels would permit the extravasation of blood. Although Buck[17] had observed that "endothelial cells of capillaries seem to possess no structure not shared by those of arteries," additional studies have emphasized the variability of the capillary endothelium of different organs.[4] Accordingly, direct observation of the microchannels which gave rise to bleeding in scurvy seemed indicated.

Accepted for publication Sept 12, 1967.

From the Department of Pathology, University Hospital, Boston University Medical Center, Boston.

Read in part at the 24th annual meeting of the Electron Microscopy Society of America, San Francisco, Aug 22-25, 1966.

Reprint requests to Department of Pathology, University Hospital, Boston University Medical Center, Boston (Dr. Gore).

Materials and Methods

Scurvy was induced in 36 male guinea pigs by feeding a commercially available (Nutritional Biochemicals Corp., Cleveland) ascorbic-acid-deficient diet for periods of one to five weeks. Half of the guinea pigs were immature, averaging about 500 gm in weight, the remainder were adults weighing from 700 to 1,000 gm. Twenty-seven animals were utilized for optical microscopy; all of them suffered from intercurrent infection and were either sacrificed in extremis or died spontaneously (Table 1). Electron microscopy was, therefore, performed upon only nine experimental pigs, three after two weeks, five

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Table 1.—Experimental Scurvy

	Weeks of Deficiency					
	1	2	3	4	5	Controls
No. of guinea pigs	1	10	10	14	1	7 *
Purpura (gross)	0	0	5	6	1	
Optical microscopy	1	7	5	14	0	4
Electron microscopy	0	3	5	0	1	3 †
Capillary lesions		1	3		1	
Junctional separation		1	3		1	
Cytoplasmic disruption		0	3		0	

* Control animals, five killed at three weeks, and two at five weeks.
† The three controls used for electron microscopy were killed at three weeks.

after three weeks, and one after five weeks of deficiency (Table 2). Seven control animals were maintained on the same diet fortified with liberal quantities of vitamin C; three of them were used for ultrastructural study, the four others were utilized for routine optical microscopy. Five minutes prior to killing the animal under intramuscular pentobarbital (Nembutal) anesthesia, each animal used for electron microscopy received an intravenous injection of thorium dioxide (Thorotrast) (1 ml/kg of a 1:1 dilution in Tyrode's solution) or colloidal carbon (1 ml/kg of a 1:3 dilution of Pelikan India Ink) to mark sites of vascular leakage.

Samples of skin, heart muscle, tongue, kidney cortex, and small intestinal mucosa were taken promptly and fixed either initially or after buffered gluteraldehyde fixation in phosphate buffered 1% osmic acid (pH 7.2). These tissues were chosen because they were richly supplied with capillaries. Dehydration through graded concentrations of ethanol permitted embedding in araldite. Sections were cut with a microtome (Porter Blum) at 1μ for light microscopic orientation. Ultra-thin sections were examined with an electron microscope (RCA EME 2E). Methylene blue was used to stain the 1μ sections, the electron microscopic sections were doubly stained with uranium[18] and lead.[19]

Table 2.—Electron Microscopy and Experimental Scurvy

Deficient Diet Weight (gm)					
Initial	2 weeks	3 weeks	5 weeks	Purpura	Capillary Lesions
650	520			0	+
570	500			±	0
760	600			0	0
715	671	650		+	+
655	634	575		+	+
630	602	500		0	+
865	790	725		0	0
480	435	445		±	0
925	825	810	700	+	
Vitamin C Supplemented Diet					
700	745	765		0	0
740	760	760		0	0
950	1,100	1,120		0	0

Observations

Contrasting with the healthy appearance and vigorous behavior of the control guinea pigs, animals maintained on the scorbutogenic diet longer than a week displayed progressive loss of appetite and weight. By the end of the second week, there was obvious hair loss, lethargy, and limitation of motion. Swelling, tenderness, and cutaneous hemorrhages, which occurred regularly about the knees pointed to involvement of the synovial tissues. Similarly, but less consistently, the appearance of diarrhea and melana were the signal of intestinal mucosal bleeding. Histologically, capillary hemorrhages were observed in the myocardium, but were more frequent and extensive in the skin, intestinal mucosa and the synovium of the knee.

The ultrastructural details of capillaries in the skin, myocardium, and tongue of control guinea pigs coincide precisely with descriptions of normal continuous channels given by Majno,[4] Bennett,[20] and Fawcett,[21-24] Fernando and Movat,[25] and Palade.[26,27] Recapitulating, however, for purposes of comparison, normal capillaries are lined by a single layer of endothelium which is extremely thin except in the portion containing the nucleus, the Golgi complex, and most of the mitochondria. Externally, separated by a relatively clear zone measuring 400 Angstroms (A) from the cell surface, there is a 700 A thick basement membrane which follows the contour of the endothelium and divides to enfold occasional pericytes. Outside the basal lamina, there are collagen fibers and fibrocytes (Fig 1). Numerous pinocytotic vesicles occupy the cytoplasm along the inner and outer plasma membranes. Ribosomes and endoplasmic reticulum are rather sparse. The apposed surfaces of adjacent endothelial cells are oblique, imbricated, and frequently inter-

Source: Medizinische Klinik 61: 1879, 25 Nov. 1966

On the Problem of Glucosuria Following High Doses of Ascorbic Acid

H. Glatzel and M. Ruberg-Schweer

In our communication "On the problem of glucosuria following high dosage of ascorbic acid" (1), we have recently reported studies which showed that, after daily addition of 1500 mg ascorbic acid to a normal diet, the excretion of glucose in the urine does not increase, as reported by Czech authors, but rather even decreases (as compared to the period prior and after the addition of vitamin C). The determination of glucose in the urine was carried out with glucose oxidase (Boehringer color test).

Since, with such small amounts of sugar, this method did not appear to be entirely reliable, we have now determined in 6 further test subjects the sugar concentration in the urine on 7 successive days prior, during and after addition of 1500 mg ascorbic acid by means of the more sensitive UV test (Boehringer Mannheim, enzymic determination of D-glucose with hexokinase/-glucose-6-phosphate dehydrogenase, UV test with TPN). By this method, added glucose (10 mg per 100 ml) could be determined in repeated batches to the extent of 80-90%. The presence of large amounts of urate crystals in the urine appears to have a disturbing effect on the determination. In any case, surprisingly small amounts of glucose were found in cloudy urine containing large amounts of urates.

In this test series, the glucose concentration of the urine was between 2 and 7 mg per 100 ml (Table 1). Renschler et.al. (2) most frequently found amounts of 8 to 9 mg per 100 ml. The differences between the period prior to administration and the administration period and between the latter and the period after administration are statistically not significant, with one exception (test subject 2, ratio of period prior to administration and administration period, $p < 0.05$).

In 3 test subjects, we also determined the daily total glucose excretion during 13 test days. In 2 of these subjects (and in 1 subject to a significant extent), the glucose excretion in 24 hours was smaller during the vitamin administration period than in the period after administration, and in the third test subject it was greater.

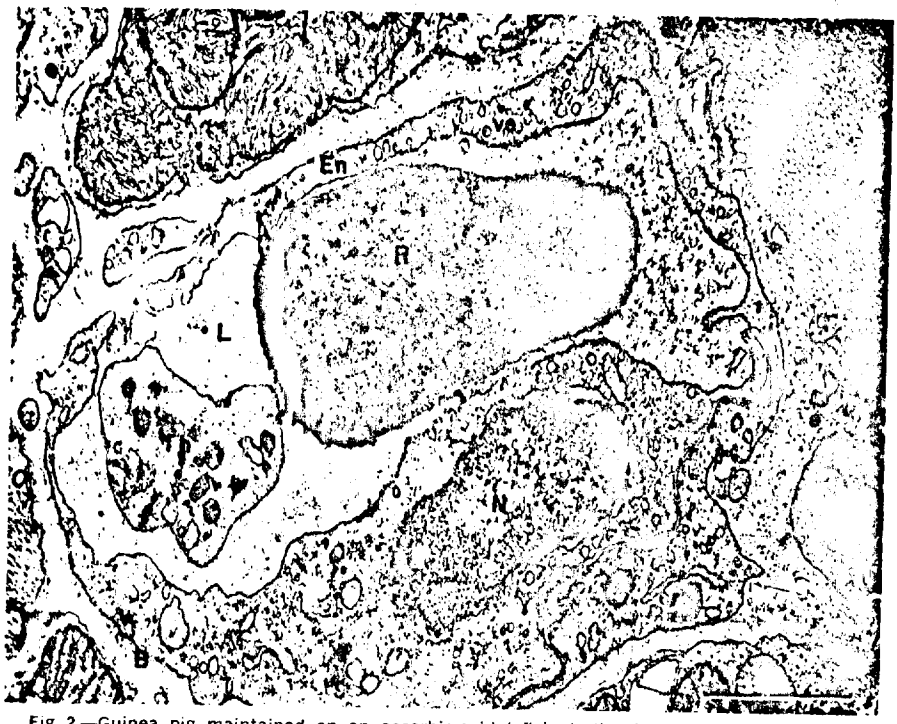
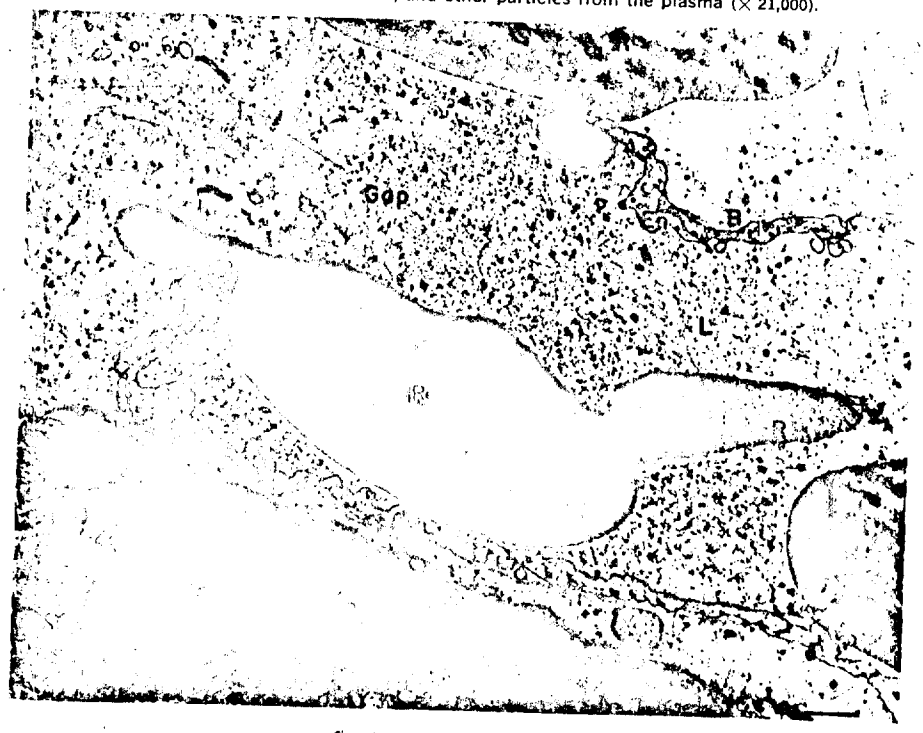


Fig 2.—Guinea pig maintained on an ascorbic-acid-deficient diet for three weeks. Note the junctional separation at the upper left portion of the capillary contrasting with the normal tight junction at the lower right. At the site of separation, only traces of the basement membrane can be seen (× 41,000).

Fig 3.—Myocardial capillary displaying junctional separation and basement depletion after two weeks of ascorbic acid deficiency. Note that thorium dioxide particles are present extravascularly (× 80,000).

Fig 4.—Junctional separation and loss of capillary basement membrane has permitted the extravasation of colloidal carbon and other particulate material, perhaps lipoprotein. Note the presence of two electron lucent structures in the lumen. These contain a few vesicles in proximity to the surface membrane and at other levels are directly continuous with endothelial cells (see Fig 9). Three-week ascorbic-acid-deficient guinea pig (× 41,000).

Fig 5.—Myocardial capillary in a three-week ascorbic-acid-deficient guinea pig. Observe the large junctional gap associated with absence of the basement membrane which allows extravasation of red blood cells, colloidal carbon, and other particles from the plasma (× 21,000).



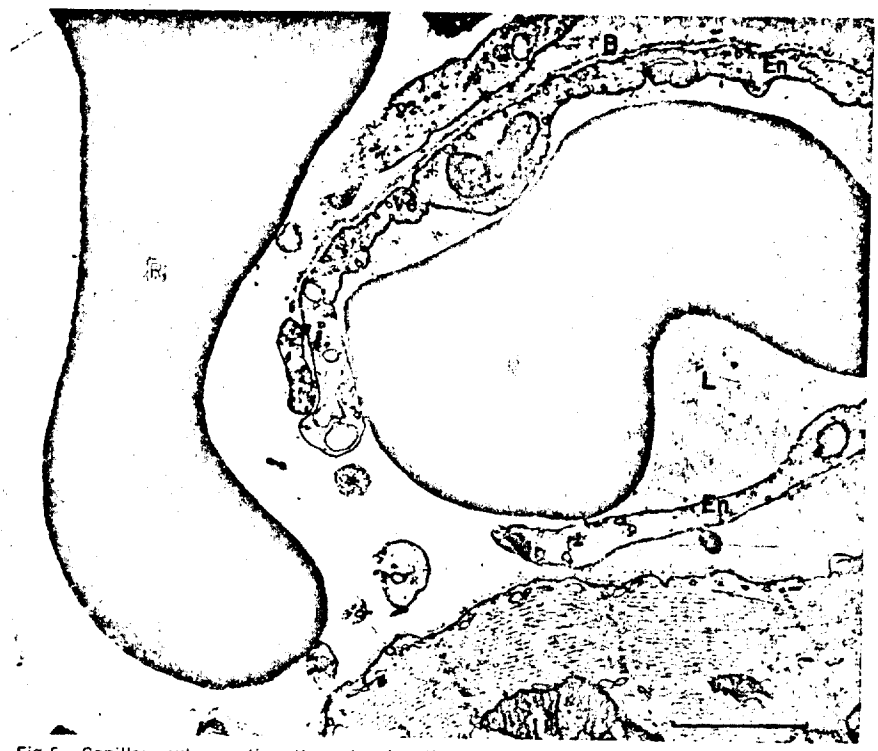
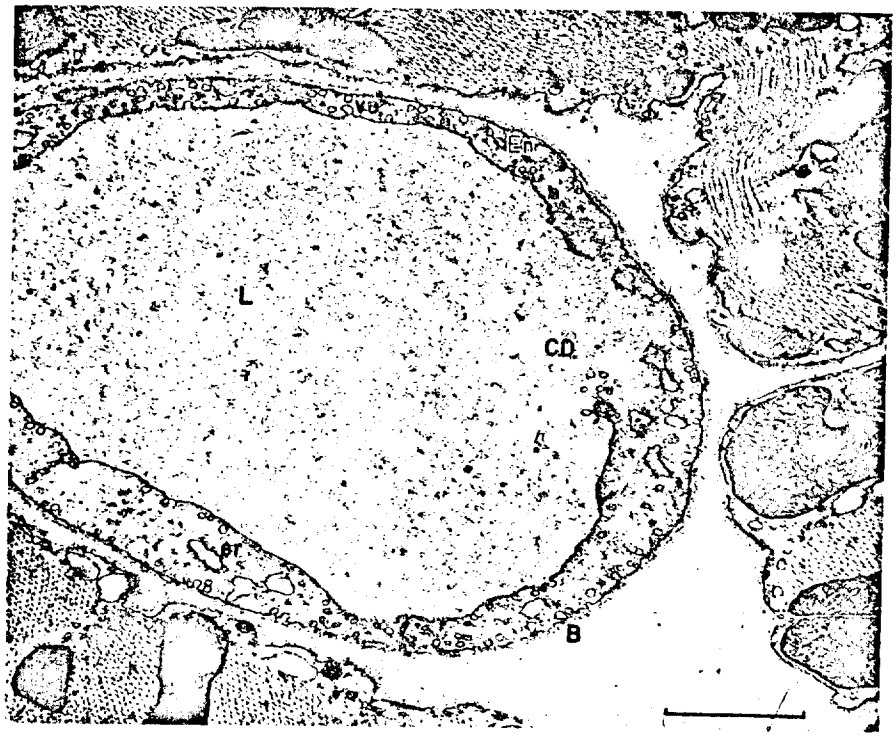


Fig 6.—Capillary extravasation, through a junctional gap in a three-week-deficient animal. Note that the deficiency of the basement membrane extends beyond the limits of the separation (× 35,000).

Fig 7.—After three weeks of ascorbic-acid deficiency, observe the focal loss of the internal cell membrane associated with loss of endothelial cell constituents; a few vesicles remain in the defect as vestiges of cytoplasmic structure (× 38,000).



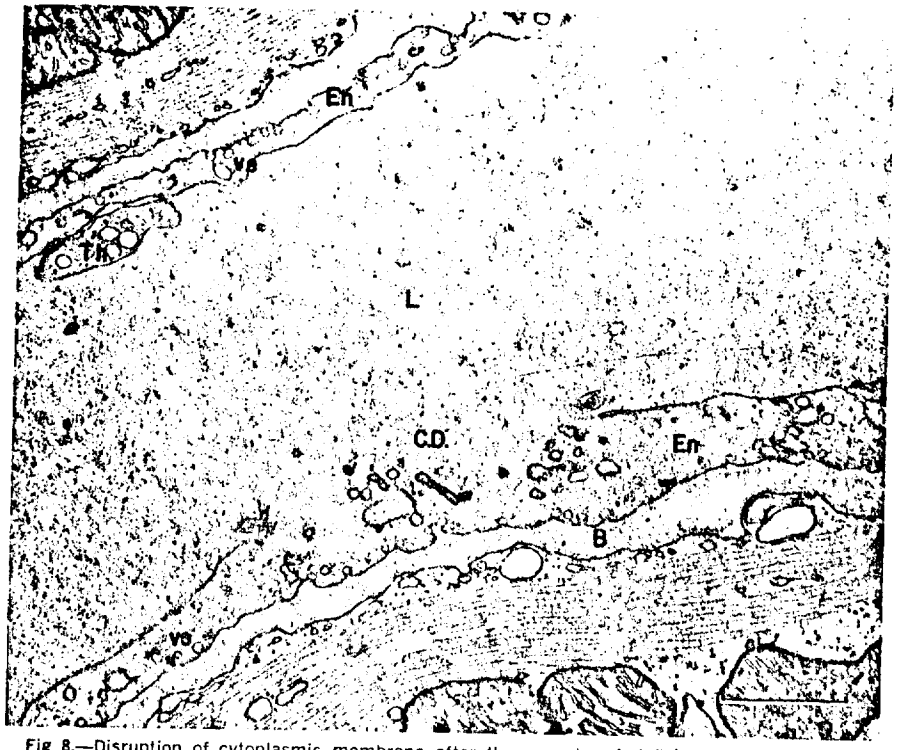


Fig 8.—Disruption of cytoplasmic membrane after three weeks of deficiency. Compared with Fig 7, a greater number of cell constituents persist in the rarefied cytoplasm of the defect. Observe the coiling of the cytoplasmic membrane at the margins of the lesion (× 34,000).

Fig 9.—A greatly swollen rarefied capillary endothelial cell found in association junctional separation and defects of the cytoplasmic membrane in a three-week-deficient guinea pig (× 43,000).

observed in and immediately external to the separations (Fig 3, 5, and 6).

The second type of lesion, found only when junctional separation was present as well (Table 1), was characterized by a defect in the luminal plasma membrane of the endothelial cell. Frequently, a tight outwardly directed curl of cytoplasmic membrane marked the margins of the disruption. Small vesicles and other organelles were observed in the gap appearing to have been washed from the cytoplasm (Fig 7 and 8). Progression of these changes with loss of the underlying basement membrane and pericapillary collagen were characterized by complete discontinuity of the cell and extravasation of blood constituents. In association with this change, other endothelial cells were observed to be enormously swollen, displaying a paucity of pinocytotic vesicles along the ballooned plasma membrane and characterized by a less dense cytoplasm containing fewer and more widely separated organelles (Fig 4 and 9).

The intact endothelial cells in the deficient animals were characterized by mitochondria somewhat larger than normal, more abundant pinocytotic vesicles, dilated endoplasmic reticulum, large vesicles, and greater prominence of the Golgi apparatus (Fig 9). The pericytes of deficient and control animals were indistinguishable. While gross defects and absence of basement membrane were limited to (and possibly determined) the two types of lesion, just described, scorbutic animals displayed a more diffuse attenuation of the basal lamina and depletion of pericapillary collagen fibrils. In contrast to these findings, structural changes were not seen in the endothelium or basement membrane of the renal glomerulus, nor in the fenestrated capillaries of the renal intertubular space.

Comment

The localization of the capillary lesions at sites of extravasation (erythrocytes and/or colloidal particles) and the presence of erythrocytes in the gaps, indicates that they had occurred in vitro and were not artefacts produced by manipulation or sectioning of fragile scorbutic tissue. Edematous swelling of endothelial cells and the elastic recoil producing the curled plasma membrane marginal to disrupted foci, as well as the attenuated and missing basement membrane characterizing both focal changes, can only be the result of the experimental procedure.

It seems probable that discontinuities of both the endothelium and the underlying basement membrane are essential for the occurrence of capillary hemorrhage.[28] Increased permeability of microchannels (venules) produced by histamine or serotonin [29] results from widening of the endothelial cell junctions. In that situation, colloidal markers pass the endothelium, but accumulate along the basement membrane. While leukocytes readily penetrate the basal lamina in acute inflammation [30] these are highly mobile cells in contrast to red blood cells. As inflammation continues, however, the release of leukocytic proteases may produce defects in the basement membrane, facilitating the diapedesis of erythrocytes too.

While it seems likely that simple mechanical tearing does not produce the observed changes in the endothelium, it is possible that loss of support by basement membrane and pericapillary collagen may facilitate their formation. Earlier studies had, of course, emphasized the importance of weakening of the capillary and pericapillary fibrillar structures.[31-33] Immunologically, capillary basement membrane is identical with the basement membrane of renal glomeruli [34,35] which has been shown to contain collagen.[36] Attenuation and focal absence of basement membrane, therefore, merely reflects participation in the generalized deficit of collagen synthesis which characterized scurvy. Although the renal glomerular basement membrane is not perceptibly altered, it is a much bulkier

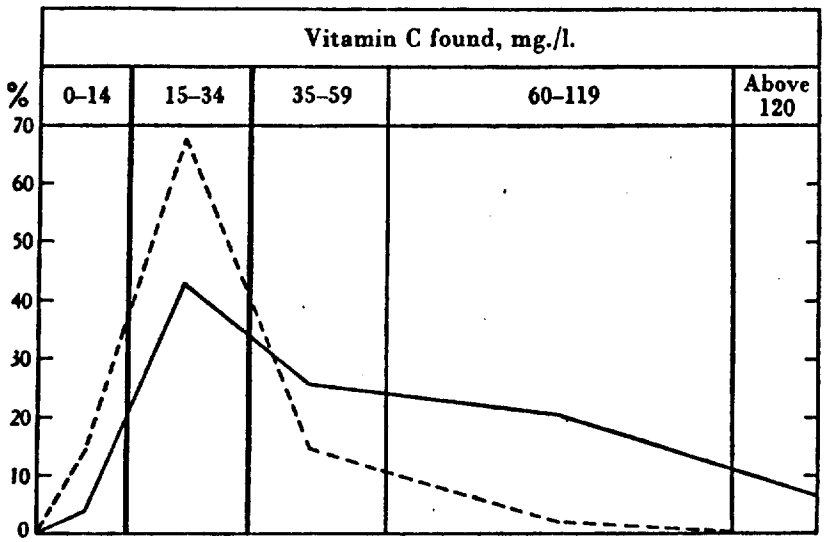


Fig. 3. Frequency distribution of quantity of vitamin C excreted in urine by 30 adults in Jerusalem (expressed in percentage of total).

——— November to May. - - - - - June to October.

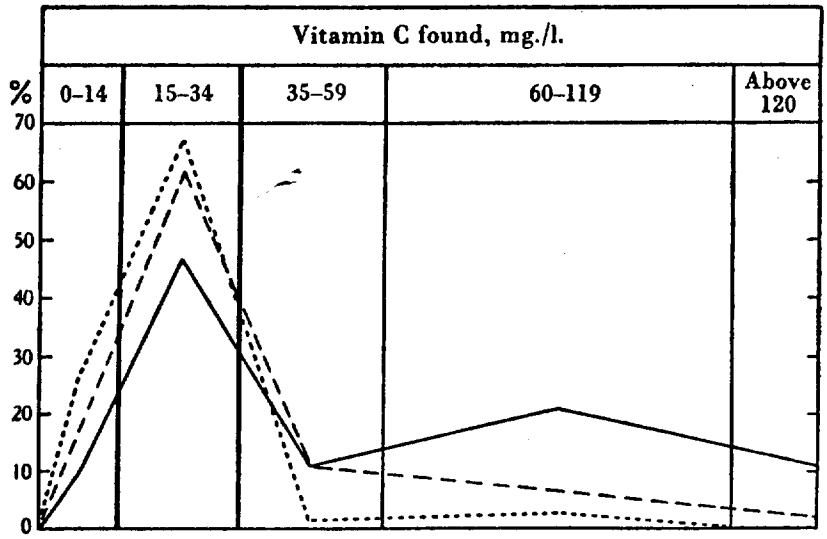


Fig. 4. Frequency distribution of quantity of vitamin C excreted in urine by 25 children in Jerusalem (expressed in percentage of total).

——— January to April. - - - - - May, November, December.
. . . . . June to October.

13. Peach, R.: An Electron Optical Study of Experimental Scurvy, *J Ultrastruct Res* 6:579-590, 1962.

14. Jorgenson, O.: Electron Microscopical Studies of Granulation Tissue Formations in Open Wounds of Ascorbic Acid Deficient Guinea Pigs, *Acta Path Microbiol Scand* 60:365-375, 1964.

15. Ross, R., and Benditt, H.P.: Wound Healing and Collagen Formation: IV. Distortion of Ribosomal Patterns of Fibroblasts in Scurvy, *J Cell Biol* 22:365-390, 1964.

16. Gore, I.; Fujinami, T.; and Shirahama, T.: Endothelial Changes Produced by Ascorbic Acid Deficiency in Guinea Pigs, *Arch Path* 80:371-376 (Oct) 1965.

17. Buck, R.C.: The Fine Structure of Endothelium of Large Arteries, *J Biophys Cytol* 4:187-190, 1959.

18. Stempak, J.G., and Ward, R.T.: An Improved Staining Method for Electron Microscopy *J Cell Biol* 22:697-701, 1964.

19. Reynolds, E.S.: The Use of Lead Citrates at High pH and on Electron-Opaque Stain in E.M., *J Cell Biol* 17:208-212, 1963.

20. Bennett, H.S.; Luft, J.H.; and Hampton, J.C.: Morphological Classification of Vertebrate Blood Capillaries, *Amer J Physiol* 196:381-390, 1959.

21. Fawcett, D.W.: "The Fine Structure of Capillaries, Arterioles, and Small Arteries," in Reynolds, S.R.M., and Zweifach, B.W. (eds.): *Microcirculation*, Chicago: University of Illinois Press, 1959.

22. Fawcett, D.W.; and Wittenberg, J.: Structural Specializations of Endothelial Cell Junctions, abstracted, *Anat Rec* 142:231, 1962.

23. Fawcett, D.W.: "Comparative Observations on the Fine Structure of Blood Capillaries," in Orbison, J.L., and Smith, D. (eds.): *The Peripheral Blood Vessels*, Williams and Wilkins, Baltimore, 1963.

24. Fawcett, D.W.: An *Atlas of Fine Structure*, Philadelphia: W. B. Saunders, Co. 1966.

25. Fernando, N.V.P., and Movat, H.Z.: The Fine Structures of the Terminal Vascular bed: III. The Capillary, *Exp Mole Path* 3:87-97, 1964.

26. Palade, G.E.: Fine Structure of Blood Capillaries, abstracted, *J Appl Physics* 24:1424, 1953.

27. Palade, G.E.: Blood Capillaries of the Heart and Other Organs, *Circulation* 24:368-384, 1961.

28. Poliwoda, H.; Schmidt-Mattheisen, H.; and Stanbesand, J.: Pathogenesis and Therapy of Increased Vascular Fragility, *Bibl Anat* 7:235-241, 1965.

29. Majno, G., and Palade, G.E.: Studies on Inflammation: I. The Effect of Histamine and Serotonin on Vascular Permeability: An Electron Microscopic Study, *J Biophys Cytol* 11:571-605, 1961.

30. Marchesi, V.T.: Some Electron Microscopic Observations on Interactions Between Leukocytes, Platelets, and Endothelial Cells in Acute Inflammation, *Ann NY Acad Sci* 116:774-788, 1964.

31. Gersh, I., and Catchpole, H.R.: The Organization of Ground Substance and Basement Membrane and Its Significance in Tissue Injury, Disease and Growth, *Amer J Anat* 85:457-507, 1949.

32. Gersh, I., and Catchpole, H.R.: The Nature of Ground Substance of Connective Tissue, *Perspect Biol Med* 3:282-319, 1960.

33. Lee, R.H., and Lee, N.R.: The Peripheral Vascular System and Its Reactions in Scurvy: An Experimental Study, *Amer J Physiol* 149:465-475, 1947.

34. Goodman, M.; Greenspon, S.A.; and Krakower, C.A.: The Antigenic Composition of the Various Anatomic Structures of the Canine Kidney, *J Immunol* 75:96-104, 1955.

35. Krakower, C.A., and Greenspon, S.A.: "The Antigens of Capillary and Venular Basement Membrane Elucidated by the Use of Lens Capsule," in Colwell, A.R., and Meyer, K. (eds.): *Small Blood Vessel Involvement in Diabetes Mellitus*, Washington, DC: American Institute of Biological Sciences, 1964.

36. Kefalides, N.A., and Winzler, R.J.: The Chemistry of Glomerular Basement Membrane and Its Relationship to Collagen, *Biochemistry* 5:702-713 (Feb) 1966.

37. Friederici, H.H.R.; Taylor, H.; and Pirani, C.L.: The Fine Structure of Capillaries of the Synovium and Other Tissues in Scrubutic Guinea Pigs, *Lab Invest* 15:1442, 1966.

38. Cliff, W.J.: Observations on Healing Tissue: A Combined Light and Electron Microscopic Examination, *Phil Trans Roy Soc Lond*, Series B, 246: 305-325, 1963.

39. Schoefl, G.I.: Studies on Inflammation: III. Growing Capillaries—Their Structure and Permeability, *Virchows Arch* 337:97-151, 1963.

40. Friederici, H.H.R., and Pirani, C.L.: The Fine Structure of Peripheral Capillaries in Experimental Nephrotic Edema, *Lab Invest* 13:250-258, 1964.

41. Merrill, T.G., and Sprinz, H.: Ultrastructural Changes of Intestinal Capillaries in Cholera, abstracted, *Fed Proc* 25:466, 1966.

42. Gore, I., et al: Aortic Acid Mucopolysaccharides and Collagen in Scorbutic Guinea Pigs, *J Nutr* 87:311-316, 1964.

Science 150: 1589-1590 1965

Fast Reactions of Ascorbic Acid and Hydrogen Peroxide in Ice, a Presumptive Early Environment

Abstract. *Nonenzymatic decomposition of hydrogen peroxide proceeded more rapidly in ice than in liquid water. At 5×10^{-7}M ferric chloride or 10^{-8}M cupric chloride, breakdown of hydrogen peroxide was significant at −11° and −18° but negligible at +1°C. Ascorbic acid oxidation was faster in ice both with or without added metal ion. Nonparallel effects of metals and pH indicate mechanism changes in ice.*

Recent work has shown that several bimolecular reactions proceed more rapidly in ice than in liquid water (*1–6*). Kinetic analyses (*4, 5*) and inhibition by substrate analogs in frozen systems (*6*) suggest that the reaction mechanism has changed on freezing and that the ice structure may participate catalytically. These findings have possible implications in areas ranging from the storage of tissues and food at low temperatures to considerations of the origin of life on earth and elsewhere. We have now extended the studies in frozen solutions to metal ion–catalyzed reactions which behave similarly to enzymatic reactions. These include the breakdown of hydrogen peroxide and the oxidation of ascorbic acid.

For determination of hydrogen peroxide breakdown, an adaptation of the catalase assay method of Bonnichsen, Chance, and Theorell (*7*) was used. Solutions for storage were prepared in deionized distilled water and contained 0.0056*M* H_2O_2, 0.02*M* tris buffer at *p*H 7.2, or 0.02*M* acetate buffer at *p*H 5.5, and the desired amounts of $CuCl_2$ or $FeCl_3$. Freezing was initiated in a dry ice–acetone bath, and the samples were then transferred to −11° or −18°C. At half-hour intervals over periods of 3 to 5 hours, samples were thawed rapidly in a bath at room temperature, in the presence of 1-percent sulfuric acid, and titration was performed with 0.006*N* potassium permanganate. The reaction was first-order at all temperatures to at least 50 percent decomposition.

Acceleration of hydrogen peroxide decomposition occurred in ice under all conditions studied. Table 1 shows that at $5 \times 10^{-5}M$ iron the rate in ice (−11°) exceeded that in water (+1°C) by a factor of 28. Both rates decreased with decreasing iron concentration, although not in parallel. At $5 \times 10^{-4}M$ iron, the rate at −18° was 10 times that at 1°C. At $5 \times 10^{-7}M$ iron, the reaction rate was still significant at −18°, but zero at +1°C. Lowering the *p*H to 5.5 lowered the rates in the liquid and frozen solutions. The fall at −18°, however, was far more abrupt than that at +1°C, again indicating dissimilar reaction mechanisms.

Table 2 compares the rates for cupric chloride–catalyzed splitting of hydrogen peroxide. At −11° copper was considerably more effective than iron, although iron catalyzed the reaction somewhat better at 1°C. At $10^{-8}M$ copper, the reaction was appreciable even at −18°C ($k = 28 \times 10^{-5}$ min^{-1}). An additional index of the rate enhancement on freezing is the extent of substrate decomposition after 4 days; at $10^{-8}M$ copper, this figure was 78 percent at −11°, 50 percent at −18°, and only 4 percent at 1°C.

Ascorbic acid oxidation, measured by the spectrophotometric method of Racker (*8*), also followed clean first-order kinetics in frozen solutions. Table 3 compares the rates in liquid and frozen systems containing $10^{-4}M$ ascorbic

Table 1. Rates of Fe(III)-catalyzed splitting of hydrogen peroxide in liquid and frozen systems.

$FeCl_3$ (*M*)	*p*H	Rate [$10^5 k_{obsvd}$ (min^{-1})]		
		1°C	−11°C	−18°C
5×10^{-5}	5.5	14.9	24.7	17.6
5×10^{-4}	7.2	23.0		227
5×10^{-5}	7.2	9.2	255	
5×10^{-6}	7.2	7.7	96.7	
5×10^{-7}	7.2	0.0	52.2	22.6

Table 2. Rates of Cu(II)-catalyzed splitting of hydrogen peroxide in liquid and frozen systems; *p*H 7.2.

$CuCl_2$ (*M*)	Rate [$10^5 k_{obsvd}$ (min^{-1})]	
	1°C	−11°C
10^{-5}	0.0	272
10^{-6}	0.0	166
10^{-7}	0.0	130
10^{-8}	0.0	61

Table 3. Rates of ascorbic acid oxidation in liquid and frozen systems. Absorbance read at 245 mμ.

$CuCl_2$ (*M*)	*p*H	Rate [$10^5 k_{obsvd}$ (min^{-1})]	
		1°C	−11°C
5×10^{-6}	5.5	15.9	56.4
0	5.5	5.0	14.2
5×10^{-6}	5.0	4.6	5.8
0	5.0	3.4	3.8

acid and 0.02*M* acetate, buffered at *p*H 5.5 and 5.0. Unlike the hydrogen peroxide decomposition, this reaction proceeded both in the presence and absence of added metal ions. The rate at −11° exceeded that at +1°C under all of these conditions. At *p*H 7.2, all of the ascorbate oxidations occurred more rapidly than at lower *p*H levels, the reaction in ice being too rapid to give satisfactory kinetics under the conditions used.

At least two chemical species participate in each of the reactions reported here. The reactants, including molecular oxygen in the case of ascorbic acid oxidation, are clearly brought by freezing into an environment conducive to reaction. Whether this environment is the ice lattice or liquid microinclusions is not clear. However, the kinetic data appear to contradict a mechanism based upon the concentration increases resulting from phase separation.

The types of chemical reactions known to be capable of proceeding at significant rates in ice now include hydrolysis (*1*, *3*), dehydration (*2*), aminolysis (*4–6*), peptide formation (*9*), oxidation, and peroxide decomposition. In view of the facility with which these reactions occur, and as a result of Urey's postulate of a low-temperature origin of the earth (*10*), there is reason to question the assumption that water present only as frost or ice precludes prebiotic chemical evolution. It is evident, for example, that the combination of restricted diffusion and low temperature fails to slow down a number of reactions of biochemical importance. Second, one of the conditions regarded as essential for prebiotic interactions is concentration of various precursor molecules (*11*); the process of freezing could accomplish this concentration. Third, a frozen environment suggests limitations to the assumption that once living organisms have appeared they would consume biochemicals arising *de novo*, thereby perpetuating existing characteristics (L-amino acids, and so forth). In ice, newly formed chemical structures would probably be sequestered from living organisms and might evolve to considerable complexity.

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References and Notes

1. N. H. Grant, D. E. Clark, H. E. Alburn, *J. Am. Chem. Soc.* **83**, 4476 (1961).
2. W. H. Prusoff, *Biochim. Biophys. Acta* **68**, 302 (1963).
3. A. R. Butler and T. C. Bruice, *J. Am. Chem. Soc.* **86**, 313 (1964).
4. T. C. Bruice and A. R. Butler, *ibid.*, p. 4104.
5. H. E. Alburn and N. H. Grant, *ibid.* **87**, 4174 (1965).
6. N. H. Grant and H. E. Alburn, *Biochemistry* **4**, 1913 (1965).
7. R. K. Bonnichsen, B. Chance, H. Theorell, *Acta Chem. Scand.* **1**, 685 (1947).
8. E. Racker, *Biochim. Biophys. Acta* **9**, 577 (1952).
9. N. H. Grant, D. E. Clark, H. E. Alburn, *J. Am. Chem. Soc.* **84**, 876 (1962).
10. H. C. Urey, *Geochim. Cosmochim. Acta* **26**, 1 (1962).
11. J. D. Bernal, in *The Origins of Prebiological Systems and of Their Molecular Matrices*, S. Fox, Ed. (Academic Press, New York, 1965), p. 71.
12. We thank S. Hixson for valuable technical assistance.

16 September 1965

J. Amer. Pharm. Assoc. 30: 613-616 1941 3172

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

A. G. DuMez, Editor, Baltimore, Maryland

Volume XXX | *December, 1941* | Number 12 Consecutive No. 23

Studies on Barbiturates. XXV. The Effect of Vitamin C Level on Barbiturate Depression in Guinea Pigs*

By Melvin W. Green† and Rade R. Musulin†

King, *et al.* (1, 2), found that a large series of organic compounds, notably aromatic ketones and alcohols of the terpene series, as well as barbiturates caused an increased excretion of vitamin C in the urine of rats kept on a diet affording minimal vitamin C synthesis. Since the vitamin C content of the tissues did not deviate from the normal, it was concluded that the increased excretion was due to an increased synthesis. Ritz, Samuels and Addis (3) found an increased urinary excretion of vitamin C in rats fed carvone or salicylates. They concluded that feeding of carvone did not deplete the tissues of ascorbic acid while salicylates did. Gruber, *et al.* (4), found an increased vitamin C excretion in rats when fed sodium diphenyl hydantoinate (Dilantin sodium), a compound chemically related to barbiturates; the increased excretion being due to a loss of vitamin C from the tissues, notably the adrenals and the liver.

These findings suggested the following problems: (*a*) Is there a correlation between the vitamin C level of the diet and the degree of depression produced by barbiturates in animals depending entirely on dietary sources for their vitamin C supply? (*b*) In such animals, or humans, on a diet just barely sufficient in vitamin C, would the oft-repeated administration of certain barbiturates produce scorbutic states of various magnitudes? If a correlation between vitamin C level was found to exist, it was hoped that these studies might throw some light on the more general problem of the mode of action of vitamin C as a detoxifying agent.

EXPERIMENTAL

(*a*) *Effect of Vitamin C Level on Barbiturate Depression.*—For these experiments, healthy guinea pigs, weighing between 250 and 300 Gm., were fed a basal diet completely devoid of vitamin C, but complete in all other respects (5). After the animals ceased to gain weight, they were divided into groups as nearly balanced as possible with respect to weight and general health. One of these groups was used as a negative control and received the basal diet only, while the other groups received 0.25, 0.5, 1.0 and 2.5 mg. of pure vitamin C daily by mouth. It will be recalled that 0.5 mg. of

* This investigation was made possible by a grant from Hoffmann La Roche, Inc., which partially defrayed the expense of this study. Presented to the Scientific Section, A. Ph. A., Detroit meeting, 1941.

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vitamin C daily is just sufficient to maintain weight and permit no gain; 1.0 mg. of the vitamin will permit virtually normal growth. Each vitamin group was divided in turn into three groups, one of which served as a control and received daily intraperitoneal injections of 1 cc. of saline, while the other groups were given sodium pentobarbital (Nembutal) and sodium phenobarbital, respectively, daily by the intraperitoneal route.

Since the entire experiment involved a total of 50 animals, the determination of the mean sleeping time was not considered expedient and a system of scoring numerically the degree of depression was devised. If animals showed no deviation from the normal, a score of 0 was given, while the presence of general lassitude was awarded a score of 1. If the animal could not maintain its normal posture and assumed a lateral position, a score of 2 was awarded. Complete abolishment of the righting reflex was scored 5. The ability to locomote also was scored as 1, 2 or 3, depending upon the degree of incoördination observed. Using such a scoring method, the highest possible degree of depression was represented by a total score of 11. At no time did any of the animals lose its corneal reflex from the doses of barbiturates employed. The injections and observations were made daily for a period of 30 days.

Table I.—Effect of Vitamin C Intake on Sodium Pentobarbital[a] Depression in Guinea Pigs

Number of Animals	Daily Intake of Vitamin C (Total Mg.)	Average Degree of Depression at 30 Min.	Average Degree of Depression at 60 Min.	Improvement in Animals During Second 30-Min. Period in Per Cent
4	0.0	9.25	9.9	−1.1
4	0.25	9.9	7.3	26.3
4	0.5	6.6	3.9	40.9
4	1.0	7.0	2.7	61.4
4	2.5	5.5	1.8	67.3

[a] 15 mg./Kg.

Table I shows the results of the experiment with sodium pentobarbital given in doses of 15 mg.[1] At the end of 30 minutes after the time of injection, the animals on the higher vitamin levels were not so depressed as the ones on the lower vitamin intake, but the difference was not a marked one. At the end of 60 minutes, however, the difference was more significant. It will be noticed that the negative controls were actually slightly more depressed at the end of 60 minutes than they were at the end of 30 minutes. In the last column of the table, the degree of recovery during the second 30-minute period is expressed in terms of percentages, *i. e.*,

$$\frac{(\text{av. depression at 30 min.} - \text{av. depression at 60 min.}) \times 100}{\text{av. depression at 30 min.}}$$

From these data it is plain that the lower the vitamin C level, the greater the depression and the more protracted the recovery.

Table II shows a similar experiment with soluble phenobarbital administered in doses from 15 to 50 mg. Regardless of the dose of phenobarbital used, it will be noticed that it is almost consistently true that the higher the vitamin C level, the less the depression. Since phenobarbital is slower to take effect and has a longer duration of action than pentobarbital, the time relations are more difficult to correlate with the vitamin level. However, in those cases where extended observations were made, the animals on a high vitamin C level recovered more quickly. Small doses of phenobarbital were used because accumulation was expected, particularly in animals on the lower vitamin levels. However, no observable accumulation occurred at any level up to doses of 50 mg. of phenobarbital.

At the end of the experiment, two of the animals in the phenobarbital series and one in the pentobarbital series which were receiving 0.25 mg. of vitamin C daily, were now given 2.5 mg. of the vitamin daily for 7 days. As the vitamin supply was thus restored, the response to both barbiturates became less severe progressively. For example, the score representing the depression of the animal receiving daily injections of pentobarbital progressively fell from 11 to 5 as the vitamin was deposited in the tissues under the influence of the higher vitamin feeding.

Table II.—Effect of Vitamin C Intake on Sodium Phenobarbital Depression in Guinea Pigs

Number of Animals	Daily Intake Vitamin C[a]	Responses to Varying Doses of Sodium Phenobarbital[b]: 15 mg. (7 Days) After 30 Min.	15 mg. (7 Days) After 60 Min.	30 mg. (10 Days) After 30 Min.	30 mg. (10 Days) After 60 Min.	40 mg. (10 Days) After 30 Min.	40 mg. (10 Days) After 60 Min.	50 mg. (4 Days) After 30 Min.	50 mg. (4 Days) After 60 Min.
4	0.0	4.3	4.8	5.3	7.6	[c]	[c]	[c]	[c]
4	0.25	2.6	3.0	3.9	4.3	6.8	6.5	5.1	7.1
4	0.5	2.8	3.6	2.9	2.7	3.7	2.9	6.5	6.7
4	1.0	1.9	2.0	1.2	0.9	1.8	1.9	2.5	2.4
4	2.5	1.1	1.9	0.9	0.6	1.6	2.2	3.0	2.8

[a] Total mg.
[b] Mg. per Kg.
[c] All animals dead.

(*b*) *Effect of Barbiturate on the Vitamin C Level.*—It was thought that the repeated administration of barbiturates might deplete the tissues of vitamin C to such an extent that animals on a bare subsistence vitamin intake would develop a scorbutic state. This did not appear to be the case, for the growth

[1] Throughout this paper, doses of barbiturates are expressed in terms of mg. per Kg.

curves of animals on either barbiturate were substantially the same as those of the saline controls. Furthermore, the vitamin C content of the adrenals and the livers of many of the animals were determined by the indophenol titration method (16). The vitamin content of these tissues was essentially the same in both groups. These results are in agreement with other data in the literature. Therefore, it seems improbable that in humans on a low vitamin C level, the frequent administration of barbiturates would cause an increased vitamin requirement.

There is considerable evidence in the literature that vitamin C may play a role in the detoxification of many types of organic compounds (3, 4, 6, 7, 8, 9, 10). One way in which vitamin C could be of assistance in the detoxification of barbiturates is by the formation of a simple barbiturate-ascorbate complex. To test such a possibility, the vitamin C content of the guinea-pig urine was determined before and after mild acid hydrolysis and was found not to vary. Since mild hydrolysis made no more vitamin available, it was concluded that no such complex was eliminated, at least in a manner analogous to glycuronate conjugation, unless the complex was capable of reacting with the indophenol dye used for titrating the urine. King, *et al.*, observed similar results with albino rats (1, 2).

Because vitamin C is a sugar acid lactone, it is conceivable that the tissues can convert it in part to a glycuronic acid. Since glycuronic acid is widely recognized as a detoxifying agent, it was thought possible that the vitamin detoxified barbiturates through glycuronate complex formation. To test such a hypothesis, the reducing substances of guinea-pig urine of barbiturized animals were determined by the Scales (11) method. The reducing substances present, however, were substantially accounted for in terms of ascorbate itself.

On the basis of the last two findings, any conjugative mechanism for the detoxification of barbiturates must be denied or it must be assumed that the conjugation takes place in the tissues and that the conjugates are so labile as to preclude their presence in the urine.

DISCUSSION

The data presented in this paper show that there is a definite correlation between the vitamin C intake and the response of guinea pigs, animals unable to synthesize the vitamin, to pentobarbital and to phenobarbital. At present, the explanation for this correlation, however, is a difficult if not an impossible task, for the specific mechanism of action of vitamin C and the modification of metabolic processes by this vitamin are unknown. Although the vitamin is capable of undergoing reversible oxidation and reduction, it has never been definitely identified with any specific enzyme system in animal tissues. The work on rats referred to above, showing that the feeding of barbiturates causes an apparent increase in the vitamin C synthesis by the tissues, makes it permissible to assume that this vitamin plays some role in the detoxification of barbiturates. Our finding on guinea pigs would lend support to this assumption if an exhaustion of the animal's vitamin depot would accompany oft-repeated administration of barbiturates. However this was not the case. Since the vitamin was not depleted from the tissues, it can be inferred that the frequent administration of barbiturates to humans is not likely to produce scorbutic states. This is further suggested by the findings of Wright, *et al.* (12), that the daily administration of 180 mg. (total dose) of phenobarbital to humans produced no change in the vitamin C concentration of whole blood, plasma or urine. The urine of our barbiturate-treated animals contained no abnormal amount of reducing substances such as sugars or glycuronates.

Koppanyi, *et al.* (15), have stated that animals in a good physiological condition can destroy more barbiturates than when they are in a state of "ill health." In observing individual animals used during this experiment it was apparent that the animals appearing to be less vigorous and in ill health were more severely depressed by the barbiturates and that the depression lasted appreciably longer. Indicative of such an empirical interpretation, Hjort, *et al.* (13), found a correlation between the mean sleeping time of mice, under the influence of a synthetic ureide, and the completeness of the diet. The nature of the deficiencies of their diets was not stated, but some were assumed to be deficient in protein, vitamin B complex and perhaps calcium.

Until the specific action of vitamin C is better understood, one is not justified in assigning a definite role to vitamin C in the detoxification of barbiturates; one must be content with stating that the general physiological state of animals is an important factor in modifying the action of hypnotics.

When this paper was completed, our attention was called to a note by Keuter, Richards and Klatt (14) on the action of barbital, pentobarbital and pentothal in vitamin C-depleted guinea pigs. These authors stated that at different levels of C avitaminosis the sleeping time from pentobarbital was markedly prolonged while that from barbital or pentothal was little affected. These authors suggest that general metabolic disturbances and changes in the liver following the C avitaminosis are responsible for the prolongation of pentobarbital narcosis. They suggest that the liver is particularly instrumental in the destruction of the short-acting barbiturates, but not of the long-acting group, pentothal being a notable exception. Thus two groups of investigators using slightly different methods of approach have found that lack of vitamin C leads to a prolongation of sleeping time in pentobarbital narcosis. Here unfortunately the agreement stops because the above investigators report that a long-acting barbiturate, barbital, is not appreciably influenced by lack of vitamin C, whereas we found that another long-acting barbiturate, phenobarbital, behaves like a short-acting barbiturate under identical conditions.

Moreover, there is some reason to question the validity of the conclusion of the above investigators because the main difference that we found between the action of barbiturates at a high and at a low vitamin C level is not so much concerned with the prolongation of sleeping time as with the magnitude of the initial depression. We are inclined to believe that a given hypnotic has a greater initial effect in animals at a lower physiological level than in animals at a higher physiological level. These initial differences were very readily observed in the individual animals.

SUMMARY

1. There is a correlation between the vitamin C level and the response produced by phenobarbital and by pentobarbital in guinea pigs. The higher the vitamin level, the less the depression produced by the barbiturates.

2. The frequent administration of pentobarbital and phenobarbital to guinea pigs did not cause a depletion of vitamin C from the tissues.

3. It is suggested that the most likely cause for the effect of vitamin C level on barbiturate depression is the altered general metabolism produced by the lack of this vitamin rather than an effect produced in major degree by direct conjugation.

The authors wish to thank Dr. C. G. King of the University of Pittsburgh for suggesting this problem, Dr. T. Koppanyi of the Georgetown University School of Medicine for guidance in the selection of the barbiturates and advice on the measurements of barbiturate depression, and Dr. R. D. Shaner, Medical Director of the Hoffmann La Roche, Inc., for his constant interest in the problem and the generous supply of vitamin C.

REFERENCES CITED

(1) Longenecker, H. E., Musulin, R. R., Tully, R. H., and King, C. G., *J. Biol. Chem.*, 129 (1939), 445.

(2) Longenecker, H. E., Fricke, H. H., and King, C. G., *Ibid.*, 135 (1940), 497.

(3) Ritz, N. D., Samuels, L. T., and Addis, G., *J. Pharmacol.*, 70 (1940), 362.

(4) Gruber, C. M., *et al.*, *Ibid.*, 71 (1941), 268.

(5) Sherman, H. C., La Mer, V. K., and Campbell, H. L., *J. Am. Chem. Soc.*, 44 (1922), 165.

(6) King, C. G., and Luck, J. M., *Annual Review of Biochemistry*, Stanford University, 8 (1939), 392.

(7) Onoyama, T., *Mitt. med. Akad. Kioto*, 27 (1939), 1187.

(8) Koike, M., *et al.*, *Osaka Igk. Z.*, 35 (1936), 1705; *Chem. Abstr.*, 32 (1938), 658.

(9) Holmes, H. N., Campbell, K., and Amberg, E. F., *J. Lab. Clin. Med.*, 24 (1939), 1119.

(10) Beyer, K. H., *J. Pharmacol.*, 71 (1941), 394.

(11) "Methods of Analysis—A. O. A. C.," 4th Edition, page 478, Washington, D. C., 1935.

(12) Wright, S. T., Callaghan, T. J., and Minnich, V., *Proc. Soc. Exptl. Biol. Med.*, 47 (1941), 490.

(13) Hjort, A. M., de Beer, E. J., and Fassett, D. W., *J. Pharmacol.*, 65 (1939), 79.

(14) Richards, R. K., *et al.*, *J. Biol. Chem.*, 140 (XXXV, Proceedings) (1941), lxxii.

(15) Koppanyi, T., Linegar, C. R., and Dille, J. M., *J. Pharmacol.*, 58 (1936), 199.

(16) Musulin, R. R., and King, C. G., *J. Biol. Chem.*, 116 (1936), 409.

J. FOOD SCI., 36(6): 940-942, 1971

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RETARDATION OF OXIDATIVE COLOR CHANGES IN RAW GROUND BEEF

SUMMARY—Ascorbic acid plus either BHA or PG effectively retarded both lipid and pigment oxidation in raw ground beef for up to 8 days of refrigerator storage in oxygen-permeable film. Both trained and consumer-type panels judged the additive-treated meat to be significantly better than controls. Percent metmyoglobin was found to be a useful objective measurement of color in raw beef, but total pigment concentration did not provide an adequate evaluation of color. Treatment with these additives did not appear to mask bacterial spoilage.

INTRODUCTION

THE COLOR of raw meat is a primary factor used by the consumer to judge meat quality. In order to reveal the fresh red color (oxymyoglobin), meat is commonly covered with an oxygen permeable wrapping in the display counter. However, this type of wrapping renders meat susceptible to metmyoglobin formation (brown discoloration) and rancidity during refrigerated storage (Landrock and Wallace, 1955; Greene, 1969). When meat color changes from bright red to brown, the meat loses much of its appeal to the consumer and is not purchased. Since meat packers wish to use central packaging, suitable methods of reducing nonmicrobial changes are needed to avoid economic loss.

Many studies, in both foods and in model systems, provide evidence that lipid and pigment oxidation (metmyoglobin formation) are closely interrelated. Ferric pigments are catalysts for lipid oxidation (Younathan and Watts, 1960; Brown et al., 1963). The free radical products of lipid oxidation are known to damage pigments (Haurowitz et al., 1941). Researchers have reported a positive correlation ($r_s = 0.73$) between metmyoglobin accumulation and lipid oxidation in raw meat (Hutchins et al., 1967), but it has not been established whether oxidation of the pigment precedes and causes lipid oxidation or whether pigments are damaged in the presence of oxidizing lipids. Greene (1969) attempted to retard rancid odor formation in refrigerated ground raw beef with the common lipid antioxidants propyl gallate (PG) and butylated hydroxyanisole (BHA). Not only were lipid oxidation and rancid odor formation retarded in treated samples, but these samples retained their red color somewhat longer than controls. Low concentrations of the common reducing agent, ascorbic acid (AH_2), have a color preservative effect, although higher levels often accelerate pigment oxidation and destruction (Watts and Lehmann, 1952; Rickert et al., 1957). A combination of additives may be extremely effective in preserving both pigments and lipids.

The objective of this work was to study the effects of antioxidants and ascorbate alone and in combination toward prolonging the shelf-life of refrigerated raw ground beef. Meat pigments and the effect lipid oxidation has on their color are of paramount importance to the packer, as is the relationship between objective color measurements and acceptability of meat to consumers; rancidity is a secondary problem which can be easily remedied if phenolic antioxidants are approved for use in fresh meat.

EXPERIMENTAL

FRESH BEEF eye of round or top round (U.S. Choice) of unknown history was purchased at a local supermarket. These cuts were removed from the intact hindquarter shortly before sample preparation. The initial pH was between 5.6–5.7. The meat was trimmed of external fat and excess marbling or connective tissue and ground twice in a stainless steel Hobart meat grinder (Hobart Co. Model 4812). Immediately after grinding, the meat was mixed thoroughly and aliquots taken for addition of the various additives. PG and BHA were used at a concentration of 0.01%; AH_2 at 0.05%. Amounts of reagents used in combination were additive. PG and AH_2 were dissolved in glass-distilled water, but BHA was dissolved in propylene glycol (propylene glycol itself was found to have no effect on color or flavor properties of the meat). Since microbiological spoilage can contribute to color change, 30 ppm chlortetracycline (CTC) was added in most variables to prevent interference from this source during storage. Hutchins et al. (1967) determined that CTC does not interfere with metmyoglobin reducing activity or oxygen consumption in raw ground beef. 80g portions from each aliquot were wrapped in highly oxygen-permeable "Prime Wrap" by Goodyear (950 ml/100 sq in./hr) after being shaped in a hamburger press to obtain uniform surface area. The meat patties were immediately stored at the test temperature of 3–5°C.

Analyses

Analyses run on samples initially and after several days of storage included:

Objective color measurement. Percentage of meat pigments as metmyoglobin (MetMb) was determined by reflectance spectrophotometry (Stewart et al., 1965) using an Hitachi Perkin-Elmer 139 spectrophotometer.

Objective lipid oxidation measurement. Milligrams of malonaldehyde per 1000g meat (TBA number) was determined by the method of Tarladgis et al. (1960).

Determination of total pigment content. Milligrams of total pigment per g meat were determined by a water extraction method developed by Rickansrud and Henrickson (1967).

Subjective judgments of rancidity in raw and cooked meat and of raw meat color. A trained panel was used to evaluate rancidity and color on an intensity scale of 6 (no detectable rancidity, very good color) to 1 (very strong rancid odor; very badly discolored). This technique has been described by Tarladgis et al. (1959). In addition to the trained panel, 12–36 freshman home economics students were asked to evaluate the color of meat samples after 8-days storage. The students were not asked to make a purchase-reject decision on each sample, but merely to indicate which samples they would purchase or reject in a self-service supermarket situation. All correlations were calculated using the Spearman rank-order correlation coefficient (r_s) (Siegel, 1956).

RESULTS & DISCUSSION

REPRESENTATIVE data for eye of round are in Table 1. All antioxidant-treated samples had TBA numbers of less than 0.5, indicating that lipid oxidation was prevented. In samples with only BHA or PG treatment, pigment oxidation was greatly reduced (approximately 50% over controls), but it was not prevented. When only AH_2 was used, both pigment and lipid oxidation were slowed. Ascorbic acid may have been able to function as a MetMb reductant until lipid oxidation free radicals became numerous and either destroyed the AH_2 activity or overpowered AH_2. Lipid oxidation in these samples may have been retarded by maintaining the pigment largely in the ferrous state.

When the combination of antioxidant and AH_2 was used, pigment oxidation was greatly retarded throughout the 8 days of storage (11% MetMb vs. 82% for control; 16% and 26% vs. 77%). Usually the effects of antioxidant and AH_2 together were greater than those of either additive alone. To determine if phenolic antioxidants were protecting AH_2 from destruction by free radicals from lipid oxidation, samples containing PG + AH_2, BHA + AH_2 alone were assayed for AH_2 loss over a period of 10 days (Loeffler

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Table 1—Effect of additives on pigment and lipid oxidation in refrigerated raw ground beef eye of round stored 8 days

Variable	MetMb %	Color score[a]	TBA number[d]	Total pigment mg/g sample	Total pigment Loss, %
Control-initial	11	–	1.2	3.19	0
Control	89	1.3	11.9	2.54	20.4
PG	47	3.6[b]	0.6	2.78	12.7
AH_2	50	3.3[b]	5.4	2.75	14.1
Control-initial	14	–	0.9	3.04	0
Control	82	1.2	10.0	2.40	21.1
PG	41	4.1[b]	0.3	2.56	15.7
AH_2	46	3.9[b]	2.8	2.67	12.2
PG + AH_2	11	5.2[b]	0.2	2.73	10.0
Control-initial	0	–	0.3	3.22	0
Control	77	–	8.9	2.57	20.2
BHA	38	3.9	0.3	2.77	13.6
AH_2	28	4.4	2.7	2.80	14.3
BHA + AH_2	16	5.3[c]	0.3	2.99	7.3
PG + AH_2	26	4.9[c]	0.4	2.89	10.5

[a]Color scores: 6 (very good color) to 1 (very badly discolored).
[b]Significant > control.
[c]Significant > BHA.
[d]Higher initial TBA numbers reflect time lapse between grinding of meat and completion of sample preparation.

Table 2—Color acceptability of refrigerated raw ground beef to a consumer panel after 8 days storage[a,b]

MetMb %	Purchase %	Reject %	Variable
11	100	0	PG + AH_2
16	100	0	BHA + AH_2
26	100	0	PG + AH_2
26	94	0	PG + AH_2
28	94	0	AH_2
32	75	8	BHA + AH_2
34	100	0	BHA + AH_2
41	48	4	PG
46	50	4	AH_2
47	55	0	PG
50	74	3	AH_2
57	17	42	BHA
59	0	100	BHA + PG
60	19	50	PG + AH_2
89	0	100	Control

[a]Data are taken from five experiments.
[b]Judges were not asked to make a purchase-reject decision on each sample, but merely to indicate which samples they would purchase or reject in a self-service supermarket situation.

and Ponting, 1942). All samples lost 10–80% of the AH_2 within 6 days. Phenolic antioxidants were not protecting AH_2. The effectiveness of the combination cannot be readily explained except as synergism.

In relating lipid and pigment oxidation to actual pigment loss, it can be seen (Table 1) that amount of pigment loss is dependent upon amount of pigment oxidation. A highly significant correlation ($r_s = 0.72$; significant at 0.0005 level) was found between % MetMb and % total pigment loss. Less overall pigment loss and less ferric pigment were found in samples containing BHA or PG plus AH_2 than in samples treated with BHA or PG alone. Ferric pigment formation and total pigment loss were greatest in control and AH_2 samples where lipid oxidation was apparent. If denaturation of globin had been the primary target of lipid oxidation free radicals, it should have been observed in the total pigment determination where a water extraction is made. Precipitation of denatured proteins or turbidity in extracts would be logical expectations, but they were not found. This suggests that lipid oxidation free radicals act by first oxidizing the heme to MetMb rather than by initially damaging the globin.

Figure 1 is a graphic representation of the relationship between color scores and % MetMb. The area enclosed within the dotted lines shows that the larger proportion of samples considered by trained judges to have a good or very good color contained between 0–40% MetMb. In Table 2, which shows acceptability to the consumer panel, the line of rejection again appears to be between 30–40% MetMb. The relationship between % MetMb and color scores (Fig. 1) is highly significant at the 0.0005 level ($r_s = 0.73$). This indicates that measurement of % MetMb by the method of Stewart et al. (1965) is a useful criterion for meat color acceptability.

No apparent relationship exists between total pigment concentration and degree of discoloration as measured by color scores. Samples with color scores below 4 (slightly discolored) had more than 45% MetMb regardless of pigment concentration. This suggests that the relative amount of ferric pigments rather than pigment concentration is the decisive factor in color acceptability.

Raw and subsequently cooked samples were rated for rancid odor by the trained panel. Results were in general agreement with previously reported findings, i.e., antioxidant-treated samples rated higher than untreated controls; sensory scores correlated significantly with TBA numbers ($r_s = 0.43$ for raw, significant at 0.005 level; $r_s = 0.59$ for cooked, significant at 0.0005 level).

In order to recommend use of these additives in fresh meat on a commercial basis (although use of phenolic antioxidants in food is generally under attack), it is important to know whether or not the additives will obscure bacterial change. Bacterial counts on each variable (Service Lab., CSU Microbiology Dept.) were included in several experiments. Samples with and without antioxidants and AH_2 and each of these variables with and without CTC were included. In general, spoiled samples were noticeably brown or green whether or not they contained the antioxidant and AH_2. Thus, it appears likely that the consumer would be able to detect spoiled meat even though the color and flavor would be protected during its unspoiled shelf life. Further studies of this type should be carried out in conjunction with use of these additives. A significant finding is the length of time required for bacterial spoilage to become

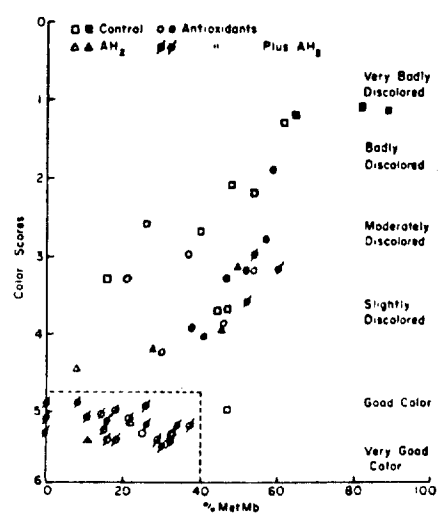

Fig. 1—Relationship of % MetMb to color scores. Unshaded symbols represent determinations at 3 days of storage; shaded at 8 days. ($r_s = 0.73$, significant at 0.0005 level.)

apparent. Meat ground to order at the supermarket contained 3–10 times the microbial flora initially found in meat ground in the laboratory. Shelf life of laboratory-ground meat was considerably longer than that of supermarket-ground beef (6 days vs. 3 days). Therefore, sanitation at the place of cutting and packaging meats play a more important role in shelf life than may be realized. (This topic is well reviewed by Brody, 1970.) CTC did not influence lipid oxidation, but it did retard pigment oxidation resulting from bacterial action.

CONCLUSIONS

THIS STUDY clearly indicates that a combination of PG or BHA and AH_2 is effective in prolonging the shelf life of refrigerated ground beef. Treated meat was judged to be significantly better in color than controls by both trained and consumer-type panels. When MetMb levels were greater than 40%, judges down graded or rejected samples for purchase. Use of these additives does not appear to mask bacterial spoilage.

REFERENCES

Brody, A.L. 1970. Shelf life of fresh meat. Modern Packag. 43: 81.

Brown, W.D., Harris, L.S. and Olcott, H.S. 1963. Catalysis of unsaturated lipid oxidation by iron protoporphyrin derivatives. Arch. Biochem. Biophys. 101: 14.

Greene, B.E. 1969. Lipid oxidation and pigment changes in raw beef. J. Food Sci. 34: 110.

Haurowitz, F., Schwerin, P. and Yenson, M.M. 1941. Destruction of hemin and hemoglobin by the action of unsaturated fatty acids and oxygen. J. Biol. Chem. 140: 353.

Hutchins, B.K., Liu, T.H.P. and Watts, B.M. 1967. Effect of additives and refrigeration on reducing activity, metmyoglobin and malonaldehyde of raw ground beef. J. Food Sci. 32: 214.

Landrock, A.H. and Wallace, G.A. 1955. Discoloration of fresh red meat and its relationship to film oxygen permeability. Food Technol. 9: 194.

Loeffler, H.J. and Ponting, J.D. 1942. Ascorbic acid. Rapid determination in fresh, frozen or dehydrated fruits and vegetables. Ind. Eng. Chem. Anal. Ed. 14: 846.

Rickansrud, D.A. and Henrickson, R.L. 1967. Total pigments and myoglobin concentration in four bovine muscles. J. Food Sci. 32: 57.

Rickert, J.A., Bressler, L., Ball, C.O. and Stier, E.F. 1957. Factors affecting quality of prepackaged meat. 2. Color studies. B. Effects of storage time, storage temperature, antioxidants, bacteria, light, freezing and fat upon color of product. Food Technol. 11: 567.

Siegel, S. 1956. "Nonparametric Statistics for the Behavioral Sciences." McGraw-Hill, New York.

Stewart, M.R., Zipser, M.W. and Watts, B.M. 1965. The use of reflectance spectrophotometry for the assay of raw meat pigments. J. Food Sci. 30: 464.

Tarladgis, B.G., Younathan, M.T. and Watts, B.M. 1959. Antioxidants in irradiated cooked pork. Food Technol. 13: 635.

Tarladgis, B.G., Watts, B.M., Younathan, M.T. and Dugan, L. 1960. A distillation method for the quantitative determination of malonaldehyde in rancid foods. J. Am. Oil Chem. Soc. 37: 44.

Watts, B.M. and Lehmann, B.T. 1952. The effect of ascorbic acid on the oxidation of hemoglobin and the formation of nitric oxide hemoglobin. Food Res. 17: 100.

Younathan, M.T. and Watts, B.M. 1960. Oxidation of tissue lipids in cooked pork. Food Res. 25: 538.

Ms received 4/24/71; revised 6/30/71; accepted 7/5/71.

Work supported by the Colorado Agricultural Experiment Station.

Proc. Soc. Exp. Biol. and Med. 32(7): 1157-1160. 1935-

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Vitamin C and Diphtheria Toxin.

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In previous work,[1] a relation between Vitamin C and diphtheria toxin was found. Freshly prepared highly labile toxin was used.

In the present work these findings were confirmed and extended using a standardized, stable toxin. In relation to this toxin, we have examined 2 different actions, (a) the effect on guinea pigs of preliminary feeding of excess Vitamin C (cevitamic acid) and of

[1] Harde, E., *C. R. de l'Acad. des Sc.*, 1934, **199**, 618; Harde, E., and Phillipe, M., *C. R. de l'Acad. des Sc.*, 1934, **199**, 738.

treatment with the vitamin,* and (b) the action *in vitro* of cevitamic acid on the toxin, on antitoxin and on mixtures of toxin and antitoxin.

A. Effect on guinea pigs of preliminary feeding of excess vitamin C (cevitamic acid) and of treatment with the vitamin. Four guinea pigs were fed excess spinach or cabbage in addition to the stock diet of hay, oats and carrots for 10 days. They were then injected with 1 M.L.D. of toxin and kept on the same diet. No increased resistance was shown as compared with controls fed the stock diet. In a second experiment, 5 guinea pigs were given 10 mg. of pure cevitamic acid in ½ to 1 cc. salt solution by mouth daily for 3 days. Three of these animals were also injected daily, intramuscularly or subcutaneously,† with 10 mg. of the cevitamic acid in a similar solution. These animals were then injected with 1 M.L.D. of standardized toxin, and the vitamin C treatment continued daily. In comparison with the control animals injected with 1 M.L.D. at the same time, no increased resistance was shown, all the animals dying in 4 to 5 days.

These results differed from those obtained in previous work using a freshly prepared toxin, in which 3 out of 6 animals survived, and 5 controls died. A freshly prepared toxin is very unstable, losing rapidly a certain part of its toxicity, and it may also be more sensitive to vitamin C.

Animals injected with toxin may be extremely sensitive to the shock of handling. Occasionally during the feeding one of these animals died suddenly. There is always the possibility of some of the liquid going into the bronchi. Autopsies have shown this in a number of cases. We have also noticed at autopsy that subcutaneous or intramuscular injections of vitamin C were not always completely absorbed.

In another series of experiments, therefore, the animals were handled with great care so that there was very little shock, only the vitamin C solution being fed and injections avoided. A salt solution of 10-20 mg. of cevitamic acid was given to each of 4 guinea pigs; after the first or second day, each was injected with 1 M.L.D. of standardized toxin, and the feeding continued daily. The controls died on the 4th day. All the treated animals lost weight and showed indura-

* We thank the Hoffman LaRoche Company for the Redoxon-Cevitamic Acid they kindly supplied us.

† These solutions were always rendered neutral to litmus using a sodium hydroxide solution and sterilizing by bringing to a boil. To some animals carotene was also given by mouth without any effect being noted.

tion, one died on the 5th day and 1 on the 6th day. To the 2 others, vitamin C feeding was continued until the 8th day. These animals survived 20 and 22 days, dying of pneumonia. A second series gave similar results. One animal died on the 3rd day with lung lesions, one on the 9th day, and the remaining 2 survived. The 4 controls died in from 3 to 5 days.

B. Action *in vitro* of cevitamic acid on toxin, on diphtheria antitoxin and on mixtures of toxin and antitoxins. To 1 or 2 M.L.D.'s of toxin in 1 cc. of salt solution was added 1 cc. of a neutral solution of cevitamic acid, 10 mg. per M.L.D. This was slowly mixed, care being taken not to bubble air through it. Four guinea pigs injected with these solutions showed no loss of weight and no local symptoms, while the controls died in 4½ days. This experiment was repeated with similar results. With 5 M.L.D.'s using 10 mg. of the vitamin per M.L.D., 2 guinea pigs survived and 2 died ten days after injection. With a toxin solution containing 10 M.L.D.'s and treated with the same amount of vitamin C and injected, 2 animals survived, while 2 animals injected with 10 M.L.D.'s to which 60 mg. of vitamin C had been added died after 4 days, and one guinea pig receiving 20 M.L.D.'s and 100 mg. of vitamin C died in 48 hours.

We are now testing whether prolonged contact or increased amounts of cevitamic acid are necessary for action on the toxin.

In the earlier experiments a partial immunity was found in animals that had survived these injections when tested with 1½ M.L.D.'s. In this work the surviving animals were injected with 2 M.L.D.'s 5 to 6 weeks after the first injection. They all died in 2 to 4 days, suggesting that there had been little if any immunity produced by the first injection.

If vitamin C is to be used clinically as an adjunct to antitoxin in the treatment of diphtheria, it is necessary to test its action on the antitoxin.

To a standard antitoxin was added 10 mg. per unit of cevitamic acid, dissolved in a neutral salt solution. This was thoroughly mixed but not aerated and allowed to remain for 24 hours at 4°C. protected from the light. Two guinea pigs were injected with this mixture to which was added ½ L+ of toxin, 2 with one L+, and 2 with 1½ L+. For controls a freshly prepared standard unit of antitoxin was used with similar amounts of toxin. The only difference in resistance between these groups was shown in the one L+ dose. The guinea pigs receiving the solution containing the vitamin C lived 2 days longer than the controls, which died between the 3rd

and 4th days. No deleterious action of Vitamin C on the antitoxin was shown. Experiments were then done to note if the vitamin had any effect on toxin antitoxin mixtures. One unit of antitoxin, one L+ of toxin, and 10 mg. of cevitamic acid in salt solution were made up to 4 cc. with a salt solution, carefully mixed without aeration and kept at room temperature protected from the light for one hour. Controls without the cevitamic acid were kept under the same conditions. Three guinea pigs were injected in each series. The controls died within 4 days while the animals injected with the cevitamic acid mixture showed no symptoms and survived.

A final experiment was made with unneutralized cevitamic acid. One hundred and fifty mg. of the vitamin was dissolved in one cc. of standardized toxin, 500 M.L.D.'s per cc. The pH was between 4 and 4.4. The mixture was kept for 18 hours at room temperature. The guinea pigs were injected with one, 1¼ and 2 M.L.D.'s of the treated mixture. They showed no effects of the injection while the controls died. Here the vitamin was neither heated nor neutralized and was only added in the proportion of one mg. to 3½ M.L.D.'s. Further experiments are necessary to determine what factors are responsible for this detoxification.

Summary. Vitamin C under certain conditions increased the resistance of guinea pigs to injection of 1 M.L.D. of a standardized diphtheria toxin. Injections of mixtures of toxin and vitamin C if the solutions had been in contact for 1 hour at room temperature before injection were less toxic. The guinea pigs that had survived these injections 5 to 7 weeks showed but slight if any immunity when tested by the injection of 2 M.L.D.'s of the toxin. Vitamin C caused no destruction of the antitoxic properties of antitoxin or of the slightly toxic toxin antitoxin mixture. Only clinical work can determine whether the increased resistance to diphtheria toxin shown by guinea pigs treated with vitamin C can be applied to human beings. While we were engaged in these experiments Dr. C. G. King told us that he and Dr. Bessey[2] were working in the same field and had also found a relationship between vitamin C and diphtheria toxin.

We wish to thank Mr. Andrew Mackey for his aid in carrying out the experiments.

[2] Bessey, O. A., and King, C. G., *J. Nutrition.* In press.

J. Hyg. 39: 35-44 1939

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INVESTIGATION ON THE SEASONAL FLUCTUATION OF VITAMIN C EXCRETION IN PALESTINE

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(With 4 Figures in the Text)

Since Harris *et al.* (1933) first showed the significance of urinary vitamin C excretion, numerous papers have been published on vitamin C excretion by man. Few of these investigations deal with excretion of vitamin C by normal individuals under natural nutritional and climatic conditions. v. Euler & Malmberg (1935) carried out a comprehensive study of the vitamin C excretion of the inhabitants of north Sweden, south Sweden and Stockholm. Their observations, however, are based on but a few random samples obtained during the winter months. Hamel (1937) investigated the daily vitamin C excretion of different classes of the population of Rostock, during a small part of the year. The investigations of van Eekelen & Wolff (1936) dealt with thirty-three Dutch families and were intended mainly to yield information on the vitamin C nutrition of the Dutch population, rather than on the seasonal fluctuation in vitamin C excretion in urine.

The aim of the present investigations was twofold: (1) to follow the vitamin C excretion in man under prevailing nutritional conditions, and (2) to ascertain the influence of the specific natural nutritional conditions of Palestine on the seasonal variations in the excretion of vitamin C.

Technique

The vitamin C excretion in the urine of thirty adult residents of Jerusalem of both sexes (mostly staff members of the Department of Hygiene and Bacteriology or students of the Hebrew University) was followed over a period of one year from November 1936 to November 1937. The urine samples were obtained at intervals of 8–10 days, mostly in the morning hours. In all, over 1000 urine samples of this class were examined.

Once a month samples of the morning urine were analysed from each of twenty-five children attending the day nursery of the Straus Health Centre at Jerusalem. These children, aged 2–5 years, are kept in the nursery throughout the day and, as they are fed there, constitute a group of fairly uniform nutrition.

Investigations were also conducted at two places which represent climatic and nutritional extremes; the workers at Kalliah at the Dead Sea, and of the

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settlements Givath-Brenner and Dederah in the Coastal Plain. Kalliah is 390 m. below the sea-level, and is distinguished by its low rainfall and high temperature—a dry tropical climate. The examinations were made once in the summer and once in winter; the summer investigation comprised fifty-eight samples and the winter investigation forty-seven. Gederah and Givath-Brenner lie in the centre of the Palestine citrus culture and enjoy a Mediterranean climate. The citrus crop ripens in the winter months, and consequently here too the tests were carried out once in winter (sixty-two tests) and once in summer (sixty-one tests). The subjects consisted of the agricultural workers and their families; the common kitchen at Givath-Brenner and Gederah provided a group of people under approximately uniform nutrition.

We limited ourselves to determinations of the vitamin C content of samples of the urine, since under the conditions of our work it was impossible to obtain 24 hr. samples of urine. From a few subjects we were able to obtain at different times of the year the 24 hr. output of urine, and these outputs furnish a general idea of the average daily excretion of urine. In adult persons the quantity varied from 1250 to 1500 c.c. during the winter months and 600 to 800 c.c. during the summer months.

Although the urine samples were collected into dark bottles and as a rule analysed the same day the addition of vitamin C stabilizers was found to be necessary. The problem of stabilization of vitamin C in various solutions is still obscure. Leibowitz & Guggenheim (1938) investigated the question of stabilization of vitamin C by acids and showed that the different acids tested provided different degrees of protection, according to the substances present in the solutions. For urine examination sulphuric acid proved to be most suitable; hence, 2 c.c. concentrated sulphuric acid were added to 100 c.c. in the dark brown bottle used for collecting the urine samples.

The vitamin C determinations were made with dichlorphenol-indophenol by Tillmans' method. The titre of the indicator was standardized against a known solution of ascorbic acid, and the vitamin C in the urine determined by the amount of urine required to bring a given amount of the dichlorphenol-indophenol solution to a faint pink. The results are expressed as mg./litre of urine.

Results

The results show that there is a regular and constant seasonal fluctuation in the vitamin C excreted in the urine; it is consistently high in winter and low in summer. The data relating to the different groups are summarized below.

(a) *Findings on thirty adult residents in Jerusalem*

Table I and Fig. 1 present the vitamin C values found. The figures represent the monthly averages of the analyses of all adult samples of urine.

The high order of values found in November to May and the low order of values found in June to October are notable. If we assume on the basis of

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data available in the literature that the standard vitamin C excretion is 15–35 mg./l. (Harris & Ray, 1935; Ippen, 1935; v. Euler & Burstroem, 1936), it is apparent that the average values for November to May are well above the

Table I. *Urinary excretion of vitamin C in Jerusalem during one year (average of samples tested)*

Month	30 adults		25 children	
	Range, mg./l.	Average, mg./l.	Range, mg./l.	Average, mg./l.
January	11–470	61	14–237	57
February	12–540	56	14–185	44
March	11–330	69	11–340	64
April	11–350	55	10– 78	41
May	11–590	61	8–144	31
June	10–100	28	10– 67	25
July	10– 62	27	10– 69	22
August	7– 60	25	8– 34	22
September	7– 50	23	11– 31	19
October	5–129	27	9– 72	23
November	13–120	42	11– 84	31
December	11–286	47	9– 74	34
January–April	—	—	11–340	51
May, November, December	—	—	8–144	31
June–October	5–129	28	8–72	22
November–May	11–590	54	—	—

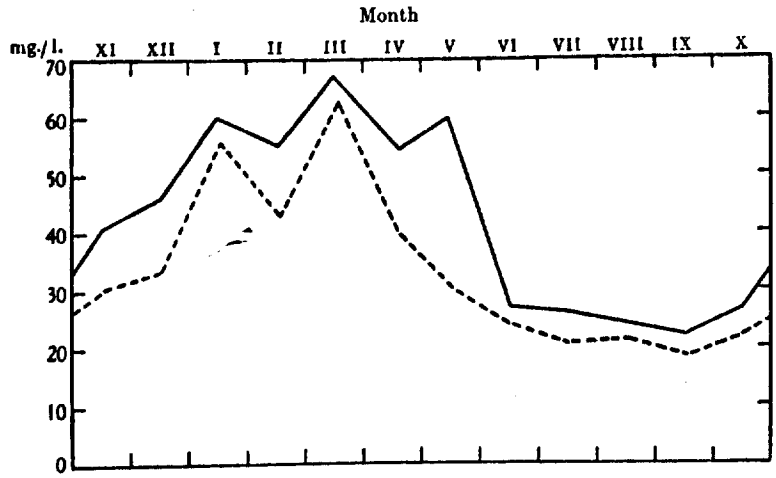

Fig. 1. Seasonal fluctuation in the monthly average urinary vitamin C excretion in 30 adults and 25 children in Jerusalem.

——— Adults. - - - - - Children.

standard. During these months the vitamin C excretion often reaches the high values of 400–600 mg./l. Although instances occurred during these months where less than 15 mg./l. were excreted, these were on the whole extremely rare, and were found only in persons who consumed very small quantities of citrus fruits. Taking the accepted standard values as a basis, 46–71 % of the

analyses during November to May yielded superstandard values, while between June to October only 8–21 % of the analyses were above standard. On the other hand, substandard values were found in only 2–5 % of the cases between November and May, and in 9–18 % of the analyses between June to October. The relationship is shown graphically in Fig. 2.

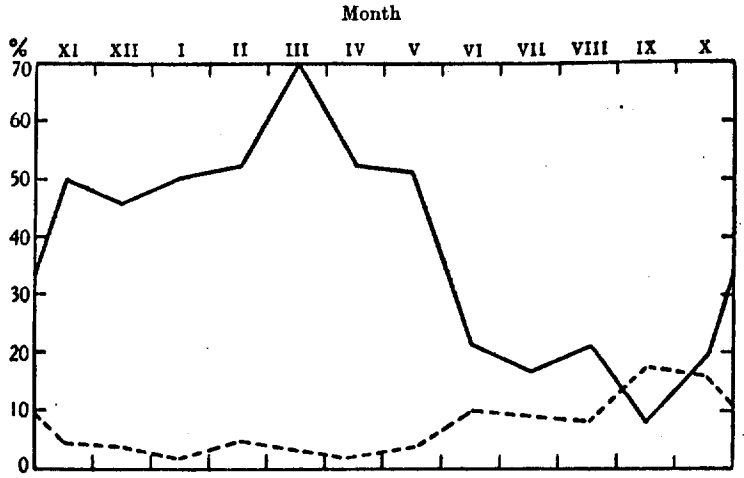

Fig. 2. Percentage distribution of super- and substandard urinary vitamin C excretion in 30 adults in Jerusalem.

——— Superstandard: excretion above 34 mg./l.
- - - - - Substandard: excretion below 15 mg./l.

The frequence distribution of the substandard, standard and superstandard values obtained during the periods November to May and June to October, respectively, gives striking curves which are shown in Fig. 3.

It will be noted that between November and May only 4 % of the values were below 15 mg./l., whereas between June and October 15 % were below this figure. On the other hand, between November and May 53 % if the values were over 34 mg./l., and between June and October only 17 % were over this figure.

(*b*) *Findings on twenty-five children, aged 2–5 years, in Jerusalem*

The results obtained among children resemble in many respects those among adults (Table I, Fig. 1), but there are certain deviations. The correspondence is evident in the high excretion during the winter and spring months and the low values found in summer. The curves for children are, however, consistently lower than those for adults. Among the children three distinct periods may be distinguished: January to April, when the average was 41–64 mg./l., May, November and December, when the average was 31–34 mg./l., and June to October, when the average was 19–25 mg./l. The percentage distribution of vitamin C excretion during the different periods of the year are shown in Fig. 4 and corresponds with curves for adults shown

structure than other capillary basement membranes, and minor changes would be less apparent.

The discontinuities which we have described in the capillary walls were not observed by Friederici and associates.[37] These investigators used immature guinea pigs rather than the more robust adults which permitted us to maintain the deficient state for a longer period. Many of their observations were made upon capillaries in inflamed synovial tissues which, as they themselves noted, presented features common to capillaries in granulation tissue.[38,39] Their estimate of endothelial cell activity judged by dilated endoplasmic reticulum, increased numbers and aggregates of ribosomes, enlarged mitochondria and prominent Golgi apparatus were biased by the hyperplastic state of the capillaries they observed and was, accordingly, greater than our own. Our findings of bullous edema of endothelial cells coincides with their own but our interpretations differ. Disruption of the cytoplasmic membranes in one type of lesion visibly allows some of the cellular organelles to be washed away and permits the cytoplasm to become infused with plasma. We are unable to ascertain whether, or to what extent, this mechanism explains all instances of endothelial cell edema. Morphologically, however, it seems distinct from the edema characterized by large cytopemptic vacuoles riddling the otherwise normal cytoplasm of capillary endothelium in experimental nephrosis.[40] Nonspecificity of the lesion seems to be suggested by Merrill and Sprinz's[41] observation of ballooned rarefied capillary endothelial cells in experimental cholera.

Friederici[37] speculated that the morphologic evidence of increased endothelial synthetic activity was associated with increased formation of abnormal acid mucopolysaccharides and increased hydration. Such cytologic evidence is far less impressive outside of inflamed synovia; histologically, increased production of acid mucopolysaccharides does not occur in scorbutic arterial endothelium.[16] The quantitative contribution of the endothelium to total aortic acid mucopolysaccharides can only be minuscule, and although these are increased in scurvy and changed in their proportions, the isolated acid mucopolysaccharides are entirely normal.[42]

This study was supported in part by grants-in-aid from the National Institutes of Health, HE-07327; and in part by the Council for Tobacco Research of the United States.

Generic and Trade Names of Drug

Pentobarbital—*Nembutal*.

References

1. Follis, R.H.: *Deficiency Disease*, Springfield, Ill: Charles C Thomas, Publisher, 1958, p 577.

2. Stolman, J.M.; Goldman, H.M.; and Gould, B.: Ascorbic Acid and Blood Vessels, *Arch Path* 72:535-545 (Nov) 1961.

3. Florey, H.W.; Poole, J.C.F.; and Meek, G.A.: Endothelial Cells and Cement Lines, *J Path Bact* 77:625-636, 1959.

4. Majno, G.: "Ultrastructure of the Vascular Membrane," in Hamilton, W.F., and Dow, P. (eds.): *Handbook of Physiology*. Baltimore: Williams & Wilkins Co., 1965, pp 2293-2375.

5. Wolbach, S.B., and Howe, P.R.: Intercellular Substances in Experimental Scorbutus, *Arch Path* 1:1-24 (Jan) 1926.

6. Gross, J.J.: Studies on the Formation of Collagen: IV. Effect of Vitamin C Deficiency on the Neutral Salt-Extractible Collagen of Skin, *J Exp Med* 109:559-569, 1959.

7. Gould, S.B., and Woessner, F.J.: The Influence of Ascorbic Acid on the Proline, Hydroxyproline, Glycine, and Collagen Content of Regenerating Guinea Pig Skin, *J Biol Chem* 226:289-300, 1957.

8. Robertson, W.V.B.; Hewitt, J.; and Herman, C.: The Relation of Ascorbic Acid to the Conversion of Proline to Hydroxyproline in the Synthesis of Collagen in the Carrageenan Granuloma, *J Biol Chem* 234:105-108, 1959.

9. Gerber, G.B.: Some Interrelated Aspects of Proline and Hydroxyproline Metabolism, *Nature* 185:767-768, 1960.

10. Mitoma, C., and Smith, T.H.: Studies on the Role of Ascorbic Acid in Collagen Synthesis, *J Biol Chem* 235:426-428, 1960.

11. Williams, G.: Some Histologic Aspects of Connective Tissue Metabolism in Acute Scorbutus, *Brit J Exp Path* 40:176-182, 1959.

12. Wolbach, S.B.: Controlled Formation of Collagen and Reticulum: A Study of the Source of Intercellular Substance in Recovery From Experimental Scorbutus, *Amer J Path* 9:689-700, 1933.

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in Fig. 3. During the winter months (January to April) 43 % of the urine samples gave values above 34 mg./l. and 10 % below 15 mg./l.; during the summer months (June to October) only 5 % of the samples contained over 34 mg./l. and 27 % below 15 mg./l. May, November and December seem to constitute transition months giving wide divergence in either direction: 21 % of the samples contained over 34 mg./l. and 17 % less than 15 mg./l.

There are two possible explanations for the somewhat lower vitamin C concentrations found in the urine samples of children both in summer and winter. One is a relatively smaller vitamin C intake; the other a higher vitamin C requirement by the growing body. Since citrus fruits, which constitute the most important source of vitamin C in Palestine, are so cheap that they form a staple food of even the poorest classes, we may reject the assumption of a lesser intake of vitamin C. It seems much more probable that the lower excretion is due to a higher vitamin C requirement by the growing body. A similar conclusion, it may be recalled, was reached by Everson & Daniels (1936). This explanation accounts for the slow rise in the vitamin C excretion during November and December, the initial months of the citrus season and the rapid fall in May at the close of the season.

(c) *Findings in Kalliah at the Dead Sea*

The winter survey was carried out on 18 January 1937, the summer survey on 28 June 1937. The population was divided on a nutritional basis into two groups: (1) workers who live together and eat in the common kitchen, and (2) workers who live with their families and take their meals at home. The results are shown in Table II. The pronounced difference between the vitamin C

Table II. *Urinary vitamin C excretion at Kalliah near the Dead Sea*

		18. i. 1937		
Subjects investigated	Age	No. of subjects	Range, mg./l.	Average, mg./l.
Workers, taking their meal in the common kitchen	17–63	12	14– 75	38
Police	21–48	5	36– 188	77
Workers and their wives, taking their meals at home	21–56	17	23– 330	93
Children	3–13	12*	142–1000	392
Total—adults	17–63	34	14– 330	69

		28. vi. 1937		
Subjects investigated	Age	No. of subjects	Range, mg./l.	Average, mg./l.
Workers, taking their meal in the common kitchen	17–63	27	22–48	28
Police	21–48	3	13–34	25
Workers and their wives, taking their meals at home	21–56	18	13–48	24
Children	3–13	10	16–54	30
Total—adults	17–63	48	13–48	26

* The extremely high values found in the urine of children are attributable to the orange juice meal which they received 2 hr. before the urine samples were taken.

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excretion in winter of canteen members and of workers who take their meals at home and receive a diet richer in citrus fruit is striking. In the summer, when the vitamin C content of the diet is smaller, this difference between the two groups tends to disappear. The extremely high concentrations found in the urine of the children at winter survey must be regarded as exceptional and are attributable to the meal of orange juice which they received 2 hr. before the urine samples were taken. No influence of climate on vitamin C excretion is indicated. The data tend rather to show the all-important role of the form of nutrition, i.e. of the amount of the citrus consumption. This conclusion is even more obvious from the figures obtained in the investigation of a group of inhabitants of the citrus zone.

(*d*) *Findings in the citrus zone*

As has been pointed out above, the investigation on the agricultural workers and their families in the citrus zone was carried out in two adjacent settlements, Givath-Brenner and Gederah. The results obtained at these points in the winter (4 January 1937) and summer (8 July 1937) are set forth in Table III. The extraordinary large amounts of vitamin C excreted during

Table III. *Urinary vitamin C excretion at Givath-Brenner and Gederah*

		4. i. 1937			8. vii. 1937		
Subjects investigated	Age	No. of subjects	Range, mg./l.	Average, mg./l.	No. of subjects	Range, mg./l.	Average, mg./l.
Man	17–38	26	15–920	375	30	13–126	30
Women	17–51	20	31–194	96	23	12– 50	31
Children	1–11	16	22–157	68	8	7– 13	11

the winter are outstanding. Whereas at Jerusalem and Kalliah no differences were noted in the vitamin C excretion of males and females, in the citrus zone there is a pronounced difference between male and female during the winter but not in the summer. The values for the females are not appreciably higher than those found in Jerusalem, but the values for the males are markedly higher. This difference is due to the fact that most of the men work in citrus plantations, where they consume large quantities of citrus fruits, whereas few of the women are occupied in this manner. The same circumstance explains also why the difference between males and females is absent in summer. The low vitamin C excretion of children in summer is notable, but the data available seem insufficient as a basis for any conclusion.

Our survey shows the significance of the consumption of citrus fruits for the vitamin C supply of Palestine's population. The citrus season begins in October, is in its full swing in December and does not end until May of the succeeding year. Among the different population groups the form of the excretion curves was similar. The minimum shown towards the close of summer (September) is characteristic, and coincides with the time of the lowest fresh fruits supply. As citrus fruits are of such great importance for the vitamin C

supply of the Palestine population the possible sources of vitamin C supply in that time of the year when citrus fruits are not available here, i.e. from June to September, seemed of interest. Accordingly we made a few analyses of the popular fruits and vegetables eaten fresh. The vitamin C content of some Palestinian fruits has been determined already by Robinson (1937); his examinations, however, were limited to bananas, chestnuts, milk and the

Table IV. *Vitamin C content of some fruit and vegetables grown in Palestine*

Food stuff	mg./100 c.c. or g.	Average values according to Eddy & Dalldorf (1937)	
		Units/oz.	mg./100 c.c. or g.
Oranges	33·0–64·0	230–235	38·0–39·0
Grapefruit	48·0–72·0	185–200	31·0–33·0
Apricots	4·4–6·3	10–70	1·7–12·0
Figs	1·1–2·3	10	1·7
Plums	0·6–1·0	30	5·1
Grapes	0·9–3·9	8–20	1·3–3·4
Pomegranate juice (iodine titration)	5·0–13·0	—	—
Melon	16·7–22·0	—	—
Watermelon	2·3–5·0	40	6·8
Cucumbers	14·1–14·9	70	12·0
Cucumbers (pickles)	2·5	—	—
Tomatoes	8·3–38·0	78–140	13·0–23·0
Egg plant	1·1–4·3	20	3·4
Endive	13·0–19·0	56	9·3
Peppers, green	70·0–84·0	155–310	25·5–52·0

juice of citrus fruit, grapes and tomatoes. The values found by us using the Tillmans' reagent are shown in Table IV. The comparison with the standard values given by Eddy & Dalldorf (1937) shows that there is no essential difference between the vitamin C content of Palestinian and foreign fruit and vegetables. The great fluctuations in vitamin C content of several kinds (grapes, tomatoes) are, however, remarkable. It remains to ascertain whether season or plant nutrition have any influence on the vitamin C content.

Discussion

As mentioned above, the only existing comparative investigations on the vitamin C excretion of different population groups are those by v. Euler & Malmberg (1935) and by Hamel (1937). v. Euler & Malmberg examined by titrating against indophenol the vitamin C concentration of urine samples sent to the laboratory during the winter months. The average values found were:

	mg./l.
North Sweden	5
Rural population of Sweden	9
South Sweden	11
Stockholm	25

Hamel (1937) investigated the total daily excretion of people in Rostock by titrating against methylene blue after treatment with oxidase. He, there-

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fore, got much lower values than with the Tillmans' reagent. His results were as follows:

	mg.
Students, warm meals twice weekly, fruit rare	1·0– 4·5
Students, mensa meals	4·5– 8·0
Normal family food (often warmed meals)	2·2– 9·6
Owners of vegetable gardens	14·0–21·0

Our results cannot be compared with those found by Hamel, but a comparison with the Swedish data shows that even the low average values in Jerusalem are as high as the Stockholm values. These figures are as high as the average vitamin C concentration of the urine given by v. Euler & Burstroem (1936) (15–25 mg./l.), Ippen (1935) (10–35 mg./l.) and by Harris & Ray (1935) (20–30 mg./l.).

It is generally assumed that deficiency in vitamin C is subject to seasonal fluctuations and is greater in winter and spring months than during the rest of the year. This view is supported by a limited number of investigations. Forsgren (1932) examined 500 patients of a Swedish sanatorium by the Goethlin capillary method, and found that about 10–20 % of the patients were subscorbutic. Stocking (1933) also found a lowered capillary resistance in the spring and attributed it to vitamin C subnutrition. Demole (1936) and Ippen (1935) found by means of the saturation tests that most people have a certain degree of vitamin C deficiency in the winter and at the beginning of spring. On the basis of this finding Demole considered the spring tiredness with the accompanying psychoneuroses was due to a prescorbutic condition. Degeller (1936) also found a seasonal fluctuation in the vitamin C content of the blood. He found the minimum during January to March and the maximum from June to October. Our investigations, however, lead us to believe that a reduced excretion is not necessarily a consequence of an increased requirement during the winter and spring months; it seems more likely that the low values found by the European investigators are indications of a lower vitamin C intake. Our results are completely the reverse of those reported in the literature due to the high intake of vitamin C during these months.

It may be asked whether the vitamin C supply of the Palestinian population is adequate in the summer, namely, between May and October. On the whole the average values (23–28 mg./l.) found among adults of Jerusalem during this time are not subnormal. It is of interest, however, that while 17 % of all samples showed a high vitamin C concentration (above 34 mg./l.), 15 % of the samples give values below 15 mg./l. The conditions were worst in September, when only 8 % of the adults gave values above 34 mg./l., and 18 % below 15 mg./l. It is apparent, therefore, that during the summer months an appreciable number of people are below standard in vitamin C.

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Summary

During the period November 1936–November 1937 the vitamin C excreted in the urine by adults and children was investigated in a number of places in Palestine. The pronounced influence of the citrus season (winter and spring) with its greater consumption of citrus fruit was noted in all the population groups investigated. In the summer, however, when the supply of fresh vegetable sources of vitamin C is remarkably poor, much lower, partly subnormal, excretion values are to be found.

The lower average excretion of vitamin C in the urine samples taken from children probably indicate a higher requirement by the growing than by the adult body.

Acknowledgements

I wish to express my indebtedness to Dr Perl of the day nursery at Jerusalem, Dr Ebel at Kalliah, Dr Glaser at Givath-Brenner, Dr Schachtel at Gederah, and to the management of the Palestine Potash Co. for their kind co-operation.

REFERENCES

Degeller, O. (1936). Dissertation, Utrecht.
Demole, V. (1936). *Schweiz. med. Wschr.* no. 29, p. 685.
Eddy, W. H. & Dalldorf, G. (1937). *Avitaminoses.* Baltimore.
v. Eekelen, M. & Wolff, L. K. (1936). *Acta brev. Neerl. Physiol.* **6**, 12.
v. Euler, H. & Burstroem, D. (1936). *Biochem. Z.* **283**, 153.
v. Euler, H. & Malmberg, M. (1935). *Biochem. Z.* **279**, 338.
Everson, G. J. & Daniels, A. L. (1936). *J. Nutrit.* **12**, 15.
Forsgren, E. (1932). *Hygiea, Stockh.*, **95**, 175.
Hamel, P. (1937). *Klin. Wschr.* **16**, 1105.
Harris, L. J. & Ray, S. N. (1935). *Lancet*, **1**, 71.
Harris, L. J., Ray, S. N. & Ward, A. (1933). *Biochem. J.* **27**, 2011.
Ippen, F. (1935). *Schweiz. med. Wschr.* no. 19, p. 431.
Leibowitz, J. & Guggenheim, K. (1938). *Z. Vitaminforsch.* (in the Press).
Robinson, P. (1937). *Harefuah* (Hebrew), **12**, 73.
Stocking, R. E. (1933). *Arch. Pediat.* **50**, 823 (*Brit. Chem. Abstr.* 1934, **A**, p. 707).

(*MS. received for publication* 3. viii. 38.—Ed.)

Note. Since this paper has been submitted for publication two studies of a similar character have appeared. Trier (*Klin. Wschr.* 1938, **17**, 976) studied the seasonal variation in the vitamin C in serum and Mueller (*Z. Vitaminforsch.* 1938, **7**, 311) studied the variation in vitamin C excreted in the urine. Both authors worked in Europe and obtained curves which are the mirror image of those obtained by us. The results emphasize the importance of the fluctuating seasonal supply of vitamin C in the diet.

J. Amer. Geriatr. Soc. 13: 561-568 1965 3251

JOURNAL OF THE AMERICAN GERIATRICS SOCIETY
Vol. 13, No. 6
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Printed in U.S.A.

FACTORS IN THE NUTRITION OF ELDERLY PEOPLE LIVING ALONE OR AS COUPLES AND RECEIVING COMMUNITY ASSISTANCE*

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An important principle in providing care for the aged is to assist them in staying as long as possible in their own surroundings in the community. Emphasis is placed on extramural care, leaving intramural services for the severely disabled aged requiring more medical attention in sheltered conditions or for those who, for other reasons, are unable to stay in the community. Thus, efforts are made to improve the functional capacity of the aged, even despite disease, in order to enable them to live an independent life.

Obviously, some indispensable conditions for comprehensive care are an adequate income, adequate housing facilities, opportunities to continue an active life, satisfactory health, and availability of services to meet individual needs. Nutrition plays an important role in the physical, mental and social well-being of the aged. Adequate nutrition is often neglected in the services provided for the aged, particularly for those living alone in the community.

In the survey described here an attempt was made to learn about the food consumption and nutritional status of a group of elderly people living alone or as married couples and subsisting mainly by public assistance. It should be emphasized that the social policy for the aged in Israel is only in its nascent stage. Our hypothesis was that in such a low-income group, the consumption of nutrients might be inadequate. The objectives of the survey were: (a) to describe a group of elderly people subsisting mainly by means of public assistance, (b) to study the consumption of food and the nutritive value of their diets, and (c) to examine the general health status of a sub-group of the study population with special consideration of their nutritional status.

MATERIAL AND METHODS

The study was conducted by means of social, dietetic and clinical examinations. A list of approximately 200 social-welfare recipients of the municipality of Jerusalem was provided. The age of the men was 65 years or more, and the age of the women was 60 years or more. Only those subjects were included who were known by the social workers of the municipality to be living alone or as married couples, and not presenting any particular health problems.

The food consumption survey included 115 subjects, but data on social status were available for only 114 of them.

* Study supported by Mr. and Mrs. Carl Newman, Los Altos, California, and by A.J.D.C.-Malben (which receives its funds from U.J.A. in the U.S.A.).

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Social workers completed a questionnaire providing information on the family status of the subjects, their relationships with members of their families, country of origin, educational level, social life, housing facilities and income. They were also helpful in explaining the purpose and introducing the nutritionists and physicians.

Each subject was visited and interviewed three times by a trained nutritionist. The interview covered the food eaten on the two previous days. Five week-days and a Sabbath were included, in view of a previous finding that the Sabbath menu differed from the week-day diet (1). A fluid measuring cup, measuring spoons and a spring balance were used as aids. Estimates of nutrient content were based on analyses of locally grown food (2) or, when these were not available, on the tables of Watt and Merrill (3). Corrections were made for vitamin losses in cooking, as suggested by the Interdepartmental Committee on Nutrition for National Defense (4).

Thirty-nine subjects (12 men and 27 women) were clinically examined by a physician. Their consumption of calories and protein did not differ much from that of the total group under study. They could, therefore, be considered as a representative sub-sample of the total. The examination comprised measurement of height (without shoes) and weight (without clothing), assessment of the general status of nutrition, measurement of skinfold thickness on the posterior arm and over the scapula, and a careful general evaluation of the face, eyes, lips, tongue, gums and skin. Blood was taken from a finger for a hemoglobin determination by the cyanmethemoglobin method (5).

FINDINGS

Socio-economic characteristics

This group of 114 subjects consisted of 76 women and 38 men. Their age distribution can be seen from Table 1.

Twenty-eight women and 31 men were married, 40 women and 3 men were widowed, and the remaining subjects were single or divorced. Thirty-five women and 17 men had children living in Israel.

Thirty-one women and 16 men were born in Europe, 7 women and 5 men in Africa, 26 women and 13 men in Asian countries except Israel, and 12 women and 4 men in Israel. Forty-two per cent of the subjects had immigrated to Israel after the establishment of the State in 1948.

The educational level of the group was very low. About half of the males and two-thirds of the females had no formal schooling; the rest had received some elementary education and 5 subjects had had vocational training. Most

TABLE 1

Distribution of 114 Subjects by Age and Sex

Age group	Males	Females
60–64	—	24
65–69	6	19
70–75	10	13
76–79	12	10
80–84	7	7
85–89	2	2
90 and over	1	1
Total	38	76

of the women were housewives, and most of the men were peddlers, unskilled laboreres and artisans.

Their social life was very limited. Less than 20 per cent attended clubs for elderly persons. More than half had no contacts with close members of their families, and more than 40 per cent had no contacts with relatives and friends.

Housing conditions were poor. Seventy-six subjects had neither bath nor shower, and only 50 had toilet facilities within the house; 13 had no kitchen.

As far as information on sources of income could be obtained, it was found that public assistance was the main or even sole source of maintenance for most of the subjects. The National Insurance allowance granted to old people amounts to the equivalent of $25 monthly for couples and $17 for single persons. The needy aged who are not eligible for the National Insurance allowance are entitled to be supported by a special fund administered by the Municipality ($21 monthly for couples and $11 for single persons). Seven women had additional income from work, and 13 women and 9 men were supported by relatives. It appears that the population sample studied represented indigent people belonging to a low socio-economic class.

Food consumption and nutritional value of diet

Table 2 presents data on food consumption for the 115 subjects studied. The men usually consumed more cereals—and particularly more brown bread, meat and eggs—than did the women. The nutritive value of their diet (Table 3) was, therefore, somewhat higher than that of the women. However, the diets of most of the subjects were inadequate when compared with the allowances of the National Research Council (6). Even the 75th percentile did not reach the Recommended Dietary Allowances (except for the intake of ascorbic acid by men).

Cereals contributed over 40 per cent of the calories, protein, thiamine and riboflavin and approximately one third of the minerals and nicotinic acid (Table 4). The relatively high amounts of calcium and riboflavin contributed by cereals were partly the result of enrichment of the flour with these nutrients—a process which is controlled by Government. Nearly half of the protein was derived from animal sources. Milk and milk products supplied 44 per cent of the calcium and 34 per cent of the riboflavin; meat, poultry and fish supplied about half of the nicotinic acid; and fruits and vegetables supplied one third of the iron and 72 per cent and 97 per cent respectively of vitamins A and C.

The food consumption of 49 women living alone was similar to that of 27 women living with spouses. Only the consumption of calcium was significantly higher by the women living alone (536 ± 29 mg as against 455 ± 18 mg). Among men, 7 lived alone and 31 with spouses. In these groups also there was no large difference in the consumption of nutrients. But men living alone consumed 32 ± 6.0 mg of ascorbic acid and 2243 ± 434 I.U. of vitamin A daily, whereas men living with spouses consumed 55 ± 6.9 mg of ascorbic acid and 3128 ± 413 I. U. of vitamin A. Only the difference in intake of ascorbic

TABLE 2

Mean Daily Food Consumption (Grams) of 115 Elderly Persons Living Alone or As Couples in Jerusalem

Food	Men (38)	Women (77)
Cereals		
White bread	145 gm	135 gm
Brown bread	52	28
Others	56	52
Total	253	215
Pulses and seeds	12	9
Fruits and vegetables		
Potatoes	62	52
Tomatoes	38	50
Citrus fruits	90	74
Others	118	112
Total	308	288
Fats and oils	15	12
Sugar, jam and sweets	34	34
Meat, poultry, fish and eggs		
Meat	21	11
Poultry	51	48
Fish	20	18
Eggs	30	23
Total	122	100
Milk and milk products		
Milk and sour milk	160	162
Cheese and cream	16	19
Total	176	181
Beer and wine	3	2

TABLE 3

Nutritive Value of the Diet of 115 Aged Persons

	Energy (Cal.)	Protein (gm)	Calcium (mg)	Iron (mg)	Vitamin A (I.U.)	Thiamine (mg)	Riboflavin (mg)	Nicotinic acid (mg)	Ascorbic acid (mg)
Men (38)									
Mean	1373	52.9	528	7.5	2965	0.75	0.91	8.5	51
Standard error	54	2.3	31	0.3	355	0.03	0.04	0.5	5.9
Percentiles									
25%	1155	42.0	362	6.1	1488	0.60	0.69	5.8	26
50%	1297	53.0	502	7.0	2443	0.71	0.87	8.6	38
75%	1653	66.1	644	9.3	3841	0.88	1.08	10.9	71
Women (77)									
Mean	1255	48.8	508	6.6	2151	0.71	0.89	7.9	47
Standard error	41	1.7	21	0.2	165	0.03	0.03	0.4	4.2
Percentiles									
25%	1019	36.7	386	5.0	1017	0.54	0.66	4.7	21
50%	1208	47.5	482	6.6	1863	0.67	0.87	7.7	38
75%	1434	60.6	610	7.8	3003	0.81	1.11	10.0	63

TABLE 4

Percentage Contribution of Various Food Groups to the Total Supply of Nutrients in the Diets of 115 Aged Persons

	Calories	Protein	Calcium	Iron	Vitamin A	Thiamine	Riboflavin	Nicotinic acid	Ascorbic acid
Cereals	51	41	38	34	—	55	42	31	—
Pulses and seeds	2	4	2	9	1	5	2	3	—
Fruits and vegetables	8	6	12	30	72	22	7	14	97
Fats and oils	8	—	—	—	7	—	—	—	—
Sugar and sweets	10	—	—	—	—	—	—	—	—
Meat, poultry and fish	8	26	2	15	—	5	9	49	—
Eggs	3	6	2	9	10	2	6	—	—
Milk and milk products	10	17	44	3	10	11	34	3	3
Totals	100	100	100	100	100	100	100	100	100

acid was statistically significant. The reason for the different intake levels of these vitamins probably lies in differences in the consumption of fruits and vegetables (except potatoes). Men living alone ate 192 gm per day, whereas men living with spouses consumed 258 gm.

Consumption of calories and protein was similar whether the subjects originated in Europe, Asia or Africa. Persons with or without living children likewise did not differ significantly with respect to consumption of these nutrients.

Status of nutrition

Thirty-nine subjects underwent a clinical examination. The weight of the 12 males was 66.2 ± 5.3 kg, and their height 161 ± 1.7 cm. Corresponding values for the 27 females were 65.0 ± 2.7 kg and 151 ± 1.2 cm. The mean combined skinfold thickness for the males was 16.1 ± 2.5 mm, and that for the females was 20.5 ± 1.7 mm. No subject presented typical symptoms suggesting nutritional deficiency. The general health status was satisfactory. The mean hemoglobin level for the men was 13.6 ± 0.53 gm per 100 ml, and for the women, 14.4 ± 0.30 gm. In 2 subjects (both males) the values were below 12.0 gm per 100 ml.

DISCUSSION

The most striking finding of our study was the low mean intake of all nutrients. The median intake was much lower than that recommended by the National Research Council (6). Food consumption was also considerably lower than that of 50 pregnant women in Jerusalem belonging to the lowest socio-economic class, who were recently studied by one of us (7). These women consumed daily 358 gm of cereals, 13 gm of pulses and seeds, 736 gm of fruits and vegetables, 29 gm of fats and oils, 35 gm of sugar and sweets, 156 gm of meat, poultry, fish and eggs, and 220 gm of milk and milk products. A comparison with the data in Table 2 shows that the elderly people consumed

much less of cereals, fruits, vegetables and fats and oils; somewhat less of animal products; and similar quantities of sugar and sweets. Since cereals and fats and oils are (in this country) cheap sources of energy in comparison with animal products, it follows that the low food intake is probably not only caused by economic limitations but also by a diminished need of energy. The fact that most of the subjects of this study lived a physically inactive life lends support to this suggestion. A low consumption of sources of energy is generally accompanied by a low intake of other nutrients. Thus, most of these diets appear to be nutritionally inadequate.

However, it is not possible to say whether the nutrient intake of the subjects studied was satisfactory or not. If the requirements for aged people are similar to the requirements for younger adults, as suggested by the National Research Council (6) and the British Medical Association (8), the intakes of most of the subjects were insufficient. However, nutritional deficiency was not clinically evident. Their mean weight appeared to be satisfactory in comparison to height, and anemia was uncommon. The mean values for hemoglobin did not differ much from those found by Hobson and Blackburn (9) in a group of elderly people, 177 males and 246 females, in Sheffield. Nine men (5.1 per cent) and 16 women (6.5 per cent) had hemoglobin levels less than 11.7 per 100 ml. In our sample, 5 per cent of the subjects had such low hemoglobin levels. Mean skinfold thickness over the arm and scapula was somewhat lower than that measured by Hejda (10) in a sample of 56 men and 85 women aged 65-101 years, living in Prague.

Studies of the food intake of elderly people show considerable variation in the findings. In Table 5 are summarized the data on 8 groups of elderly per-

TABLE 5

Consumption of Nutrients by Elderly People As Reported in the Literature

No. of Subjects	Age	Sex	Energy (Cal.)	Protein (gm)	Calcium (mg)	Iron (mg)	Vitamin A (I.U.)	Thiamine (mg)	Riboflavin (mg)	Nicotinic acid (mg)	Ascorbic acid (mg)	Authors (Reference Number)
40	50–91	F	1448	50		8.8	2090	0.82	0.92	7.9	14	(13)
24	60–85	M	2136	75		14.4	3430	1.32	1.45	13.6	35	(13)
16*	66–85	F	1037	29	340	4.5	1395	0.47	0.67	5.1	28	(12)
125	over 67	M	2096	69	800	13.0	2351	1.10	1.10	8.0	18	(14)
178	over 62	F	1746	57	800	10.0	2115	0.90	1.00	7.0	21	(14)
20	77–97	F	935	38								(11)
23	60–69	F	1930	61	810	11.0						(15)
32	65–86	F	1696	66	810	10.1	4885	1.10	1.68	21.0	102	(16)
38	65–93	M	1373	53	528	7.5	2962	0.75	0.91	8.5	51	Present study
77	60–92	F	1255	49	508	6.6	2151	0.71	0.89	7.9	47	Present study

* Debilitated.

sons, some living at home and some in institutions; these results compared with the results of the present study. The average consumption of energy varied between 935 and 2136 calories per day. Moreover, the lowest energy consumption was reported from an old age home (11) where the subjects had free access to food without economic limitations. The subjects studied by Vinther-Paulsen (12) also had a very low intake of nutrients. They were debilitated patients of a geriatric department, and many of them exhibited signs of nutritional deficiency. Their consumption of nutrients was much below that of the subjects of the present study. But even groups of healthy people who consumed higher quantities of calories than did the subjects of our study, did not necessarily have higher intakes of vitamin A, nicotinic acid and ascorbic acid (13, 4). It appears, therefore, that the allowances of nutrients recommended for elderly persons afford large margins of safety.

Nevertheless, the limitations of the interview as a tool in surveys of food consumption should be considered when the subjects are of a very low educational level.

SUMMARY

A study was made of 115 elderly people (77 women and 38 men) in Jerusalem, living alone or as married couples and subsisting mainly by means of public assistance. The factors investigated were food intake (all subjects), socio-economic status (114 subjects), and the nutritional state (39 subjects).

Thirty-one of the men were married. Over half of the women were widows. The educational level of all subjects was very low, and all belonged to a low social class. Housing conditions and income were poor.

Food consumption was low and the mean intake of all nutrients was below the allowances recommended by the National Research Council. Men living alone consumed less fruits and vegetables and, therefore, less vitamins A and C than did men living with their spouses. No marked difference in food consumption was found among subjects originating from different parts of the world.

Clinical examination of a sub-sample of 39 subjects did not reveal any overt symptoms suggesting nutritional deficiency.

It is concluded that the nutritional allowances for old and physically rather inactive persons afford large margins of safety.

Acknowledgments

The authors are indebted to Mrs. B. Azoulay, Head of the Social Welfare Services of the Municipality of Jerusalem, and her staff for their close cooperation; to Dr. E. Perez, Dr. I. Altshuler, Mr. T. Halbersberg and Mr. M. Ansbacher for their help in the medico-social investigation; to Mrs. Z. Flexer, Miss K. Ron and Miss D. Ben Shemesh for their assistance in the food consumption study; and to Dr. D. Drutman, Statistician of A.J.D.C.-Malben, for the statistical elaboration of the results.

REFERENCES

1. Guggenheim, K.; Ilan, J.; Vago, T., and Mundel, G.: Nutritional status and food consumption of pregnant women in a development area of Israel, *Brit. J. Nutrit. 14:* 347–354, 1960.

3251

2. Halevy, S.; Koth, H., and Guggenheim, K.: The vitamin and mineral content of fruits and vegetables grown in Israel, *Brit. J. Nutrit. 11:* 409–413, 1957.
3. Watt, B. K., and Merrill, A. L.: Composition of Foods, Raw, Processed, Prepared. Agriculture Handbook No. 8, U. S. Department of Agriculture, Washington, D. C., 1950.
4. Interdepartment Committee on Nutrition for National Defense: Manual for Nutrition Surveys. Washington, D. C., U. S. Gov't Printing Office, 1957, p. 117.
5. Crosby, W. H.; Munn, J. F., and Furth, F. W.: Standardizing a method for clinical hemoglobinometry, *U.S. Armed Forces M. J. 5:* 693–703, 1954.
6. National Research Council, Food and Nutrition Board: Recommended Dietary Allowances (revised 1963). Washington, D. C., National Research Council Publication No. 1146, 1963.
7. Guggenheim, K.; Kark, S. L., and Abramson, J. H.: Diet, social class, and neighbourhood in Jerusalem, Israel, *J. Am. Dietet. A. 45:* 429–432, 1964.
8. British Medical Association: Report of the Committee on Nutrition. British Medical Association, London, 1950.
9. Hobson, W., and Blackburn, E. K.: Haemoglobin levels in a group of elderly persons living at home alone or with spouses, *Brit. M. J. 1:* 647–649, 1953.
10. Hejda, S.: Skinfold in old and longlived individuals, *Gerontologia 8:* 201–208, 1963.
11. Kapp, H., and Fischer, H. R.: Erhebungen ueber den Nahrungsmittelverbrauch im Alter, *Internat. Ztschr. Vitaminforsch. 27:* 103–109, 1956.
12. Vinther-Paulsen, N.: Investigations of the actual food intake of elderly chronically hospitalized patients, *J. Gerontol. 5:* 331–338, 1950.
13. Pyke, M.; Harrison, R.; Holmes, S., and Chamberlain, K.: Nutritional value of diets eaten by old people in London, *Lancet 2:* 461–464, 1947.
14. Bransby, E. R., and Osborne, B.: A social and food survey of the elderly, living alone or as married couples, *Brit. J. Nutrit. 7:* 160–180, 1953.
15. Durnin, J. V. G. A., and Blake, E. C.: A comparison of food intake of elderly women estimated by the individual inventory and the National Food Survey methods, *Brit. J. Nutrit. 16:* 261–265, 1962.
16. Fry, P. C.; Fox, H. M., and Linkswiler, H.: Nutrient intakes of healthy older women, *J. Am. Dietet. A. 42:* 218–222, 1963.

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J. Pharm. Pharmacol. 17:387,

LETTERS TO THE EDITOR, *J. Pharm. Pharmacol.*, 1965, **17**, 387

Anti-anaphylactic effect of vitamin C in the guinea-pig

SIR,—Hoffman (1942) reported the anti-anaphylactic effect of vitamin C in mice. Naranjo (1952) and Herxheimer (1955) found that vitamin C has no protective effect against histamine shock in the guinea-pig at the specified dose levels.

The effect of vitamin C in histamine shock and anaphylaxis is here reported.

Guinea-pigs, 375–425 g at the beginning of the experiment, received 50 mg vitamin C per day in the drinking water for 21 days. Each week the animals were subjected to histamine or antigen aerosol using the technique of Herxheimer (1952), and animals found suitable in this way were chosen for the test. The histamine aerosol, 0·25 mg base/ml, was sprayed into a glass box. The animal was allowed to remain in the box until the first sign of coughing or choking occurred, and the preconvulsion time was recorded in seconds. The intracardial injection of the vitamin was not used, because the severe stress to the animals might have interfered with the test. The preconvulsion times (sec) for the following conditions were: control (injected with saline), 130 (s.d. 9·84); 200 mg vitamin C i.p., 20 min before anaphylaxis, 293 (s.d. 26·16); 200 mg vitamin C i.m., 2 hr before anaphylaxis, 223 (s.d. 18·63). Each figure is an average of 5 animals.

A group of 5 animals was tested for histamine resistance as above and then put on a scorbutic diet for 21 days and tested again. The preconvulsion times (sec) were: normal, 118 (s.d. 9·08); 8–12 day scurvy, 77 (s.d. 6·1); 18–21 day scurvy, 297 (s.d. 11·5).

Anaphylactic shock in vivo. The guinea-pigs were sensitised to commercial egg albumen by the intraperitoneal injection of 100 mg as a 5% w/v solution in normal saline. Three weeks later the animals were exposed to an aerosol of 50 mg of the antigen 1% w/v. The end-point was fixed at 10 min after which the animal was taken out of the box. When 200 mg vitamin C was administered intraperitoneally 20 min before being shocked, 7 out of 16 survived. In the control guinea-pigs only 2 out of 16 survived. When the dose of the antigen which caused shock was given intracardially, all the animals died. The time before death, however, was delayed 3–4 times in animals given the vitamin, compared with the controls.

No protective effect of vitamin C was found with oral administration of the vitamin or after 6 hr from the injection. The finding of Naranjo and Herxheimer that low doses of the vitamin had no protective effect, was also confirmed.

It is concluded that vitamin C has an anti-anaphylactic effect in the guinea-pig with the doses and under the conditions specified. At the early stage of scurvy there is a significant drop in the resistance of the animal to histamine aerosol and a marked rise above normal at the late stage of scurvy.

I thank Dr. P. B. Marshall for his help and advice.

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April 2, 1965

References

Herxheimer, H. (1952). *J. Physiol.*, **117**, 25.
Herxheimer, H. (1955). *Brit. J. Pharmacol.*, **10**, 160.
Hoffman, V. (1942). *Arch. exp. Path. Pharmacol.*, **199**, 631.
Naranjo, P. (1952). *Proc. Soc. exp. Biol., N.Y.*, **81**, 111.

THE USE OF BACTERIA IN THE CHEMICAL DETERMINATION OF TOTAL VITAMIN C

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(Received for publication, September 22, 1941)

Based on the discovery of Esselen and Fuller (3) that certain strains of *Bacterium coli* are able to reduce dehydroascorbic acid, a simple rapid method has been worked out for the determination of vitamin C in food products. After 15 minutes incubation with a suspension of resting cells the total vitamin C in the form of ascorbic acid is titrated directly with 2,6-dichlorophenol indophenol dye.

The procedure, as applied to milk, is as follows: 10 cc. of milk are measured into a test-tube containing 0.5 to 1.0 cc. of *Bacterium coli* suspension. After mixing, the contents of the tube are brought to 40° and held 15 minutes. The tube is then emptied into a beaker and rinsed with 20 cc. of 0.1 N H_2SO_4. The acidified solution is titrated immediately with dye to an end-point remaining definitely pink for 30 seconds (7). Various acids may be used for acidification and the solution may be filtered before titration, although this additional step is usually unnecessary. For the determination of total vitamin C in food products other than milk, the extract or juice should be brought to pH 6.2 before reduction. The bacteria are more active if 1.0 cc. of cell suspension is mixed with 1.0 cc. of 10 per cent glucose 15 minutes before addition of the extract to be analyzed. Pretreatment of the bacterial suspension with glucose is not necessary when milk is the material for analysis.

The *Bacterium coli* suspension is prepared as follows: An 18 to 24 hour culture of *Bacterium coli* is added to 5 liters of broth containing 1.0 per cent peptone, 0.1 per cent glucose, and 0.3 per cent secondary potassium phosphate and allowed to grow

14 to 24 hours at 37°. The culture is centrifuged in a continuous centrifuge, the cells washed once with 0.03 M neutral phosphate, then suspended in 50 cc. of the neutral phosphate, and stored in a refrigerator. There is no appreciable deterioration in activity of the cell suspension in 1 month if it is kept cold.

Approximately one-half the strains of *Bacterium coli* isolated from human feces are active in reducing dehydroascorbic acid but the individual strains show considerable differences in activity. A strain designated as "Crookes," originally presented to the authors through the kindness of Dr. Esselen, has proved consistently active. Besides *Bacterium coli* certain strains of the closely related *Bacterium cloacae* were also found to reduce dehydroascorbic acid. Negative results were obtained with fifteen strains of streptococci and with bakers' yeast.

Bacterium coli is to a certain extent a specific biological reagent for the determination of oxidized vitamin C in that no oxidation product of ascorbic acid is reduced except dehydroascorbic acid. Oxidation of ascorbic acid with hydrogen peroxide, or treatment of dehydroascorbic acid with alkali or heat yields products which no longer can be reduced with these bacteria. However, the reducing action of *Bacterium coli* is not entirely specific for oxidized vitamin C, because the oxidation product of *d*-isoascorbic acid can also be reduced. See Table I.

The necessary conditions for the quantitative reduction of dehydroascorbic acid have been elaborated. The sensitivity of the acid to heat and alkali offers the chief difficulty. If conditions of pH and temperature for the most rapid bacterial action are selected, the dehydroascorbic acid disappears so quickly that complete reduction is not obtained.

The more alkaline the pH and the higher the temperature the more rapidly the reduction must be carried out to avoid loss. In milk at pH 6.6 and 40° quantitative reduction can be brought about in 10 to 15 minutes. In fruit juices adjustment to pH 6.2 makes possible complete reduction with minimal quantities of bacterial suspension. Sufficient cells must be used to insure rapid reduction. If the reduction is not 100 per cent complete within 15 minutes, it never will be complete. The amount of cell suspension needed in a determination should be tested each time the cells are grown and every few days during storage.

J. Biol. Chem. 141: 853-858, 1941.

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1.0 per cent of cucumber juice concentrate was carried out at 10° in 0.05 M phthalate buffer of pH 5.0. As soon as the oxidation was complete, 1.0 cc. of the dehydroascorbic acid solution was added to 10 cc. of phosphate buffer of pH 6.2 containing bacterial suspension and glucose. Reduction was allowed to take place at 40°. In sixteen determinations the average per cent reduction was 98.6 ± 3.7 maximum deviation. This figure is slightly lower than 100 per cent because of unavoidable loss of dehydroascorbic acid during the time required for oxidation.

TABLE II

Comparison of Reducing Action of Bacterium coli and H_2S after Oxidation of Ascorbic Acid by Non-Specific Reagents

All solutions contained 0.88 mg. of ascorbic acid per cc. and solutions with H_2O_2 contained 0.3 mg. of H_2O_2 per cc.

Oxidizing agent	Ascorbic acid after partial oxidation	Ascorbic acid after reduction by *Bacterium coli* 15 min. at pH 6.2	Ascorbic acid after reduction by H_2S 20 min. at pH 4.0	Ascorbic acid after reduction by H_2S 24 hrs. at pH 4.0
	mg. per cc.	*mg. per cc.*	*mg. per cc.*	*mg. per cc.*
H_2O_2, unbuffered	0.046	0.14	0.13	0.21
O_2, "	0.61	0.69	0.69	0.73
H_2O_2, pH 5.0	0.008	0.054	0.054	0.096
O_2, " 5.0	0.60	0.69	0.70	0.73
H_2O_2, " 7.0	0	0	0.004	0.009
O_2, " 7.0	0.41	0.43	0.42	0.44
H_2O_2, " 10.0	0.079	0.082	0.086	0.086
O_2, " 10.0	0.19	0.19	0.19	0.24

The method generally used at present for the chemical determination of total vitamin C is by means of reduction with hydrogen sulfide (1,2,5,8). Mack and Tressler (6) have observed a non-specific action of hydrogen sulfide on biological materials by which reducing agents capable of reacting with the dye are produced. We have compared the reduction by *Bacterium coli* with the reduction by H_2S. If the reduction by H_2S is carried out at pH 4.0 in pure buffered solutions (free of biological materials), the two methods give the same results (see Table II). This agreement between the two methods holds even when non-specific oxidizing agents such as copper or hydrogen peroxide cause the destruction of the ascorbic acid. We have no evidence to support the suggestion of Tillmans (8) and Woessner, Elvehjem, and Schuette (9) that non-specific oxidizing agents besides causing the irreversible destruction of ascorbic acid will also cause the formation

TABLE III

Ascorbic Acid and Dehydroascorbic Acid in Commercial Milk

The values are given in mg. per liter.

	Days after milking							
	1		2		4		6	
	Ascorbic acid	Dehydroascorbic acid	Ascorbic acid	Dehydroascorbic acid	Ascorbic acid	Dehydroascorbic acid	Ascorbic acid	Dehydroascorbic acid
Raw	14.9	6.3	11.4	4.8	5.1	7.9	1.7	4.7
Pasteurized	14.9	3.6	10.4	4.8	3.8	7.9	2.4	2.6
" 0.1 mg. Cu per liter	7.9	8.6	2.1	8.9	0	5.1	0	1.0
Pasteurized, 0.5 mg. Cu per liter	0	12.6	0	7.0	0	3.4	0	1.0
Pasteurized, 1.0 mg. Cu per liter	0	9.6	0	4.9	0	3.1	0	0.7

TABLE IV

Reduced and Total Vitamin C in Commercial Canned Juices

The values are given in mg. per cc. of undiluted juice.

Juice	pH, undiluted juice	0.2 N NaOH to bring 1 cc. to pH 6.2	Immediately on opening can		After aeration and 5 days storage in glass at 1°			
			Ascorbic acid	Total vitamin C	Ascorbic acid	Total vitamin C	Total vitamin C after addition of 0.335 mg. dehydroascorbic acid	Recovery of added dehydroascorbic acid
		cc.						*per cent*
Orange	3.8	0.48	0.393	0.394	0.364	0.395	0.716	95.8
Grapefruit	3.8	0.92	0.344	0.342	0.307	0.321	0.652	98.9
Lemon	2.5	4.35	0.330	0.330	0.170	0.302	0.635	99.4
Pineapple	3.6	0.53	0.084	0.092	0.075	0.084	0.419	100
Apple	3.5	0.46	0.004	0.006	0	0.016	0.349	99.4
Sauerkraut	3.9	0.36	0.153	0.007	0.037	0	0	0
Composite vegetable	4.4	0.22			0.013	0	0.031	9.3

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of substances other than dehydroascorbic acid which, when reduced with H_2S, are capable of instantaneous reaction with indophenol at pH 4.0.

Tables III and IV show application of the method of bacterial reduction to milk and canned fruit juices. In the case of commercial milk samples titration of the reduced ascorbic acid alone is not even an approximate measure of total vitamin C. The method of bacterial reduction is not applicable to all biological materials. For example, in sauerkraut and a composite vegetable juice the *Bacterium coli* failed to cause any reduction of the added dehydroascorbic acid but acted instead as an oxidation catalyst to decrease the ascorbic acid present. This reversal of the action of the bacteria in some biological fluids has not been explained. The bacterial reduction method for total vitamin C can be applied satisfactorily to urine. In a typical analysis 1 cc. of urine was found to contain 0.027 mg. of ascorbic acid and 0.053 mg. of total vitamin C. Added dehydroascorbic acid was recovered to the extent of 99.4 per cent.

SUMMARY

The quantitative determination of total vitamin C in biological materials can be accomplished by reduction of dehydroascorbic acid to ascorbic acid with a resting suspension of *Bacterium coli*, followed by direct titration of the ascorbic acid with 2,6-dichlorophenol indophenol in acid solution. The reduction can be carried out in 15 minutes at pH 6.2 to 6.6 and 40° with a suspension of the bacteria in the presence of glucose.

The method has been applied to milk, fruit juices, and urine.

BIBLIOGRAPHY

1. Barron, E. S. G., DeMeio, R. H., and Klemperer, F., *J. Biol. Chem.*, **112**, 625 (1936).
2. Borsook, H., Davenport, H. W., Jeffreys, C. E. P., and Warner, R. C., *J. Biol. Chem.*, **117**, 237 (1936).
3. Esselen, W. B., and Fuller, J. E., *J. Bact.*, **37**, 501 (1939).
4. Kohman, E. F., and Sanborn, N. H., *Ind. and Eng. Chem.*, **29**, 1195 (1937).
5. Kon, S. K., and Watson, M. B., *Biochem. J.*, **31**, 223 (1937).
6. Mack, G. L., and Tressler, D. K., *J. Biol. Chem.*, **118**, 735 (1937).
7. Sharp, P. F., *J. Dairy Sc.*, **21**, 85 (1938).
8. Tillmans, J., Hirsch, P., and Dick, H., *Z. Untersuch. Lebensmittel*, **63**, 267 (1932).
9. Woessner, W. W., Elvehjem, C. A., and Schuette, H. C., *J. Nutrition*, **20**, 327 (1940).

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Chimie et industrie 40:891-892, 1938

Vitamin C Metabolism and Chronic Benzol Poisoning

J. Hagen, Arch Gewerbepath. 1936, 8(5): 541-569

Chronic benzol poisoning must be considered as a hypovitaminosis C. But the determination of ascorbic acid in urine cannot be used to establish an early diagnosis of poisoning.

The administration of vitamin C in the course of severe poisoning brings about great clinical and hematological improvement. According to the severity of the poisoning, daily doses of from 75 to 400 mg of vitamin C are necessary.

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Le métabolisme de la vitamine C et l'intoxication chronique par le benzol. — J. Hagen. — *Arch. Gewerbepath.*, 1936. T. 8, N° 5, p. 541-569.

L'intoxication chronique par le benzol doit être

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considérée comme une hypo-vitaminose C. Mais la détermination de l'acide ascorbique dans l'urine ne peut pas être utilisée pour établir le diagnostic précoce de l'intoxication.

L'administration de vitamine C au cours de l'intoxication grave se manifeste cliniquement et hématologiquement par une grande amélioration. Suivant l'état de gravité de l'affection, des doses quotidiennes de 75 à 400 mgr. de vitamine sont nécessaires.

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Biochem. J. 15: 521-522

LXIV. THE ANTI-SCORBUTIC PROPERTIES OF CONCENTRATED FRUIT JUICES. PART IV.

By ARTHUR HARDEN and ROBERT ROBISON.

(*Received July 27th, 1921.*)

In earlier communications [1919, 1920] we have shown that orange juice can be evaporated to a dry residue without suffering any loss of the anti-scorbutic accessory factor, and that the product retains its potency in a very considerable degree after two years' storage in a dry atmosphere at room temperature.

The effect of storage at higher temperatures (29° C.) has now been investigated and, from the results obtained, it is evident that the accessory factor is much more rapidly destroyed under such conditions.

This particular sample of orange juice was dried in December 1919 and was found to afford complete protection from scurvy when fed to guinea-pigs in daily doses of 0·15 g., equivalent to 1·5 cc. of the original juice. The very hygroscopic solid was transferred as rapidly as possible to a screw-top glass jar, which was then placed in a desiccator for a few days before being closed down. In spite of these precautions it is probable that the material absorbed some moisture, for on placing the jar in the incubator, slight softening was seen to take place and the top layer of the substance gradually darkened in colour, owing presumably to oxidation. The jar was kept at a uniform temperature of 29° until February 1921, *i.e.* during 14 months, after which further animal tests were begun. The material was then in the form of a hard toffee-like mass but was found to contain 8 % of water.

A number of guinea-pigs were fed on the usual basal ration of oats, bran and autoclaved milk, together with doses of the dried juice of 0·15 g., 0·3 g., 0·45 g., and 1 g., equivalent to 1·5 cc., 3 cc., 4·5 cc., and 10 cc. of the original juice. All the animals developed scurvy but those receiving the highest dose did not show any symptoms until the 24th day and were still alive on the 63rd day of the experiment. Some measure of protection was therefore given by this amount of the dried juice, but it is evident that more than 85 % of the anti-scorbutic accessory factor had been destroyed during storage under the above conditions. Tests were also carried out with some of the same batch of dried juice that had been stored in a desiccator at room temperature during a similar period, 14 months. Two guinea-pigs, each receiving 0·15 g. daily, showed normal growth for about 50 days but thereafter remained practically stationary in weight. They were killed on the 81st day and symptoms of scurvy were found. A guinea-pig which received a daily ration of 0·3 g.,

equivalent to 3 cc. of juice, showed no signs of scurvy after 81 days, though this animal also failed to exhibit normal growth during the last 20 days of the experiment.

In the experiments previously described it was found that a daily ration of 0·5 g. of dried juice that had been stored during two years at room temperature afforded complete protection from scurvy. It would appear, therefore, that under the conditions detailed above the loss of the anti-scorbutic accessory factor is of the order of 50 % of the amount originally present in the juice.

REFERENCES.

Harden and Robison (1919). *J. Roy. Army Med. Corps*, 48.
—— (1920). *Biochem. J.* 14, 171.

(the so-called 'static measurement', § II (1), p. 12). Both standard dye and diluted extract are at pH 3·5. By means of the pumping device (*C*), operated by an ordinary filter-pump, the two solutions are forced through the mixing chamber (*D*), and into the reaction tube (*DE*). Instantaneous mixing occurs at the moment when the solutions first impinge on one another head-on, at *D*. By means of the two manometer devices (*F*, *G*) the rate of flow of the solutions out of the two bottles is kept identical, and at any desired figure. Thus the reactants after mixing flow along the reaction tube

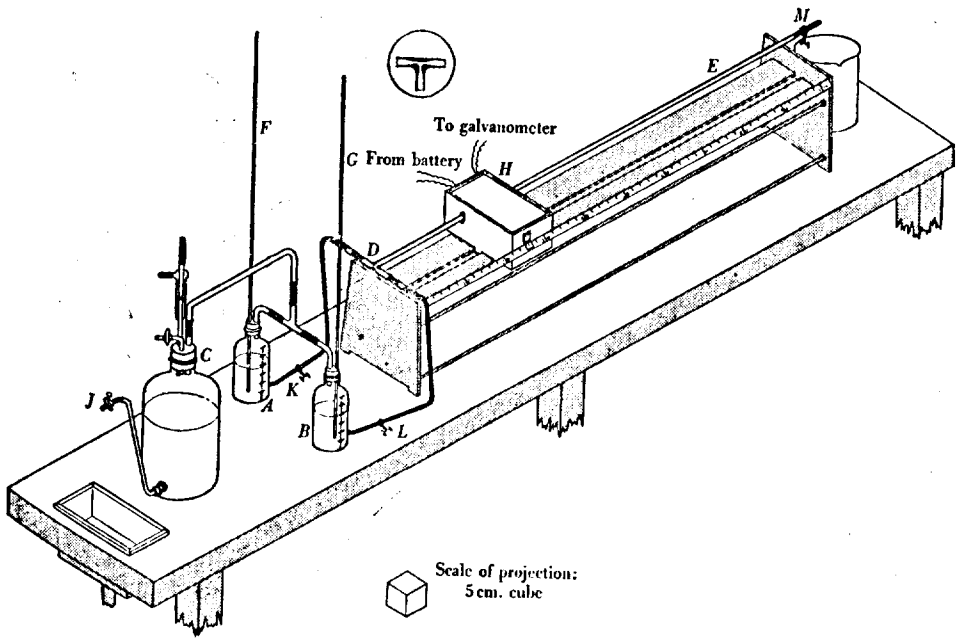

Fig. 1. Apparatus used for determination of ascorbic acid by the continuous-flow method. Isometric projection. Inset shows plan of mixing chamber on double scale.

in equivalent amounts and at a constant velocity. In consequence distances along the tube will also represent the time intervals of the chemical reaction. If, for example, the mixture flows along the tube at the rate of x cm./sec., then at distances x, $2x$ and $3x$ cm. along the tube, the reaction will have proceeded for 1, 2 and 3 sec. respectively. Now the extent of the reaction at any point is indicated by the degree of decolorization of the dye. This is determined by means of a photo-electric colorimeter (*H*) arranged to slide alongside the reaction tube, and parallel with it. Hence by plotting *colour intensities* against *distance along tube*, a continuous record of the time course of the reaction is obtained. When two separate chemical reactions are occurring simultaneously and at slightly different rates, as in the case of the ascorbic acid and the interfering substance both competing for the dye, it is possible to interpret the time curve in such a way as to measure the extent to which each of the two reactions has gone within a given time limit. From this we are able to measure the quantities of both ascorbic acid and interfering substances present.

Dissolve 4 g reagent grade KI in H_2O and dilute to 100 ml.

(c) *N-bromosuccinimide reagent.*—0.01% (w/v). Add 200 mg N-bromosuccinimide and about 100 ml H_2O to a 200 ml volumetric flask. Warm and shake until dissolved. Cool and dilute to 200 ml. This stock solution (0.1%) is stable for a few days at 4 C. Dilute stock solution (1+9) just before use. 1 ml of this 0.01% reagent is approximately equivalent to 0.1 mg ascorbic acid.

(d) *Standard ascorbic acid solution.*—About 1 mg/ml. Weigh accurately about 50 mg Reference Standard Ascorbic Acid. Transfer to a 50 ml volumetric flask and dilute to mark with 3% acetic acid. Standardize 0.01% N-bromosuccinimide at once as described below.

N-Bromosuccinimide Standardization

Transfer 2.0 ml freshly prepared ascorbic acid standard (about 2 mg) to a 100 ml beaker. Add 5 ml 4% KI solution and dilute with 3% acetic acid to about 45 ml (or enough to cover electrodes). Titrate to point of maximum inflection, using 2 platinum electrodes with polarizing current.

Stir at maximum rate without creating a strong vortex or splashing. After each addition of the titrant, re-adjust the voltage. Determine the volume of titrant that was used to reach equivalence point. Using this value, express concentration of standard reagent in terms of its ascorbic acid equivalent. (Example: 1 ml 0.01% N-bromosuccinimide = 0.0979 mg ascorbic acid.)

Determination

Assay sample immediately after opening container. Filter through cotton if necessary. Transfer an aliquot equivalent to 1–3 mg ascorbic acid to a 100 ml beaker (do not take aliquot greater than 25 ml). Add 5 ml 4% KI solution and dilute with 3% acetic acid to about 45 ml (or enough to cover electrodes). Titrate with standard 0.01% N-bromosuccinimide as in *N-Bromosuccinimide Standardization*. ml titrant × equivalent = mg ascorbic acid in aliquot.

Discussion and Recommendation

N-bromosuccinimide is a highly selective oxidizing agent. In the titration process of Barakat, *et al.*, free iodine is liberated by a second selective oxidation of KI after ascorbic acid has been totally oxidized to the dehydroascorbic acid stage.

Barakat, El-Wahab, and El-Sadr report that a number of substances that might be expected to interfere, including reductones, reductic acid, and iron salts, have no influence on the titration process.

The method described has been used for drug mixtures with good results. Sample and standard were prepared by the method in USP XVI, p. 67. Assays on three such mixtures agreed with the 2,6-dichlorophenol indophenol method within 2%. The method has also been used for routine analysis of drinks enriched with vitamin C, with apparently good results.

By changing the concentration of the N-bromosuccinimide reagent to 0.001%, amounts as low as 100 μg of ascorbic acid have been determined. This range has been tried only with the pure substance and not with sample material.

It is recommended that the method be studied collaboratively.

Acknowledgments

The author is indebted to George Schwartzman and George Keppel, Cincinnati District, for their helpful suggestions.

This report of the Associate Referee was presented at the Seventy-seventh Annual Meeting of the Association of Official Agricultural Chemists, Oct. 14–17, 1963, at Washington, D.C.

This recommendation of the Associate Referee was approved by the General Referee and by Subcommittee A, and was accepted by the Association. See *This Journal*, 47, 121 (1964).

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Determination of Ascorbic Acid in Presence of Interfering Substances by the 'Continuous-Flow' Method. 1. Preliminary Control Tests. By L. J. HARRIS and L. W. MAPSON

Dehydrated vegetables, caramelized by exposure to unduly high temperatures during either processing or storage, may contain reducing agents of the nature of reductones, which, since they react rapidly with indophenol dye, will interfere in the chemical estimation of ascorbic acid if the ordinary method of direct titration is used. A procedure has been devised for the differential determination of both ascorbic acid and reductones present in such materials, and has been used satisfactorily for routine determinations. It depends on the use of an apparatus employing the principle of the continuous flow,* by which the complete time course of a reaction can be followed.

Control tests with mixtures of equal volumes of ascorbic acid (0·000228 M) and 2, 6-dichlorophenolindophenol (in equivalent strength) at pH 3·5 and temperature 20° indicate that after 0·28, 0·55, 0·78 and 1·1 sec. respectively, 64, 80, 84 and 87–89 % of the reaction is complete, whereas with 'gluco-reductone' (mixture of substances formed by heating a solution of glucose with alkali) in place of ascorbic acid the corresponding values are 26, 34, 37·5 and 38·5 %. With 'amino-reductone' (mixture of substances formed by heating a solution of glucose with glycine and alkali), they are 30, 38, 41 and 44 %. The values when plotted lie on well-separated smooth curves, and thus ascorbic acid can be readily distinguished from these interfering substances. Moreover, mixtures of gluco-reductone and ascorbic acid give curves in intermediate positions, in proportion to the relative amounts of each present: hence both substances can be accurately estimated when present simultaneously. In recovery tests, ascorbic acid and gluco-reductones could be determined under the above conditions with an error of less than 2 % of the total reducing value.

Other substances were examined in the same way and their velocity constants determined. Cystein and sulphite could be readily distinguished from ascorbic acid by their much slower rates of reaction, while dihydroxymaleic acid, thiosulphate, and the ferrous ion were distinctive in having relatively slow rates at pH 3·5 but much faster at pH 1·4. Reductic acid had half the velocity constant of ascorbic acid, while hydroxytetronic acid, alone of the substances examined, had approximately the same constant: there is, however, no evidence that either of these substances, or dihydroxymaleic acid, occur in natural products.

* Hartridge & Roughton (1923). *Proc. roy. Soc.* A, **104**, 395.

Determination of Ascorbic Acid in Presence of Interfering Substances by the 'Continuous-Flow' Method. 2. Tests with Dehydrated Vegetables, and with Various Raw and Cooked Fruits and Vegetables. By L. J. Harris and L. W. Mapson

The reductants (interfering substances) formed when dehydrated vegetables are caramelized resembled gluco-reductones closely in their rate of reaction with 2, 6-dichlorophenol-indophenol at pH 3·5, and no indication was obtained of the presence of the other types of reductants mentioned in Part 1. Both the ascorbic acid and the interfering substance could therefore be estimated by the use of the 'continuous-flow' technique, the experimental evidence for this conclusion being as follows.

(1) Various dehydrated vegetables known to be devoid of antiscorbutic activity were heated for differing periods at 100° so as to cause caramelization to varying extents: application of the 'flow' test then accurately showed the presence of interfering substance and no ascorbic acid. (2) On the other hand, dehydrated vegetables which had known antiscorbutic activities, but had not been subjected to caramelization were shown by the test to contain ascorbic acid and no interfering substance. (3) Furthermore, in recovery tests, it was shown that a quantitative estimate was possible of the ascorbic acid or gluco-reductones, or both, added to various dehydrated vegetable preparations already containing either (*a*) ascorbic acid without the interfering substances produced on caramelization, or (*b*) the interfering substances without the ascorbic acid, or (*c*) both present simultaneously. (4) Results of determinations of ascorbic acid in caramelized dehydrated vegetables by the constant-flow method agreed well with those obtained by the formaldehyde method.*

The limit of error was low, the ascorbic acid or interfering substance, determined in recovery tests, ranging from about 97 to 103 % of the theory (expressed as a percentage of the total reducing content).

No significant amount of interfering substances was found in numerous fresh vegetables or in certain common fruit juices, nor in vegetables cooked by the usual methods of boiling or steaming, or processed or dehydrated in absence of caramelization. Hence, for the usual run of fruit and vegetable materials it is confirmed that the ordinary indophenol titration is accurate, since it gives the same result as that obtained by the 'constant-flow' method. Only with plant products heated in the presence of alkali, or heated to exceptionally high temperatures—as, for example, in the preparation of chip potatoes—are interfering substances encountered. In doubtful cases, or when new types of products are being examined, the flow method provides a rapid and convenient means of ascertaining whether or not certain interfering substances are present.

* Mapson (1943). *J. Soc. chem. Ind.* **62**, 223.

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Determination of Ascorbic Acid in Presence of Interfering Substances by the 'Continuous-Flow' Method*

By LESLIE J. HARRIS AND L. W. MAPSON
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(*Received* 19 *November* 1946)

Degree of applicability of indophenol test. It is now generally acknowledged that the results of biological and chemical assays for vitamin C on numerous common foodstuffs are in good agreement. Such comparisons of the biological and chemical values are available for virtually all the better known sources of the vitamin, including large numbers of fruits and vegetables, as well as for various less active or inert foods. Likewise there is no evidence that the chemical method is any less satisfactory when used for fruits or vegetables as normally canned, or as cooked by either boiling or steaming. Examples of this kind of correspondence between the chemical and biological data have been cited in previous papers (Harris & Ray, 1933 *b*; Birch, Harris & Ray, 1933; Harris & Olliver, 1942).

In claiming that the chemical method is thus reliable for all such ordinary purposes it is of course understood that the titration with 2:6-dichlorophenolindophenol is carried out by the standard procedure, and that the usual precautions are taken: these include the inactivation of oxidases and the prevention of vitamin loss during the preliminary process of extraction, the final titration itself being rapidly performed, and in a strongly acid reaction, in order to rule out reduction of the indophenol dye by glutathione and certain other substances (e.g. Harris & Ray, 1933 *a*; Birch *et al.* 1933; Harris & Olliver, 1942).

Products containing interfering substances. To this generalization, that for ordinary foodstuffs the simple method of titration gives accurate results, there is probably only one exception of any importance, namely, with certain caramelized and fermented products (Harris, 1933; Mapson, 1943 *a*; Wokes, Organ, Duncan & Jacoby, 1943 *a*). These may contain reducing agents of the nature of reductones, which since they react rapidly with the dye in acid solution will interfere in the titration unless a modified procedure is used. We need not refer here to animal products, including urine, except to mention that the presence in the latter of thiosulphate or other interfering substances does not militate against the practical applicability of the method of determining nutritional status in the human subject by counting the number of days taken to saturate (Harris & Ray, 1935; Harris, 1943; Nutrition Society, 1945). We intend to return to a discussion of the specificity of indophenol titrations for animal materials in a later publication. Also our concern here will be primarily with caramelized foodstuffs rather than fermented preparations.

Methods for use with caramelized products. At this Institute one of the authors had

* Communicated to the Biochemical Society, 29 September 1944 (Harris & Mapson, 1944).

been concerned in a study of the nutritional value of dehydrated foods. These, it was found, under exceptional conditions of preparation, namely, if exposed to unduly high temperatures during either processing or storage, were liable to become caramelized with the consequent production of the reductone-like substances. Thus a method for differentiating between the ascorbic acid and the interfering substances in them became necessary. The only quantitative test hitherto available was one depending on the different rates of condensation with formaldehyde (Lugg, 1942; Mapson, 1943*b*). This method, however, had the disadvantage of being time-consuming and relatively laborious. It was felt, moreover, that the results in the formaldehyde test would gain in conviction if they could be shown to agree with those obtained by a second method based on an entirely different principle.

The other author had previously been interested in the possibility of applying to this problem of differentiation between ascorbic acid and interfering substances the principle of the 'continuous-flow' apparatus. This apparatus had been first devised by Hartridge & Roughton (1923) for their physico-chemical investigations on haemoglobin, and provides a means whereby the rate of very rapid chemical changes may be accurately measured. It will be recalled that it was used over 10 years ago by Millikan (1935) for obtaining presumptive evidence of the identity with ascorbic acid of the reducing substances present in the wall of the gut (Hopkins & Slater, 1935); he did not, however, apply it to the actual estimation of ascorbic acid or its differentiation from other substances, which is the problem with which we have been concerned.

We undertook, therefore, a somewhat detailed study of the rates of reaction of ascorbic acid and other reducing substances, as influenced by various changes in the experimental conditions (such as pH, concentration of reactants, solvent, temperature, etc.), using the continuous-flow apparatus for the purpose. As a result we feel able to recommend a reliable and convenient technique which may be used, first, to detect whether or not the interfering substances are present, and secondly, if so, to make a quantitative estimation both of them and of the ascorbic acid. It will be shown that biological tests confirm the accuracy of this new method, and that the results agree also with those obtained by the formaldehyde method.

I. GENERAL ACCOUNT OF PROCEDURE AND FINDINGS

For simplification of presentation, a brief outline will first be given of the analytical procedure which was ultimately adopted, together with a short statement of some of the more important conclusions reached; the more detailed descriptions of the construction of the apparatus, of the technique of its use, and of the various theoretical studies, control tests and checks, can then follow more conveniently in later sections.

The continuous-flow apparatus. The equipment is shown in Fig. 1. Of two exactly similar bottles, one (*A*) contains a solution of indophenol dye of standard strength (1 ml. = 0·04 mg. of ascorbic acid), and the other (*B*) an extract of the 'unknown' made up at such a dilution as to be precisely equivalent in its total reducing capacity to the strength of the dye. The degree of dilution necessary is ascertained by means of a preliminary measurement of the total reducing titre, determined photo-electrically

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Potentiometric Determination of Vitamin C in Highly Colored Products

By WILLIAM S. HARDESTY (Food and Drug Administration, 1141 Central Parkway, Cincinnati, Ohio 45202)

The method of Barakat and co-workers for determination of vitamin C in colored products has been modified for potentiometric use. The procedure is simple and rapid: An aliquot of sample containing 1–3 mg of ascorbic acid is titrated in a 3% acetic acid medium in the presence of KI with previously standardized 0.01% N-bromosuccinimide. The potentiometric titration uses two Pt electrodes with a polarizing current. Results were compared with the USP XVI method and the AOAC 2,6-dichlorophenol indophenol method. Recoveries, obtained from seven commercial products, four of which were vitamin C-enriched, ranged from 96 to 101%. The method also works well on drug mixtures containing ascorbic acid. It is recommended that the method be studied collaboratively.

It is often necessary to determine vitamin C (ascorbic acid) in products not adaptable to the USP and AOAC methods. These products may contain extensive coloration or chemically interfering ions. Excessive coloration or pigmentation excludes any method relying on an internal indicator.

Barakat, El-Wahab, and El-Sadr (*Anal. Chem.*, 27, 536 (1955)) have described an N-bromosuccinimide titration, applicable to a variety of substances, in which the reaction of liberated iodine with an internal starch indicator produces a visual end point. This starch-iodide end point, however, is masked by coloring matter in many products. The procedure of Barakat, *et al.* was modified by the Associate Referee for potentiometric use.

Recovery experiments were made on seven commercial products, four of which were enriched with vitamin C. To obtain recovery data, the product was first assayed for ascorbic acid content; then a known amount of the vitamin was added to an aliquot and this aliquot was again assayed. The difference in content before and after addition of the vitamin was considered to be the ascorbic acid recovered. Recovery values ranged from 96 to 101% (Table 1).

METHOD

Special Apparatus

(a) *Titrating apparatus.*—Fisher Titrimeter, Model 35, or equivalent, equipped with a constant stirring assembly.

(b) *Two platinum wire electrodes.*

Reagents

(a) *Acetic acid.*—3% (v/v). Dilute 15 ml glacial acetic acid to 500 ml with H_2O.

(b) *Potassium iodide solution.*—4% (w/v).

Table 1. Recovery experiments of ascorbic acid from commercial juices and drinks[a]

Product	mg Ascorbic Acid per 5 ml Prepared Sample		Ascorbic Acid Recovered	
	Present	Added	mg	%
Orange juice	1.68	0.86	0.86	100
Enriched cranberry juice	1.46	1.52	1.48	97
Enriched vegetable juice	0.55	2.96	2.99	101
Enriched grape juice	1.23	2.00	1.98	99
Concentrated grape juice	0.08	2.24	2.25	100
Tomato juice	1.37	2.00	1.92	96
Enriched vegetable juice	0.76	2.33	2.30	99

[a] Ascorbic acid content of various commercial products was determined before and after the addition of known amounts of the vitamin. Submitted data represent averages of duplicate and triplicate determinations.

Method of determining ascorbic acid in the presence of 'gluco-reductone'. Under the standardized conditions described, the reaction between gluco-reductone* and the dye is found to proceed considerably more slowly than that between ascorbic acid and the dye (Fig. 2). After 1·1 sec. it will be seen that only 38% of gluco-reductone has reacted, whereas with ascorbic acid the reaction is almost complete. With mixtures of ascorbic acid and gluco-reductone, the curve is found to lie in an intermediate position, depending on, and in proportion to, the relative amounts of the two substances present (Fig. 2). Hence from the position of the curve it is possible to deduce how much of either constituent is present. For this purpose, and when other interfering substances

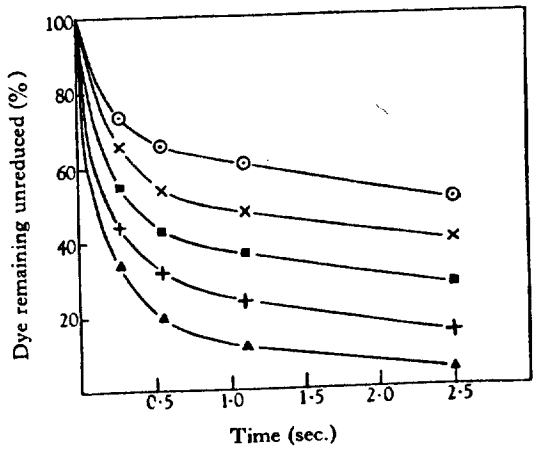

Fig. 2. Rates of reaction of ascorbic acid, gluco-reductone, and mixtures of the two, with the indophenol dye. (pH 3·5, 20°, strength of indophenol dye and total reductants ≡ 0·04 mg. of ascorbic acid/ml.) ⊙——⊙ Gluco-reductone 100%, ascorbic acid 0%; ×——× gluco-reductone 75%, ascorbic acid 25%; ■——■ gluco-reductone 50%, ascorbic acid 50%; +——+ gluco-reductone 25%, ascorbic acid 75%; ▲——▲ gluco-reductone 0%, ascorbic acid 100%.

are absent, all that is necessary is to have a simple reference curve (Fig. 3) relating the proportion of each in the mixture with the percentage of the reaction which is completed after some suitable time limit, e.g. 1·1 sec. Recovery tests indicate that this method gives results of satisfactory accuracy, viz. under the above-mentioned conditions, ascorbic acid and gluco-reductone could be determined with an error of less than 2% of the total reducing titre.

Rates of reaction of various substances with dye. The questions next arise: (1) What other substances are known which resemble ascorbic acid in their ability to reduce the dye? (2) Can they be distinguished from ascorbic acid by differences in their rates of reaction? (3) Do any of these substances actually occur in natural products or processed foods?

To answer these questions, tests will be described in § II (4) (see Fig. 8 and Table 4) to determine the velocity coefficients of the reaction of various known reductants with the dye. The conclusions reached are: (*a*) that it is almost always possible to distin-

* The term gluco-reductone is used throughout this paper to designate the several reductones which are produced when glucose is heated in alkaline solution. Reductone is used to mean the specific substance, enol-tartronaldehyde.

guish between ascorbic acid and other reductants by differences in their rates of reaction, but (*b*) that the matter is of little more than academic interest as there is no evidence of caramelized food containing reducing agents other than those of the gluco-reductone type already referred to.

Tests on ordinary foodstuffs. In the numerous raw fruits and vegetables which we have examined, the figures for vitamin C, as estimated by the flow method, agreed within the experimental error with those determined by the ordinary indophenol test. This again confirms the accuracy of the latter method, which is in agreement, moreover, with the results both of biological assays and of titration by the formaldehyde method. For steamed and boiled fruits and vegetables our conclusions are the same, although our experience here has been less extensive (see § II (5) and Tables 5 and 6).

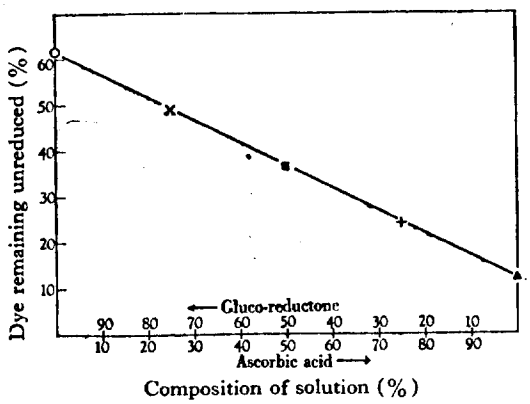

Fig. 3. Reference graph for determining relative amounts of ascorbic acid and gluco-reductone present in mixtures of the two, from the amount of dye found to be reduced in a given time. (Time 1·1 sec., 20°, pH 3·5, solutions of mixed reductants and dye each equivalent to 0·04 mg./ml. of ascorbic acid.)

Caramelized products. Our conclusion that in the case of caramelized dehydrated vegetables the interfering substance resembles gluco-reductone and that both it and the ascorbic acid can be estimated by the flow test, is based on the following observations, among others:

(1) Certain vegetable products devoid of vitamin C, after first being caramelized, were examined by the flow method, and found to respond in the test in a way resembling that of gluco-reductones but not like ascorbic acid.

(2) Conversely, biologically active vegetable products, which had not been subjected to caramelization, reacted in the way typical of ascorbic acid, and not of gluco-reductones.

(3) In recovery tests, ascorbic acid or interfering substance (either artificially prepared gluco-reductone, or the products present in caramelized dehydrated foods), or both, were added to natural foodstuffs, and it was shown that the amounts of both so added could be accurately determined.

(4) The results agreed with those obtained by the formaldehyde method. Also they were checked in a series of biological assays specially designed to be as crucial as possible.

Applicability of the new method. The general conclusion is that the method described can be used, first in a *qualitative* way; that is, to ascertain, when some new type of product is being examined for the first time, whether there is any evidence of interfering substances being present. If the test shows that the velocity of the reaction is the same as that for ascorbic acid, with the corresponding characteristic changes for pH (and if necessary for other variables), then the assumption may fairly be made that ascorbic acid only is present and further search for interfering substances is unnecessary. This is likely to be the case with all ordinary foodstuffs, and then the usual simple method of titration can be regarded as permissible and much labour is saved. If, on the other hand, as may happen with caramelized products, interfering substances are found to be present, then the method may be used in a *quantitative* way to estimate the amounts both of ascorbic acid and of interfering substance.

II. TECHNICAL SECTIONS

(1) *Initial determination of total indophenol-reducing capacity (static measurement)*

In the experiments to be described, velocities of reaction have always been determined by passing exactly equivalent amounts of the given reductant and of the indophenol dye through the flow apparatus. An accurate method for estimating the total indophenol titre of the reductant was therefore the first necessity.

With substances which, like ascorbic acid, react very rapidly, the value could be conveniently determined by simple titration in metaphosphoric acid against indophenol in the ordinary way. With other reductants, such as cysteine, which react relatively slowly, we found it better to titrate against iodine (see Lavine, 1935). With the special foods in which we were interested, however, neither of these procedures was permissible, as the extracts might have contained substances which were oxidized by iodine but not by indophenol, or which reacted only slowly with the latter. We found that the most satisfactory procedure for use in this case (as also in control tests with solutions of reductone and gluco-reductone) was to determine the total indophenol titre by means of a photo-electric colorimeter, used essentially according to the method described by Mindlin & Butler (1937) and by Bessey (1938), sufficient time being allowed, and sufficient excess of the dye added, to ensure the reaction going to completion. By this method the end-point could be determined more accurately in the presence of substances reacting slowly with the dye than by either the visual or the potentiometric methods. A further advantage over the visual method was that it could be operated with deeply pigmented solutions.

(*a*) *Photo-electric colorimeter and method of use.* A photo-electric colorimeter containing a contrast green Wratten filter, 61 N, stable to light, with an absorption range of 490–600 mμ and with a maximum transmission at 520 mμ was used. All solutions were examined in glass cells of 10 ml. capacity.

The principle of the method is to add to the unknown a constant amount of dye, in excess of that required for completing the reaction, and to estimate photo-electrically the amount of it left unchanged after the reaction. The difference between the amount of dye first added and that remaining at the end gives the measure of the total reducing titre.

(*b*) *Reference curve for galvanometer.* In order to be able to convert galvanometer readings into terms of excess of dye, reference charts were constructed as follows.

To 5 ml. of 0·5 M-phosphate-citrate buffer at pH 3·5, 1 ml. of ascorbic acid solution was added, in strengths varying from 0·0 to 0·08 mg./ml. To this mixture 4 ml. of a standard solution of 2:6-dichlorophenolindophenol (equivalent to 0·02 mg./ml. of ascorbic acid) were then added. In each instance the galvanometer reading was recorded after an interval of 45 sec. from the time of the addition of the dye. From the data so obtained a calibration chart was drawn, the ordinates giving the galvanometer readings, and the abscissae the amount of dye left unreduced, and hence by difference the reducing titre (amount of ascorbic acid present).

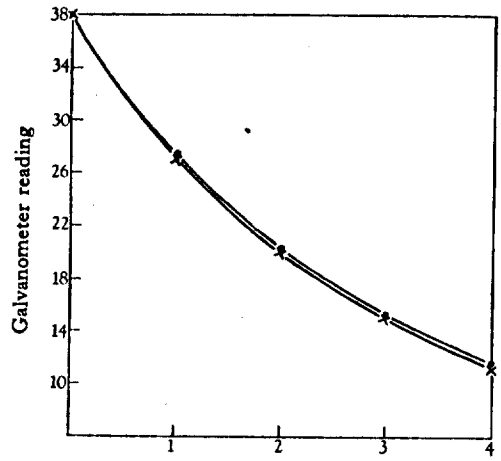

Fig. 4. Static measurement: reference chart for determining excess of dye from galvanometer reading. (Standard dye is equivalent to 0·04 mg. of ascorbic acid/ml.) ×——× After 45 sec. standing; ●——● after 5 min. standing. Spontaneous fading of dye is allowed for in these curves.

(*c*) *'Blank' correction for fading of dye.* The pH of 3·5 was purposely chosen because it is sufficiently acid to ensure a reasonably specific and rapid reduction of the dye by the ascorbic acid, but not too acid to cause any excessive rate of its destruction by bleaching. Indeed, within the time limit of 45 sec. referred to above the amount of fading was in fact ascertained to be negligible. However, in tests on food extracts, to be described below, a wait of longer than 45 sec. was sometimes necessary for the full reduction to occur. Hence an allowance for fading of the dye had to be made, although this correction was still relatively small. This correction was determined for standard periods of (*a*) 3 min. and (*b*) 5 min. For this purpose the same procedure was repeated as that described in the last paragraph, using standard solutions of ascorbic acid and dye, except for the longer periods of standing. In this way three separate reference charts (two of which are shown in Fig. 4) were prepared, one for 45 sec., one for 3 min. and one for 5 min., the two latter automatically correcting for the spontaneous fading of the dye within these respective time intervals.

(*d*) *'Blank' correction for natural colour of unknown.* In applying these reference

charts to determining the total reducing titres of 'unknowns', allowance had sometimes to be made for another factor, namely, any natural pigment present in the extracts. The procedure used was as follows. Five ml. of phosphate-citrate buffer, pH 3·5, were mixed with 1 ml. of the 'unknown', also adjusted to pH 3·5, and 4 ml. of the standard indophenol dye were added. The conditions were thus comparable with those employed for constructing the reference curves. The reading on the galvanometer scale after a chosen time interval (see below) was recorded. The excess of dye was then immediately decolorized by the addition of one drop of a strong solution of ascorbic acid, and the reading of the galvanometer for the corresponding 'blank' again ascertained. Finally, the amount of dye remaining unreduced was determined as the difference between the two galvanometer readings on the reference curve for the appropriate time interval. The difference between this figure and the amount of dye originally added is the amount of dye reduced in the given time interval.

Control tests confirm the accuracy of this procedure (see Table 1).

Table 1. *Estimation of ascorbic acid in coloured solutions*

Foreign dye present	Effect of added dye on percentage transmission ($100I/I_0$)	Ascorbic acid added (mg./ml.)	Ascorbic acid estimated (mg./ml.)	Error of estimation (%)
Edicol red	72	0·0387	0·0390	+0·8
Edicol red	84	0·0196	0·0196	0
Alizarin red	84	0·0388	0·0388	0
Alizarin red	70	0·0388	0·0392	+1·0
Brom thymol blue	85	0·0388	0·0390	+0·5
Brom thymol blue	77	0·0388	0·0392	+1·0
Thymol blue	76	0·0160	0·0157	−2·0
Cresol green	82	0·0160	0·0156	−2·5
Tropaeolin O	44	0·0160	0·0164	+2·5
Edicol brown	86	0·0387	0·0394	+1·8
Edicol brown	86	0·0194	0·0192	−1·0
Fuchsin	90	0·0387	0·0385	−0·5
Fuchsin	90	0·0194	0·0190	−2·0

(*e*) *Conditions necessary for completion of reaction (time; concentration of dye).* Since the object of this static measurement is to determine the total reducing capacity of the unknown, it is essential to be assured that the reaction has in fact gone to completion. The important factors are (1) that the time allowed should be sufficient, and (2) that adequate excess of dye should be added at the start.

To demonstrate the effect of concentration of dye on the rate of the reaction with gluco-reductone the following experiments were carried out. The solutions analysed contained 1 ml. of the gluco-reductone solution at pH 3·5. The appropriate amount of buffer solution (pH 3·5) was added, followed by varying amounts of standard indophenol dye (≡0·02 mg./ml. of ascorbic acid), to give a final volume of 10 ml. in each case. The amount of dye reduced was determined in the usual way by the photoelectric colorimeter.

Under these conditions the period of time necessary for complete reduction was found to diminish with every increase in the amount of indophenol dye in excess

(Fig. 5). For the estimation of total reducing power, a period of 45 sec. was too short unless very large excess of dye was added. The latter alternative, however, was not to be recommended since the percentage error of the estimation then became much greater, because of the lack of sensitiveness of the galvanometer to measure the small marginal diminution in the colour of the dye, in proportion to the large total amount present. Our procedure for estimating total indophenol-reducing capacity, therefore, has been to allow a uniform period of 5 min. to elapse after the start of the reaction, and to ensure that, at the end, an amount equivalent to at least 1·5 ml. of the standard indophenol dye is in excess. Unless such precautions are adopted variable values will be obtained, even if a fixed period is allowed for the reaction.

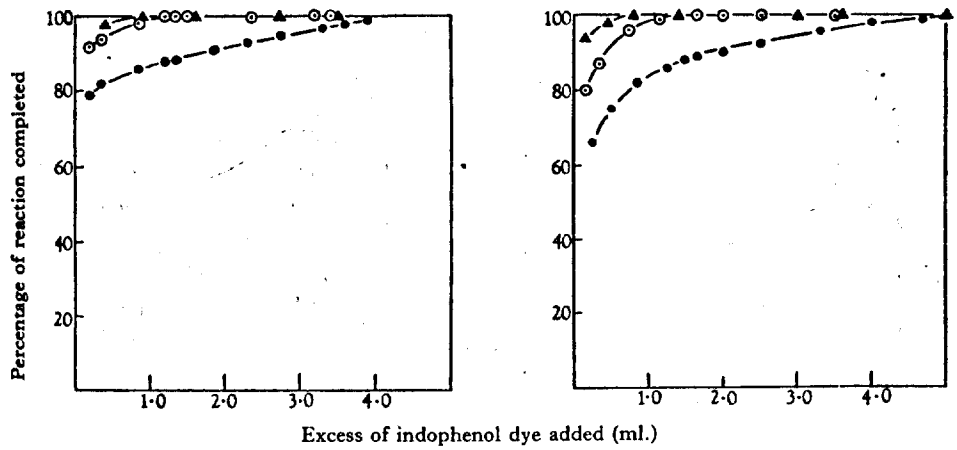

Fig. 5. Reduction of indophenol dye by gluco-reductone, as influenced by: (*a*) the excess of dye added, and (*b*) the time allowed for the reaction. ▲——▲ Reduction time of 5 min.; ⊙——⊙ reduction time of 3 min.; ●——● reduction time of 45 sec. *Left*, 'excess of dye' (abscissa) is expressed as the amount in excess of that which would be needed to complete the reaction; *right*, as the amount in excess of that actually found to have reacted within the given time limit.

(*f*) *Conditions for completion of the static reaction with caramelized vegetables.* Extracts of inert caramelized dried vegetables in metaphosphoric acid were found to reduce indophenol dye relatively slowly, compared with ascorbic acid. In their rate of reaction they resembled gluco-reductone. Moreover, as was also the case with gluco-reductone, the rate of reaction was increased by each increase in the concentration of the dye. Fig. 6 shows the relation between the percentage of the reaction completed and the concentration of dye used, in the case of an extract prepared from caramelized carrots. Other vitamin C-free caramelized vegetables behaved similarly.

As with gluco-reductone, 5 min. was found to be the most convenient period within which to estimate the total reducing titre, shorter periods involving the use of too large an excess of dye.

(2) *The continuous-flow apparatus*

The apparatus we have used is essentially the same in principle as that introduced by Hartridge & Roughton (1923), and we have modified it where necessary to meet our special requirement, namely, a determination of the velocity of reaction of indophenol

dye with ascorbic acid and with various other reductants. The salient points of the apparatus have already been described (p. 8 and Fig. 1). In order to obtain reproducible results, the conditions of use must be accurately standardized, and these will next be described.

(*a*) *Control of rate of flow*

The two reactants, indophenol dye and reductant respectively, are contained in two graduated glass reservoirs (aspirator bottles), *A* and *B* (Fig. 1), of identical shape and size (capacity about 200–400 ml.). Through the neck of each reservoir is attached a manometer tube and an inlet connected to a large aspirator (*C*) attached to a filter-

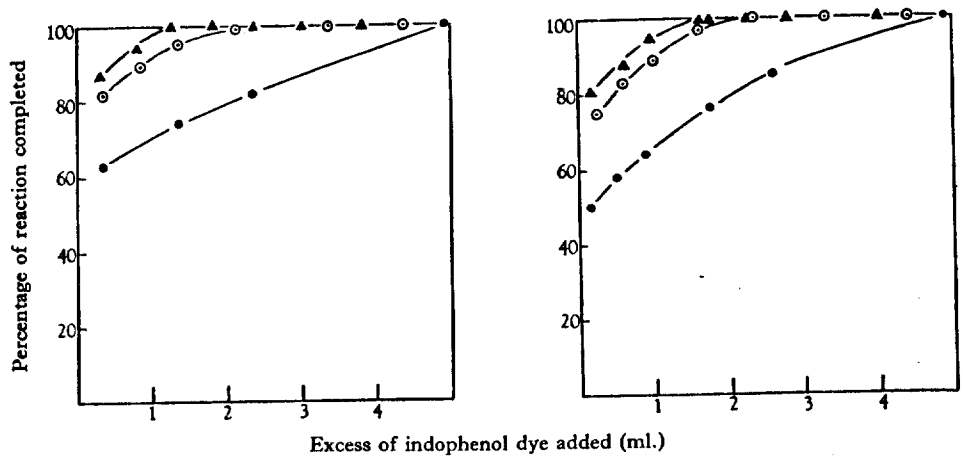

Fig. 6. Reduction of the dye by an extract of caramelized dried carrot, as influenced by: (*a*) the excess of dye added, and (*b*) the time allowed for the reaction (cf. Fig. 5). ▲——▲ Reduction time of 5 min.; ⊙——⊙ reduction time of 3 min.; ●——● reduction time of 45 sec. *Left*, 'excess of dye' is expressed as the amount in excess of that which would be needed to complete the reaction; *right*, as the amount in excess of that actually found to have reacted within the given time limit.

pump. The lower outlets lead to the side arms of the T-shaped reaction tube. The pressure in the aspirator and thus also in the two reservoirs is controlled primarily by means of a screw clip (*J*) attached to the outlet of the aspirator.

Before an estimation is begun, a slightly greater pressure is developed, by means of the pump, than that necessary to force the liquids through the reaction tube at the required rate. Clips (*K* and *L*) on the rubber tubes connecting the reservoirs with the reaction tube are next released, and then the clip (*M*) at the end of the latter. The sudden flow of liquids results in a momentary small drop in pressure; this is immediately adjusted to that required by means of the screw clip (*J*). This pressure must then be kept constant throughout the estimation.

The rate at which the solutions are delivered from the two reservoirs may be varied within wide limits by an alteration of pressure in this manner. In practice we have had occasion to use rates of flow varying from 400 to 1000 ml./min., enabling measurements of chemical change occurring in as short a time as 0·05 sec. to be measured. For most of this work a pressure producing a rate of flow of 430 ml./min. was em-

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ployed. With the conditions of our apparatus, and this rate of flow, a distance of 40 cm. along the reaction tube represented a time interval of 1·1 sec. In constructing a new apparatus where conditions may not be exactly the same (as regards volume, interval, pressure, etc.), appropriate rates of flow must first be ascertained.

(*b*) *Equalization of rates of flow of dye and reductant*

In estimating the velocity of reaction between indophenol dye and a reductant, it is clearly of prime importance that the volumes of the two solutions passing through the mixing chamber in any period of time should be equal. Experiments showed that the rate of flow of water from both bottles was nearly proportional to the square root of the pressure applied. From this it can be deduced that the rate of flow was limited more by the kinetic-energy-pressure-drop of the liquid in the mixing chamber than by the viscosity of the solutions in the tube. This implies that the volumes of the two solutions passing from the reservoir were influenced more by their relative densities than by their relative viscosities.

The 2% solution of metaphosphoric acid, used as extractant, had a specific gravity of 1·013, whilst that of the indophenol dye solution was 1·000. This difference in density was sufficient to cause a small but significant difference in the rates of flow from the respective reservoirs. To obtain an equal rate of flow under any pressure, the resistance to the flow of the dye solution was increased slightly by introducing, between the mixing chamber and the reservoir containing the dye, a piece of glass tubing of somewhat smaller bore than that between the mixing chamber and the reservoir containing the metaphosphoric acid.

Procedure with solutions of high density. The densities of solutions, in 2% metaphosphoric acid, of the various pure reducing substances examined, or of extracts of many food materials, did not differ significantly from that of the 2% acid itself, and such solutions passed through the flow apparatus at a rate equal to that of the dye. Extracts of certain food materials may, however, have a higher density (see below), and when this is so additional measures must be taken to ensure equal rates of flow of dye and extract. There are several methods whereby this may be achieved. That which we found most convenient was to add sucrose to the dye solution until its specific gravity differed from that of the extract by no more than the normal margin. This normal difference, between the specific gravity of indophenol dye and of 2% metaphosphoric acid, is 0·013.

Use of sugar. This method was found to be legitimate in use, for the added sucrose had little effect on the rates of reaction of the substances studied. For example, the rate of reaction of ascorbic acid with indophenol dye was slowed by only 1–2% when sucrose was used in concentrations as high as 10%, and we have seldom found it necessary in practice to add as much sucrose as this. If solutions of higher density were to be encountered, it would seem preferable to equalize the flow by the introduction of a further resistance to the flow of dye, in the shape of the tube of smaller bore, already referred to.

An example of an experiment, showing the effect of adding sucrose to equalize the flow, follows.

Dried carrot was extracted with a small volume of metaphosphoric acid. The specific gravity of the resulting extract was 1·042. This solution was forced through the apparatus alongside indophenol dye dissolved in water, under a given pressure, the volume of extract and dye delivered from each reservoir being recorded. A similar experiment was then carried out after sufficient sucrose had first been added to the dye solution to bring its specific gravity to 1·030. The following are the respective volumes of dye and extract delivered during a flow period of 1 min.:

		Volume passing through flow apparatus (ml.)
(a)	Indophenol dye (sp.gr. 1·000)	215
	Extract (sp.gr. 1·042)	197
(b)	Indophenol dye + cane sugar (sp.gr. 1·030)	200
	Extract (sp.gr. 1·042)	199

Alternative procedures. Another method whereby equalization of flow may be obtained is to alter the relative pressures in each reservoir. We have not found this procedure as simple to operate as the first.

It should be emphasized, however, that the need to take these special measures to equalize the rates of flow only arises exceptionally, when the extract to be examined happens to be of abnormally high specific gravity. In practice this will occur only (*a*) with foodstuffs unusually rich in sugar, such, for example, as jams, or (*b*) with materials of such a low reducing activity that particularly strong extracts have to be prepared from them.

(*c*) *Measurement of rate of flow*

In all instances, no matter whether the special measures just mentioned are needed or not, it is always essential to have a system for standardizing and checking the rates of flow. This consists of: (1) the use of calibrated manometers, and (2) an actual measurement of the volume of each liquid passed into the reaction tube in the course of the experiment.

(1) *Calibration of manometers.* The rates of flow corresponding with various heights of the liquid in the manometer tube are first determined, and plotted for later reference.

When a solution of more than usual density is being used, the complication arises that the rate of flow is lowered for the application of a given pressure. Hence a slightly higher pressure than normal has to be applied to the reservoir to obtain the standard rate of flow. It is therefore convenient to have separate calibration curves for the manometers corresponding with solutions of varying densities.

(2) *Check of volume delivered.* As already indicated, the two reservoirs (*A* and *B*, Fig. 1) are graduated. Before each experiment, a trial run is carried out to ensure that equal volumes of dye and extract are being passed from the reservoir at a constant rate into the mixing chamber. Again, after each experiment the amounts delivered from each reservoir are checked, to ensure that they are equal.

(*d*) *Photo-electric colorimeter*

The photocolorimeter used with the flow apparatus is built so that it can slide alongside the reaction tube. The source of illumination is an electric bulb, operated from a 6 V. accumulator. This is used in conjunction with an appropriate lens which passes the light through an oblong shutter and focuses it on the reaction tube. The shutter is made so that its aperture can be controlled by a vernier screw. By this means, a small rectangular section of the tube (4 mm. in height × 9 mm. in length) is uniformly illuminated. The amount of light reaching the photocell can be controlled by alteration of the size of the aperture. A Wratten filter, 61 N, is incorporated in the instrument.

(*e*) *Galvanometer*

We have found a moving-coil suspension galvanometer provided with an optical lever to be suitable. The galvanometer used has a resistance of 20 Ω, and a sensitivity of 125 mm./μA. at 1 m. distance, and a period of swing of 3 sec.

(*f*) *Determination of strength of dye from galvanometer readings*

To calibrate the photo-electric colorimeter for work both at pH 1·4 and 3·5, the following procedure is used. Indophenol dye of varying strengths (see below) is placed in one reservoir, and a solution of 2% metaphosphoric acid at pH 1·4 or of 2% metaphosphoric acid, adjusted with sodium citrate to pH 3·5, in the other. With no light passing through the apparatus, the galvanometer needle is adjusted to zero on the scale. The reaction tube is then filled with water and the intensity of light falling on the photocell adjusted by means of the movable shutter, so as to bring the galvanometer pointer to the top of the scale. With the instrument so adjusted, dye and metaphosphoric acid solution is run through and corresponding readings recorded. These readings are taken at several parts of the tube; they should not differ significantly for the same dye solution.

The relationship between concentration of dye and galvanometer reading is then determined in the following manner. A solution of dye, of standard strength (exactly equivalent to 0·04 mg. of ascorbic acid/ml.), is passed through the reaction tube from one reservoir, with an equal rate of flow of the acid from the other, and the galvanometer reading is recorded. This is repeated with varying dilutions of the dye down to 10% of the standard strength. From the corresponding galvanometer readings a chart is plotted, which shows that there is an almost linear, inverse relation between the logarithm of the galvanometer reading and the strength of the dye. This is plotted on semi-logarithmic paper and used as a reference curve in subsequent work, for calculating the strength of dye at a given point in the reaction tube from the observed galvanometer reading (Fig. 7). The slope of this curve will differ for each apparatus or for each change in experimental conditions. Alternatively, the relationship can be expressed by the formula:

$$\text{strength of dye (as \% of standard)} = K\,[\log G_w - \log G_d],$$

where G_w = galvanometer reading for tube filled with water, and G_d = galvanometer

reading for dye solution of given strength. In this way the value of K may be ascertained, and used subsequently for the calculation of dye strength from the galvanometer deflexions.

(*g*) *Method of use of flow apparatus for determining reaction velocities*

In carrying out an estimation of the rate of the reaction of a reductant with indophenol dye, the following general procedure was adopted.

The zero adjustment, with no light passing through the apparatus, and the adjustment to the illumination, so as to bring the galvanometer reading to the top of the scale, are made as described in the last section. With the instrument so set and the

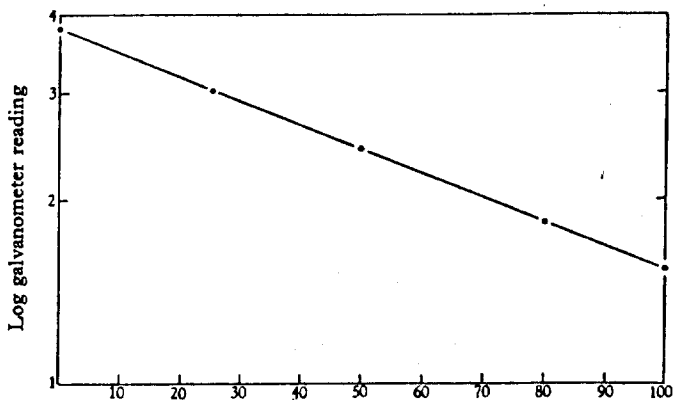

Fig. 7. Example of a reference curve, for determining strength of dye at any point in reaction tube from the galvanometer reading. (Standard strength is equivalent to 0·04 mg. of ascorbic acid/ml.)

photocell at that position on the tube at which a reading is to be recorded, the solution of the reductant and indophenol dye in equivalent strengths are then forced through the reaction tube, at equal rates of flow. First a sufficient quantity of the mixed solutions is allowed to flow through the tube to wash out all remaining water from it. The flow is next arrested and sufficient time permitted to elapse for the reductant to reduce the dye completely. This time will vary from one reductant to another and can be determined by taking periodic readings of the stationary mixed solutions until no further change in potential is registered by the galvanometer. This galvanometer reading for the 'blank' (G_b), as thus obtained, is recorded. (The difference between this reading and that given by water i.e. ($G_w - G_b$) is a measure of the natural colour of the solution under examination.) Immediately the blank value (G_b) has been determined, the reading of the galvanometer is observed during an actual flow (G_f). The amount of unreduced dye left at this point in the tube is obtained as the difference between these two readings on the calibration chart. Alternatively, it can be calculated by the formula:

$$\text{dye remaining unreduced } (\%) = K\,[\log G_b - \log G_f],$$

K being the constant previously determined during the calibration (§ II (2*f*)). By subtracting it from the total dye originally present, the amount reduced is obtained.

From this figure for amount of dye reduced and from a knowledge of the total reducing capacity (static measurement, § II (1), the extent to which the reaction has proceeded at the given point is determined. From the known rate of flow, and the distance along the tube, the time interval may be calculated. By repeating this same procedure at other points along the tube, the proportion of the total reaction which has been completed during different intervals of time can be determined, and hence the velocity of the reaction calculated.

(3) *Estimation of ascorbic acid in presence of gluco-reductone by the continuous-flow apparatus*

(*a*) *Comparison of reaction velocities of ascorbic acid and gluco-reductone.* In the control tests to determine the rates of reaction of ascorbic acid and of gluco-reductone with the dye, the standardized conditions already specified were followed (viz. equal volumes of ascorbic acid, gluco-reductone and dye solutions; dilutions of each equivalent to 0·04 mg. of ascorbic acid/ml.; temperature 20°; pH 3·5). As has already been noted (Fig. 2) the reaction proceeds much more quickly with ascorbic acid than with gluco-reductone. Thus with ascorbic acid, after intervals of 0·28, 0·55, 0·78 and 1·1 sec. respectively, 64, 80, 84 and 87–89% of the reaction is complete, whereas with gluco-reductone the corresponding figures are 26, 34, 37·5 and 38·5%. With mixtures of the two substances (Fig. 2, p. 10), the resultant rate is in proportion to their relative amounts. It is this fact which makes possible a quantitative estimation of both when they are present simultaneously.

(*b*) *Determination of ascorbic acid in presence of gluco-reductone.* The most convenient procedure for estimating the amounts of ascorbic acid and gluco-reductone in mixtures of the two, on the above principle, was to take readings after one single time interval (1·1 sec.) and to read the answer from a specially constructed reference curve (Fig. 3, p. 11). The latter, it will be understood, relates only to the given standard conditions (namely, total reducing strength equivalent to 0·04 mg. of ascorbic acid/ml., dye at the same relative dilution, temperature at about 20°, reaction normally at pH 3·5 and a time interval of 1·1 sec.), and is not applicable to other conditions. For use with more dilute solutions (equivalent to 0·02 mg. of ascorbic acid/ml.) a second, very similar but not quite identical reference chart, was constructed.

The time interval of 1·1 sec. is chosen because after that period the reaction with ascorbic acid is largely completed (viz. to the extent of 89%), while that with gluco-reductone has not yet gone too far, i.e. there is a sufficiently wide gap between the two individual curves.

(*c*) *Recovery tests.* The validity of this procedure was checked by recovery tests. These showed that both ascorbic acid and gluco-reductone could be determined in mixtures of the two, under the conditions described, with an error of less than 2% of the total reducing titre (Table 2). With more dilute solutions (0·02 mg. of ascorbic acid/ml.) the percentage error was, of course, somewhat greater. Reference is made later (§ II (7) and Table 8) to recovery tests in which known amounts of ascorbic acid and gluco-reductone were added either (1) to inert materials, e.g. oatmeal, or (2) to

extracts of fresh vegetables in which all the reducing material behaves like ascorbic acid, and in which satisfactory recoveries were also obtained.

(*d*) *Results with 'amino-reductone'*. With what we have termed 'amino-reductone', a mixture of substances formed by heating together a solution of glucose, glycine and alkali, the curve of the reaction was similar to that for gluco-reductone, but went slightly faster (Table 3), viz. 29, 38, 41 and 44% reduction after 0·28, 0·55, 0·78 and 1·1 sec. (cf. values in § II (3*a*)).

Table 2. *Determinations of ascorbic acid in presence of gluco-reductone by the continuous-flow method*

Total reducing titre, in equivalents of ascorbic acid (mg./ml.)	Percentage composition: Present: Gluco-reductone (%)	Percentage composition: Present: Ascorbic acid (%)	Percentage composition: Found Ascorbic acid (%)
0·04	90	10	10
	85	15	16
	79	21	20
	61	39	38·5–41*
	60	40	38
	50	50	49·5–50·5*
	43	57	55·5
	40	60	61
	26·5	73·5	75
	15	85	87·5
0·02	70	30	34
	80	50	47–54*
	35	65	68
	30	70	74
	10	90	87
	57	43	44
	72	28	30
	85	15	16

* Range of values in multiple observations; remaining values are averages of two or three observations.

(*e*) *Effect of change of pH on velocity of reactions*. In the cases of ascorbic acid, gluco-reductone and amino-reductone, a change in the reaction from pH 3·5 to 1·4 had no appreciable effect on the curves of the reaction. This is important in view of the fact that other substances (see below) are subject to pH shifts.

(4) *Reaction velocities of various known substances*

In order to discover whether certain other reducing agents might interfere when present, and if so whether they could be allowed for in a similar fashion, measurements of reaction rates were made in the same way, and the velocity constants calculated (Table 4 and Fig. 8).

It was found that *cysteine* and *sulphite* could both be readily distinguished from ascorbic acid by their much slower rates of reaction. *Dihydroxymaleic acid*, *thiosulphates* and *ferrous* salts could be distinguished by having relatively slow rates at pH 3·5 but much faster at pH 1·4. *Reductic acid* had a velocity constant about one-half that of ascorbic, while *hydroxytetronic acid* alone of the substances examined had

TABLE 1. Effects of ethionine and carbontetrachloride on tissue content and urinary excretion of ascorbic acid.

Treatment	Dose (mg/kg)	Tissue content (μg/g) Liver	Kidney	Liver/Kidney ratio	Urinary excretion (μg/100g/day)
1) Controls		296 ± 3(16)	150 ± 4(16)	1.95	402 ± 43(15)
2) Ethionine	50	203 ± 7(8)	146 ± 6(8)	1.39	989 ± 124(12)
3) Ethionine	200	158 ± 5(16)	134 ± 7(16)	1.18	1158 ± 148(12)
4) CCl_4	80	270 ± 10(8)	123 ± 5(8)	2.19	165 ± 29(8)
5) CCl_4	300	253 ± 9(8)	108 ± 7(8)	2.34	133 ± 18(8)
6) CCl_4	600	225 ± 9(7)	95 ± 4(7)	2.47	121 ± 21(7)

Difference and significance :

	Tissue content Liver Diff. (%)	Signif.	Kidney Diff. (%)	Signif.	Liver/Kidney ratio Diff. (%)	Urinary excretion Diff. (%)	Signif.
1)——2)	−31	p<0.01	−3	N.S.	−29	+146	p<0.01
1)——3)	−46	p<0.01	−11	N.S.	−39	+188	p<0.01
1)——4)	−8	N.S.	−18	p<0.01	+12	−59	p<0.01
1)——5)	−14	p<0.01	−28	p<0.01	+20	−67	p<0.01
1)——6)	−23	p<0.01	−37	p<0.01	+27	−70	p<0.01
3)——6)	+42	p<0.01	−29	p<0.01	+109	−89	p<0.01

Each value represents the averages ± standard error from 7–16 rats. The numerals in the brackets indicate numbers of animals used. Ethionine and CCl_4 were given intraperitoneally for 4 days and the rats were killed 24 hours after the last injection.
The urines were collected for 24 hours started immediately after the last injection.

and kidney was increased. For example, liver/kidney ratio of ascorbic acid was decreased by 29% and 39%, respectively, in the rats dosed 50 mg/kg and 200 mg/kg of ethionine, while the ratio was increased by 12%, 20% and 27%, respectively, in the rats dosed 80 mg/kg, 300 mg/kg and 600 mg/kg of carbontetrachloride.

Moreover, the administration of ethionine markedly increased the urinary excretion of ascorbic acid, while the administration of carbontetrachloride markedly decreased the urinary excretion of ascorbic acid. For example, the urinary excretion of ascorbic acid was increased by 146% or 188%, respectively by the administration of 50 mg/kg or 200 mg/kg of ethionine. In contrast, the urinary excretion of ascorbic acid was decreased by 59%, 67% or 70%, respectively by the administration of 80 mg/kg, 300 mg/kg or 600 mg/kg of carbontetrachloride.

These results suggested that ethionine may release ascorbic acid from liver and thus it increase the urinary excretion, while carbontetrachloride may inhibit the biosynthesis of ascorbic acid in liver and thus it decrease the urinary excretion.

2. *Tissue content of ascorbic acid after the administration of ascorbic acid in rats pretreated with ethionine or carbontetrachloride*

To proof the above-mentioned hypothesis ascorbic acid (200 mg/kg, i.p.) was given 4 hours before sacrifice to the rats pretreated with ethionine or carbontetrachloride.

(*a*) L-*Ascorbic acid* (Fig. 8). The reaction is bimolecular, and the velocity constant calculated on that basis (0·001 N, 20°, pH 1·4 or 3·5) was found to be 30 ± 4. This agrees well with Millikan's (1935) value, 30 to 34 ± 5.

(*b*) iso*Ascorbic acid* (D-*arabo-ascorbic acid*). This is an artificial product, a stereoisomer of L-ascorbic acid, not known to be present in any natural foodstuff, but having some biological activity. Its velocity constant at pH 1·4 or 3·5 was identical with that of ascorbic acid.

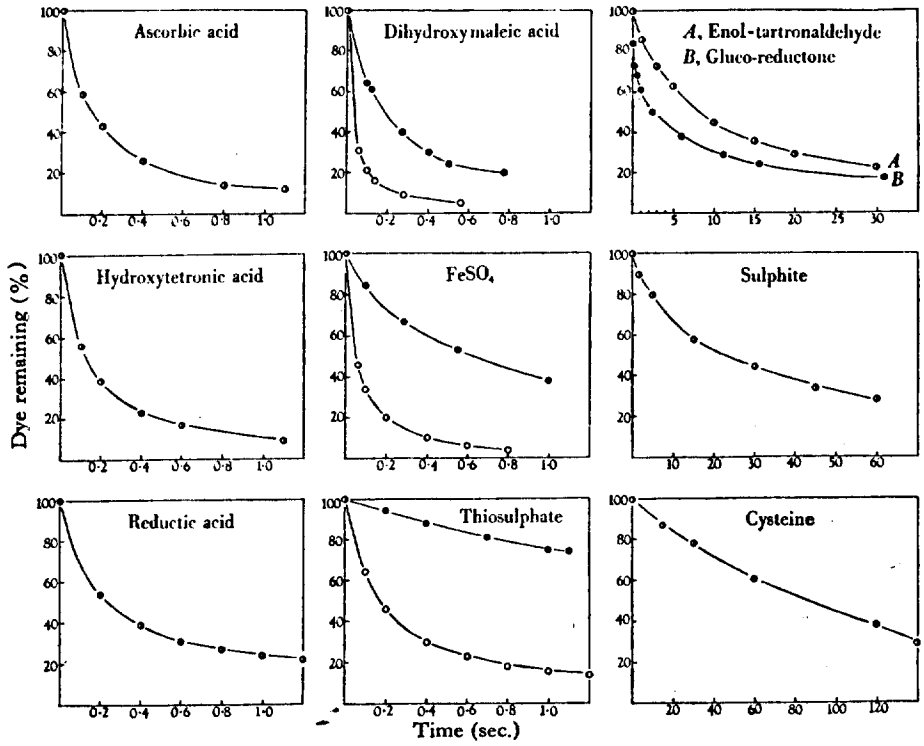

Fig. 8. Rate of reduction of 2:6-dichlorophenolindophenol by ascorbic acid and other substances, under standardized conditions. ●——● at pH 3·5; ○——○ at pH 1·4; ◑——◑ at pH 3·5 and 1·4. Indophenol dye and each reductant present in equivalent strengths (viz. ≡ 0·04 mg. of ascorbic acid/ml.)

(*c*) *Cysteine.* It has previously been pointed out (Harris, Mapson & Wang, 1942) that free cysteine, which is not normally present in fresh foodstuffs, does not in any case introduce any serious error in the estimation of the vitamin, provided that the titration is done rapidly according to the standard procedure (Harris & Ray, 1933 *a*, *b*; Birch *et al.* 1933; Harris & Olliver, 1942). It was, nevertheless, of interest to determine the precise value of the velocity constant. As will be seen (Table 4) it reduces the dye at a rate some 600 times slower than that of ascorbic acid, under the conditions studied.

(*d*) *Sulphite and thiosulphate.* Sulphite is of interest because of its presence in many preserved foods. Methods are already available for estimating it and ascorbic acid in

presence of each other (Harris *et al.* 1942; Mapson, 1942). Thiosulphate is said to be present in urine, and methods have likewise been described for its differential estimation (e.g. van Eekelen & Emmerie, 1935). It will be noted that at pH 1·4 the reaction of thiosulphate is nearly as rapid as that of ascorbic acid, but at pH 3·5 much less rapid; sulphite reduces much less slowly at both pH values.

(*e*) *Ferrous salts.* Since the ferrous ion is slowly autoxidized even in acid solution, it was necessary to take special measures for its protection. These consisted of dissolving $FeSO_4.7H_2O$ in 2% HPO_3, previously rendered free of oxygen, and after checking the strength by the static titration against indophenol, determining the velocity coefficient immediately in the flow apparatus. The distinguishing feature again is the much slower rate of reaction compared with that of ascorbic acid at pH 3·5, although at pH 1·4 the rate of reaction is faster than that of ascorbic acid. When ferrous salts are present together with ascorbic acid, several alternatives are of course available for estimating both, e.g. the formaldehyde method (Mapson, 1946).

(*f*) *Reductone.* A specimen was isolated from its lead salt from a caramelized solution of glucose (Euler & Martius, 1933) and recrystallized five times from ethanol. The rate of reaction (Table 4) is some 50 times slower than that of ascorbic acid, and is unaffected by change of reaction from pH 1·4 to 3·5.

(*g*) *Gluco-reductone.* A solution was prepared: (*a*) by treating a 2% solution of glucose with a few mg. of KCN, and heating at 90° for 2½ min. in presence of 0·17N-NaOH (Euler & Martius, 1933), or (*b*) by heating a 0·5% solution of glucose with 0·05N-NaOH for 10 min. at 80° (Kertesz, 1934). Variations in the time of heating or in the strength of the soda, within these limits, were found to produce no significant changes in reducing properties. The solutions were acidified with 20% HPO_3, cooled, brought to pH 3·5 by addition of sodium citrate solution, and diluted to approximately the correct reducing titre when tested by the static method, and finally more accurately adjusted.

To determine the rate of reaction after periods greater than 2·5 sec. the flow was arrested in the reaction tube, and readings taken at intervals at a fixed point.

It will be noted (Figs. 8, 9) that with gluco-reductone the reaction is relatively rapid during the first sec., and that a slower reaction then follows. This indicates again that a solution of this kind behaves differently from a single, pure substance. Apparently at least two reductants are present. For this reason it is not possible to calculate a velocity constant.

In comparison with reductone itself (enol-tartronaldehyde), the rate of reduction of the gluco-reductone mixture is greater initially. There was a scarcely perceptible increase in the rate of reaction on changing the pH from 3·5 to 1·4.

(*h*) *Other reductones.* Similar reducing solutions were prepared from xylose, fructose, galactose, mannose and arabinose by heating with alkali. These solutions reduced the indophenol dye at a rate not significantly different from that of gluco-reductone.

(*i*) *Amino-reductone.* The production of reductones is known to be influenced by the presence of amino-acids in solution (Enders, 1943). A solution of amino-reductone was prepared by treating a solution of glucose (1%) with a few mg. of KCN and NaOH

(0·17N) at 90° for 2½ min. The product was acidified and the concentration adjusted to standard strength as found necessary after the usual static measurement (§ II (1)). This solution differed from gluco-reductone by (1) the development of an appreciable orange or brown colour on standing, and (2) a slight fall in total indophenol titre during the first 1–2 hr. after preparation. The rate of reaction was a little greater than that of gluco-reductone at pH 3·5, and was increased slightly by a change of pH from 3·5 to 1·4.

(*j*) *Reductic acid.* This substance is formed when certain sugars are heated in presence of strong mineral acid (Reichstein & Oppenauer, 1933; cf. Snow & Zilva, 1944). From its structure it might be expected to react with indophenol in a manner similar to ascorbic acid. The constant was found to be about half that of ascorbic acid, and it was not affected by change of pH from 3·5 to 1·4.

(*k*) *Dihydroxymaleic acid.* Determination of the velocity constant was rendered difficult by the fact that this substance decomposes into glyco-aldehyde and CO_2 in acid solution (Franke & Brathuhn, 1931). In order to minimize the decomposition, a strong solution was prepared in ethanol, a small portion of it diluted with sufficient HPO_3, and the strength quickly determined, and then diluted to the necessary degree. The noteworthy feature of the reaction was the great increase in the velocity constant with raised acidity (Table 4).

(*l*) *Hydroxytetronic acid.* Again, as was to be expected from the closely similar chemical structure, the rate of reaction of hydroxytetronic acid was almost the same as that of ascorbic acid, and was unaffected by a change of reaction from pH 3·5 to 1·4 (Table 4).

(5) *Tests on fresh and cooked fruits and vegetables*

Fresh fruits and vegetables

Extracts prepared from various fresh fruits and vegetables were examined by the procedure which we have described above. It was found that the reducing substances present reacted with indophenol dye, both at pH 3·5 and 1·4, at a rate indistinguishable from that of ascorbic acid (Table 5). This gives further evidence for the view that in such foods there are no significant amounts of indophenol-reducing substances other than ascorbic acid (Birch *et al.* 1933; Harris & Olliver, 1942; Mapson, 1943*b*).

Experimental details. The technique used was as follows. Extracts of the fresh material were made by grinding up a weighed quantity of the fresh fruit or vegetable with a small amount of 20% metaphosphoric acid. Sufficient water was then added to reduce the concentration of metaphosphoric acid to 2%, and the reaction adjusted to pH 3·5 by sodium citrate solution. The total reducing activity of the extract was determined by the static measurement (§ II (1)), and the solution diluted to the extent necessary to make it equivalent to 0·04 mg. of ascorbic acid/ml. The rate of reaction was then determined in the flow apparatus. The same procedure was repeated with the reaction at pH 1·4.

Reducing substance in black-currant skins. The only exception which we have so far

encountered to the generalization that the reducing substance in fresh fruits and vegetables has the characteristics of ascorbic acid, is that a small amount of some other unidentified reductant was found in the skin of black currants but not in their juice. Since the skin of the fruit represents so small a proportion of the total berry, this finding, although of interest, seems of little practical significance.

Table 5. *Rates of reaction of ascorbic acid, and of extracts made from fresh fruits and vegetables, with indophenol dye* (20° *and* pH 3·5)*

Material tested	Percentage of reaction completed after			
	0·28 sec.	0·55 sec.	0·78 sec.	1·1 sec.
Ascorbic acid	64	80	84	87–88
Parsley	65	78	84	86
Brussels sprouts	65	80	85	89
Marrow-stem kale	63	79	84	86
Potatoes	63	78	83·5	86
Curly kale	64	79	84·5	86·5
Broccoli leaf	64	76	83	87
Spring cabbage	66	78	83	86
Broccoli	63·5	77	82	86·5
Spinach	66·5	79·5	84	88
Lettuce	65	79	83·5	88
Peas	65	80	84	88
Nasturtium leaves	64	80	83·5	88
Carrot tops	64	78	83·5	86
Orange juice	65	79	84	88·5
Tomatoes	63	78	83·5	86
Cress	66	79	84	87
Lemon juice	64	77	82	86·5
Grass	64·5	79	84	87
Gooseberries	66	79	84	88
Strawberries	63	80	84	88
Raspberries	64·5	78	—	86
Red currants	65	77	83	86
Black currants: whole (sample 1)	62	73	80	83
whole (sample 2)	58	72	—	81
skinned	64·5	78	83	87
Black-currant skins: sample 1	44	59	—	73
sample 2	41	48	—	62
Walnuts, unripe whole: sample 1	64·5	78·5	84	87
sample 2	64	78	—	87

* Concentrations of reactant and dye as in Table 3.

Cooked vegetables

A few experiments were conducted with vegetables cooked by the ordinary methods of boiling or steaming, and with various canned fruits and vegetables. The results (Table 6) give no evidence of the presence of any interfering substance.

It is recognized, of course, that when vegetables are cooked in the presence of excess of alkali with the pH above 8·5 (Allen & Mapson, 1944), or raised to a sufficiently high temperature to cause caramelization, as, for example, in the preparation of chip potatoes, interfering substances resembling gluco-reductones are formed. These may be differentially estimated by the technique described in this paper.

(6) *Tests on caramelized dehydrated vegetables*

The object of this section will be to describe control experiments in which caramelized dried vegetables were used as the test material.

Dried vegetables were taken which contained no vitamin C, or in which, if present, it could be destroyed by exposure to light. This dried product was then caramelized by heat, with concomitant production of the interfering substances. These, it was shown (e.g. Fig. 9 and Table 7) closely resembled gluco-reductone or amino-reductone in their rate of reaction with indophenol dye and could be estimated in the same way.

Table 6. *Rates of reaction of extracts made from cooked fruits and vegetables with indophenol dye* (20° *and* pH 3·5)*

Cooked fruit or vegetable	Percentage of reaction completed after 0·28 sec.	0·55 sec.	1·1 sec.
Spring cabbage (boiled)	65·5	78·5	87·5
Spring cabbage cooking liquor	65	79	87·5
Savoy cabbage (boiled)	64	78·5	86·5
Brussels sprouts (boiled)	65	78	87
Brussels sprouts cooking liquor	65	78	87·5
Brussels sprouts (steamed)	64·5	78	87
Bottled gooseberries	64	78·5	86
Spinach (boiled)	62·5	76·5	86

* Concentrations of reactant and dye as in Table 3.

Materials used. The dried vegetables studied included carrots, swedes, potatoes and cabbage. In the case of the three first-mentioned products, specimens were specially selected in which the amount of indophenol-reducing substance was nil or negligible. In the case of the cabbage, and where necessary with the other products also, the ascorbic acid present was destroyed by the following procedure. The powdered dried vegetable, after being well moistened with water, was exposed to a 500 W. lamp. The ascorbic acid rapidly fell to an insignificant figure (e.g. after 12–15 hr. illumination to less than 6 mg./100 g. of dried cabbage) without the production of other indophenol-reducing substances.

Production of caramelization. The reducing substances with which we are concerned are produced when dried vegetables are (*a*) dried at abnormally high temperatures or (*b*) stored for moderate periods at temperatures above 25° (Mapson, 1943*b*). The amount formed varies for each vegetable and is affected by other factors (Brightwell & Mapson, 1946); at 100° the formation is rapid.

In our experiments, vitamin C-free dried products, as described above, were finely ground, sieved, and heated on shallow trays in an oven at 100° for periods varying from 1 to 48 hr. Under these conditions browning quickly occurred (see also Tomkins, Mapson, Allen, Wager & Barker, 1944). With carrots and swedes the product was a dark brown powder, and with cabbage and potatoes light brown. These powders were extracted with 2% metaphosphoric acid. The colour of the resulting filtrates ranged from light yellow to deep brown. The amounts of reducing substance produced are shown in Table 7, second line of figures. It is unlikely that in practice any more highly

caramelized vegetables would be encountered than those here described. The reducing titres of the metaphosphoric-acid extracts decreased slowly on standing, unless the solution was rendered free of oxygen (as by means of a stream of nitrogen).

Reaction curves of the interfering substances present. The total indophenol-reducing capacities of the metaphosphoric-acid extracts of these caramelized dried vegetables

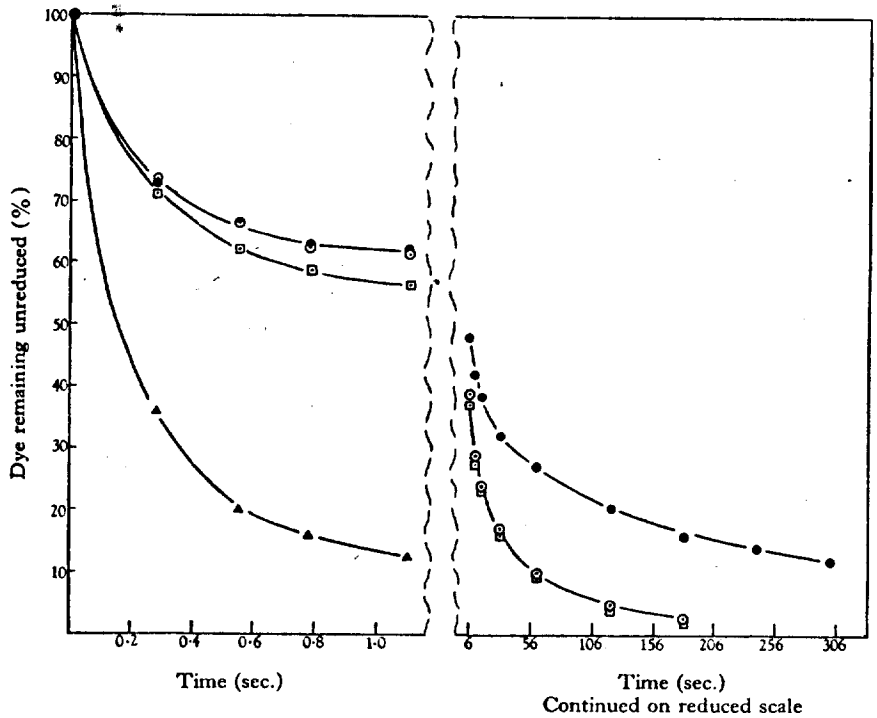

Fig. 9. Rates of reaction of gluco-reductone, amino-reductone, extract of caramelized carrot, and ascorbic acid for comparison. (pH 3·5, 20°, strength of indophenol dye and total reductant ≡ 0·04 mg. of ascorbic acid/ml.) ⊙——⊙ Gluco-reductone; ⊡——⊡ amino-reductone; ●——● extract of caramelized carrot; ▲——▲ ascorbic acid.

were first determined in the static photo-electric colorimeter (§ II (1)), and the extracts diluted as necessary to bring them to the standard strength (equivalent to 0·04 mg. of ascorbic acid/ml., § II (2)). The reaction curves were then followed in the flow apparatus at pH 1·4 and 3·5.

It was found, as with gluco-reductone, and in contrast with ascorbic acid, that the rate of reaction was slightly faster at pH 1·4 than at 3·5. Moreover, within the first 1–2 sec. of the reaction at pH 3·5, the ıction curve was almost indistinguishable from that of gluco-reductone, and after the standard time period of 1·1 sec. the percentage reduction was identical with it within the experimental error.

Although, as just mentioned, the reaction curve of the caramelized material at pH 3·5 so closely resembled that of gluco-reductone during the first second or two of the reaction, an appreciable difference could be detected after more prolonged periods, the reaction at this stage proceeding somewhat more slowlv (Fig. 9). Also, the longer

Table 7. *Rate of reaction of extracts made from inert caramelized dried vegetables with indophenol dye, at* pH 3·5 *and* 1·4 (20°)
(For comparison with values for gluco-reductone and amino-reductone, see Table 3.)

Time after start of reaction (sec.)	pH	Percentage of reaction completed. Product (with no. of hours heated at 100°, and indophenol titre)*														
		Swede					Carrot				Potato			Cabbage		
		1½ hr. 1·36 mg.	2 hr. 1·56 mg.	3½ hr. 2·30 mg.	4½ hr. 2·45 mg.	10–16 hr. 3·10 mg.	3 hr. 0·92 mg.	6 hr. 1·57 mg.	12 hr. 1·77 mg.	24 hr. 2·24 mg.	16 hr. 0·95 mg.	29 hr. 0·99 mg.	48 hr. 1·00 mg.	1 hr. 0·75 mg.	3 hr. 1·27 mg.	27 hr. 2·20 mg.
0·28	3·5	28	28	28	28	33	27	29	30·5	33·5	29	29	30	—	—	30
0·55		33·5	34·6	33	36	38	33·5	34·7	36	39·5	36	36	37	—	—	36
0·78		37	37	37	37	39	37·5	—	—	—	37	37	—	—	—	—
1·1†		38	38	38·5	38	40	38·5	37·5	39	42	39	38·5	40	37	36·5	39
6·0		48	49	51	48	53	52	48	49	53	53	53	53	51	51	53·5
11		55	55	59	55	59	58	54·5	55	59·5	61	60	59·5	58	58	60
16		59	60	61	59	62	61·5	57·5	59	62·6	65	65	63	62	62	63
31		66	65·5	68	65	68	68	63	65	68·6	71	70	69	68	68	68·5
60		74	74	75	74	74	73	70	69·5	75	76	76	74·5	75	79	75
120		80	81	81	81	81	79·5	79	79	81	81	80	81	82·5	83	81
180		84	84	84	84	86	83·5	83	83	86	84·5	83	85	88	87	86
240		88	89	88	88	88	86	86	88	89	87	88	87	—	90	88
300		89	90	90	90	90	88	88	90	92	90	90	90	94	92	90·5
0·28	1·4	—	34	—	35	40	—	35·5	—	40	36	40	39	—	—	—
0·55		—	40	—	42	46	—	44	—	46	46	47	46	—	—	—
0·78		—	41·5	—	—	—	—	46	—	49	—	—	—	—	—	—
1·1		—	43	—	45	50	—	47	—	52	52	53	53	—	—	—
6·0		—	56	—	59	65	—	59	—	68	69	67	70	—	—	—
11		—	62	—	65	73	—	66	—	75	75	74	75	—	—	—
16		—	66·5	—	69	78	—	70	—	78	80	78	79	—	—	—
31		—	71	—	76	85·5	—	77	—	84	86	84	86	—	—	—
60		—	81	—	82·5	90	—	83	—	88	89	87	88	—	—	—
120		—	90	—	91	96	—	91	—	96	95	95	95	—	—	—
180		—	96	—	96·5	99	—	96	—	99	97	99	99	—	—	—
240		—	—	—	—	—	—	97	—	—	—	—	—	—	—	—
300		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

* As equivalent of mg. of ascorbic acid/g. of heated product (estimated in static, photo-electric colorimeter 5 min. after addition of dye).
† Standard time interval, for comparison with the corresponding values in Table 3.

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the dried vegetable had been heated, and the greater the consequent degree of caramelization, the more closely did the reductant produced resemble, in the initial stages of the reaction at pH 1·4, an amino-reductone solution, and the less a simple gluco-reductone solution (Table 7). However, even with highly caramelized products, the rate of reaction at pH 3·5 within the first 1–2 sec. is not appreciably different from that for those which are less highly caramelized, or from that of a gluco-reductone solution itself; and, in any case, such excessively caramelized products are not likely to be encountered in practice, because the maximum time of heating dried vegetables, such as swede, carrot or cabbage, is not likely to exceed 6 hr. at 100°.

We are thus able to conclude that, as with gluco-reductone solutions, a reaction of pH 3·5 and a time interval of 1·1 sec., as in Fig. 3, serve for the quantitative differentiation of the interfering substance in caramelized vegetables from the ascorbic acid.

(7) *Validity of results. Recovery tests. Biological comparisons*

The evidence so far presented indicates:

(*a*) That gluco-reductone could be quantitatively distinguished from ascorbic acid by comparing their reaction-time curves with indophenol dye, most simply by measuring the extent of the reaction after 1·1 sec. at pH 3·5 (§ II (3*a*)).

(*b*) That other reducing agents, particularly those known to be present in natural foods, could generally be distinguished from both ascorbic acid and gluco-reductone by the reaction curves, also at pH 3·5 and 1·4 (§ II (4)).

(*c*) That in fresh, or boiled, steamed or canned fruits and vegetables, the time curve is characteristic of ascorbic acid, and there is no evidence of the presence of interfering substances (§ II (5)).

(*d*) That in caramelized products the interfering substance formed resembles gluco-reductone (or amino-reductone), and no evidence could be obtained of the presence of other types of reductants (§ II (6)).

This leads us to the conclusion that both the ascorbic acid and the interfering substances mentioned in the last paragraph should be capable of estimation by the procedure we have described. Further evidence for this conclusion will be presented in this section, as follows:

(*e*) Whereas caramelized dehydrated products known to be devoid of vitamin C on being submitted to test by the flow method were shown to contain the interfering substance but no ascorbic acid, other dehydrated vegetables of known antiscorbutic activity, but not caramelized, were shown by the result of the flow test to contain ascorbic acid but no interfering substance.

(*f*) In recovery tests, a quantitative estimate was found possible of ascorbic acid and gluco-reductone or both added to various dehydrated vegetable products or other foodstuffs already containing:

(1) ascorbic acid without the interfering substances,

(2) the interfering substances without the ascorbic acid, or

(3) both ascorbic acid and interfering substances together.

(*g*) The results of the determination of ascorbic acid, both in caramelized dehydrated foods in presence of the interfering substance, and also in certain other foods, agreed

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with the results obtained by the independent methods of (1) formaldehyde test and (2) biological assay.

Limits of error. Some account must also be given of the percentage accuracy of the method. It was found that, under the conditions we employed, the recovery of added ascorbic acid or interfering substance was of the order of 97–103% of the theoretical, expressed as a percentage of the total reducing titre.

Table 8. *Recovery of added ascorbic acid and gluco-reductone*

Extract	Reducing activity, percentage of total*		
	Gluco-reductone added	Ascorbic acid added	Ascorbic acid found
Oatmeal	0	100	100
	100	0	0
	50	50	49
	20	80	79
	80	20	19
	Gluco-reductone added	Natural ascorbic acid present	Ascorbic acid found
Kale	0	100	99
	48·5	51·5	55
	64	36	35
	84	16	15
	20	80	80
Brussels sprouts	0	100	99
	53	47	50
	41	59	59
Potatoes	0	100	98
	29	71	69

* Total concentration of reductants and dye as in Table 3.

Recovery tests: experimental details. Dehydrated vegetables containing no ascorbic acid were heated at 100° for varying periods of time. Extracts of the caramelized products were made in 2% metaphosphoric acid, and brought to pH 3·5 with sodium citrate buffer. Their total indophenol-reducing activity was estimated in each case in the photo-electric colorimeter at pH 3·5, and the solution then diluted to strengths equivalent to 0·04 mg. of ascorbic acid/ml. To these solutions known amounts of solutions of ascorbic acid (0·04 mg./ml.) were added, and the mixtures examined in the flow apparatus, the standard strength of indophenol dye being used. The amount of dye left after 1·1 sec. was determined in the usual way and the reference curve (Fig. 3) used to estimate the amount of ascorbic acid present.

For the other recovery tests (p. 31) the technique used was similar. The findings are given in Tables 8 and 9.

The results of individual analyses generally showed an agreement within about ±3% (Tables 8, 9). In practice, however, duplicate or even triplicate analyses on each solution were made, and an average taken. In most instances these average values, so determined with the use of the standard reference curve, agreed with the amount of ascorbic acid added to within ±2%.

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Agreement with formaldehyde method. Comparisons of the results by the flow or formaldehyde method were made, with a large number of fresh, processed and dried caramelized and uncaramelized dehydrated fruits and vegetables and with certain other foods. Some of these preparations had been processed or stored in such a way as to produce caramelization of varying degrees; others were subjected to heat treatment in the laboratory. The agreement between the results of the two methods (see

Table 9. *Estimation of ascorbic acid added to caramelized dehydrated vegetables*

Caramelized material	Serial no.	Ascorbic acid as percentage of reducing titre		
		Added	Found	Av. found
Dried carrot, heated 24 hr. at 100°	1	11·5	11, 9, 10	10
	2	28	26, 27·5, 29	27·5
	3	46	47·5, 48·5	48
	4	63	61·5, 62·5	62
	5	82	80, 80	80
Dried carrot, heated 3 hr. at 100°	1	10	13, 12·5, 13	13
	2	19·5	19, 18·5	19
	3	40	40, 42	41
	4	71·5	71, 72·5	72
Dried potato, heated 36 hr. at 100°	1	12	10, 10	10
	2	20	17·5	17·5
	3	37·5	32·5, 34·5	33·5
	4	63	65	65
	5	84	87, 84	85·5
Dried swede, heated 12 hr. at 100°	1	12	16, 11·5	14
	2	26·5	28·5, 26·5	27·5
	3	42	43, 41·5	42
	4	75·5	74·5, 73·5	74
	5	90	93, 91	92
Dried swede, heated 1½ hr. at 100°	1	7·5	7, 6·5, 7	7
	2	10·5	8·5, 8, 8	8
	3	15	12, 14	13
	4	19	14·5, 14·5, 15·5	15
	5	32·5	29·5, 32	31
	6	72·5	74, 70, 75	73
	7	80	78, 81·5, 82·5	81

Table 10) seems very satisfactory, and illustrates also that a large amount of interfering substance may sometimes be produced in the caramelized foods, but not in the other products examined.

Comparison with biological assays. Probably the best criterion of the accuracy of any new chemical method of analysis is its agreement with results obtained in biological assays. The fuller details of the method of technique used in the biological assays (mostly by the semi-curative growth test (see Harris & Olliver, 1942, p. 168)) are given by Cruickshank, Harris & Mapson (1947), but it may be stated here that those materials were chosen for test which were considered to afford as stringent a check as possible of the reliability of the new method. Among these (either assayed afresh, or tested on previous occasions) were: (1) raw, fresh fruit or vegetable foods containing only ascorbic acid and no interfering substance, (2) cooked or processed foods, likewise containing ascorbic acid only, (3) caramelized vegetable foods containing varying amounts both of the interfering substance and of the vitamin, (4) caramelized products

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containing only the interfering substance and no vitamin, and (5) finally, food preparations devoid of both.

The results (Table 11) seem to be in very satisfactory agreement.

Table 10. *Estimation of ascorbic acid in caramelized and non-caramelized dehydrated foods. Comparison of results by* HCHO *and the continuous-flow method*

Dehydrated material (with code no.)	Treatment	Titration result expressed as equivalents of ascorbic acid (mg./100 g.) Total	By HCHO method	By continuous-flow method
Cabbage (A. 584*b*)	Stored in N_2 at 37° for 5 months	265	180	184
Cabbage (A. 584*c*)	Stored in N_2 at 25° for 5 months	435	374	357
Cabbage (A. 584*d*)	Stored in N_2 at 25° for 5 months	450	342	334
Cabbage (A. 584*d*)	Stored in N_2 at 37° for 5 months	149	81	88
Cabbage (A. 640–8*d*)	Stored in N_2 at 28° for 6 months	360	335	324
Cabbage (A. 640–10*d*)	Stored in N_2 at 28° for 6 months	310	282	274
Cabbage (A. 667*a*)	Stored in N_2 at 37°	101	5	11
Cabbage (A. 667*b*)	Stored in N_2 at 37°	117	29	27
Cabbage (A. 667*c*)	Stored in N_2 at 37°	95	34	32
Cabbage (A. 667*d*)	Stored in N_2 at 37°	116	13	7
Cabbage (A. 730)	Stored in air at 37°	70	17	16
Cabbage (A. 730)	Stored in air at 37°	59	8	3
Cabbage (A. 662*c*)	Stored in N_2 at 25°	296	250	251
Cabbage 1	Stored at 15° for 2 months	243	209	211
Cabbage 1	Heated 1 hr. at 100°	193	37	38
Cabbage 2	Freshly dried	400	371	367
Cabbage 2	Heated 30 min. at 100°	346	220	224
Cabbage 2	Heated 60 min. at 100°	300	174	172
Cauliflower (A. 559*b*)	Stored in N_2 at 25°	89	55	41
Meat and vegetable soup	Stored at 28°	38	2	2
Tomato 1	Sun-dried	120	0	5
Tomato 2	Roller-dried flakes	145	6	4
Tomato 3	Spray-dried powder	79	34	38
Guava 1	—	920	740	760
Guava 2	—	1800	1420	1440
Carrot	Stored at 37° for several months	61	1	3
Swede, raw (A. 720*a*)	—	170	148	144
Swede (A. 663*c*)	Stored in N_2 at 37°	43	7	7
Swede (A. 663*d*)	Stored in N_2 at 37°	46	15	11
Swede 1	Freshly dried	136	122	122
Swede 1	Heated 30 min. at 100°	97	64	66
Swede 1	Heated 60 min. at 100°	68	4	5
Whole orange	—	235	194	192
Whole orange	Sulphited	357	310	325
Spinach	Dried 65° without scalding	202	111	122
Spinach	Dried 65° after scalding	186	147	149
Whole grapefruit 1	—	284	249	250
Whole grapefruit 1	Heated at 80° for 2½ hr.	169	128	120
Whole grapefruit 2	Sulphited	320	263	272
Cape gooseberries	—	38	31	28

III. WORKING DIRECTIONS FOR ROUTINE USE OF THE FLOW METHOD

The following procedure is suitable for use with caramelized dehydrated vegetables and similar products.

(*a*) A weighed sample is ground in presence of sand with a small quantity of 20% metaphosphoric acid, and the mixture diluted with water to reduce the concentration

of the acid to 2%. After filtration, with spinning if necessary, the reaction of the clear fluid is adjusted to pH 3·5 by sodium citrate and the total reducing titre estimated as described in § II (1).

(*b*) On the basis of this analysis the extract is diluted to be equivalent in total reducing strength to 0·04 mg. of ascorbic acid/ml., and passed through the flow apparatus with an equal rate of flow of indophenol dye of exactly equivalent concen-

Table 11. *Examples of agreement with biological assays*

Test material	Category (see p. 33)	Presence of: Ascorbic acid	Presence of: Interfering substance	Result of chemical analysis (mg./100 g. (av.)): Ascorbic acid	Result of chemical analysis (mg./100 g. (av.)): Interfering substance (expressed as equiv. of ascorbic acid)	Result of biological assay, ascorbic acid (mg./100 g.)	Biological value as % of chemical value	Re er
ew potatoes	(1) Raw	+	0	35	0	37	106	(
bbage water	(2) Cooked	+	0	25	—	25	100	(
k-currant purée	(2) Processed	+	0	120	—	138	115	(
bbage, dehydrated	(2) Processed (not caramelized)	+	0	460	0	463	101	(
bbage, dehydrated	(3) Excessively caramelized	+	+	115	160	114	99	(
rot, dehydrated	(4) Excessively caramelized and inactivated	0	+	0	220	Nil	—	(
ied egg yolk	(5) —	0	0	0	0	Nil	—	(

Gluco-reductone, reductic acid and hydroxytetronic acid were tested biologically and found inactive (*b*).

References: (*a*) Harris & Olliver (1942).
(*b*) Cruickshank *et al.* (1947).
(*c*) Unpublished work.

tration. The percentage of dye remaining unreduced after a reaction period of 1·1 sec. is determined as described in §§ II (2) and II (3). From this value and the reference graph (Fig. 3) relating percentage of unreduced dye to the proportion of ascorbic acid and gluco-reductone in the mixture, as previously determined for the apparatus used, the percentages of ascorbic acid and of interfering substances (expressed in terms of gluco-reductone) present in the mixture are obtained.

In the case of weak solutions where the reducing strength may have to be made up to 0·02 mg. instead of 0·04 mg./ml. the dye has also to be made up to the same equivalent concentration, and the reference curve (Fig. 3) to be determined afresh. Strengths of less than 0·02 mg. are not recommended, however, and the standard concentration of 0·04 mg. should always be used where possible. The curve must also be redetermined for each change in experimental conditions, and should be rechecked from time to time.

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IV. APPLICABILITY OF METHOD

The technique here described was devised in the first place for estimating ascorbic acid in caramelized vegetable foods. It has, however, some wider uses, which can be classified as (1) qualitative and (2) quantitative.

(1) *Qualitative use.* In the first place, the method enables a rapid qualitative test to be done on any food to ascertain whether the indophenol-reducing material has the properties of ascorbic acid, or whether significant amounts of interfering substances can be shown to be present. It deserves to be emphasized that, of the known organic reductants examined, hydroxytetronic acid alone cannot be differentiated from ascorbic acid or its isomers, but that there is no evidence that this substance is produced in any normal method of food processing. If, therefore, the test on any food extract shows that the velocity of the reaction with the dye at pH 1·4 and 3·5 is identical with that of ascorbic acid, it seems a fair assumption to make that ascorbic acid alone is present. In this case the ordinary simple method of titration with indophenol can be used straight away.

(2) *Quantitative application.* Secondly, the method may be used to determine ascorbic acid quantitatively in caramelized dried vegetable foods. In addition, with many dried fruits (e.g. guava, orange, grapefruit, tomato, see Table 10) the results have been in close agreement with those obtained independently by the formaldehyde method, and also with those of a number of biological tests. It will, of course, be necessary to test a more extended range of dried fruits before it can be claimed to be of universal applicability to them all. Further work (now in progress) will be needed also to check the applicability of the method to other types of processed foods, particularly to fermented products (Harris, 1933). In such cases the available evidence suggests the possibility that materials distinct from reductones may be present (cf. Wokes *et al.* 1943*b*). Finally, the only other limitation of the method that can be foreseen at present is that the procedure cannot be used with foods so poor in ascorbic acid, or other reducing substances, that their extracts contain less than about 0·02 mg. equivalent/ml.

SUMMARY

1. Dehydrated vegetables, caramelized by exposure to unduly high temperatures during either processing or storage, may contain reducing agents of the nature of reductones, which, since they react rapidly with indophenol dye, interfere in the chemical estimation of ascorbic acid if the ordinary method of direct titration is used. A procedure has been devised for the differential determination of both ascorbic acid and reductones present in such materials, and has been used satisfactorily for routine determinations. It depends on the use of an apparatus employing the principle of the continuous flow (Hartridge & Roughton, 1923), by which the complete time course of a reaction can be followed.

2. Control tests with mixtures of equal volumes of ascorbic acid (0·04 mg./ml.) and 2:6-dichlorophenolindophenol (in equivalent strength) at pH 3·5 and temperature 20° indicated that after 0·28, 0·55, 0·78 and 1·1 sec. respectively, 64, 80, 84 and 87–89%

of the reaction was complete, whereas with gluco-reductone (the mixture of substances formed by heating a solution of glucose with alkali) in place of ascorbic acid the corresponding values were 26, 34, 37·5 and 38·5%. With amino-reductone (the mixture of substances formed by heating a solution of glucose with glycine and alkali), they were 30, 38, 41 and 44%. The values when plotted lay on well-separated smooth curves, and thus ascorbic acid could readily be distinguished from these interfering substances. Moreover, mixtures of gluco-reductone and ascorbic acid gave curves in intermediate positions, in proportion to the relative amounts of each present; hence both substances could be accurately estimated when present simultaneously. In recovery tests, ascorbic acid and gluco-reductones could be determined under the above conditions with an error of less than 2% of the total reducing value.

3. Other substances were examined in the same way and their velocity constants determined. Cysteine and sulphite could be readily distinguished from ascorbic acid by their much slower rates of reaction, while dihydroxymaleic acid, thiosulphate, and the ferrous ion were distinctive in having relatively slow rates at pH 3·5 but much faster at pH 1·4. Reductic acid had approximately half the velocity constant of ascorbic acid, while hydroxytetronic acid, alone of the substances examined, had approximately the same constant; there is, however, no evidence that either of these substances, or dihydroxymaleic acid, occurs in natural products.

4. The reductants (interfering substances) formed when dehydrated vegetables are caramelized resembled gluco-reductones closely in their rate of reaction with 2:6-dichlorophenolindophenol at pH 3·5, and no indication was obtained of the presence of the other type of reductants mentioned in paragraph 3 above. Both the ascorbic acid and the interfering substance could therefore be estimated by the use of the continuous-flow technique, the experimental evidence for this conclusion being as follows:

(*a*) Various dehydrated vegetables known to be devoid of antiscorbutic activity were heated for differing periods at 100° so as to cause caramelization to varying extents; application of the flow test then accurately showed the presence of interfering substance and no ascorbic acid.

(*b*) On the other hand, dehydrated vegetables which had known antiscorbutic activities, but had not been subjected to caramelization, were shown by the test to contain ascorbic acid and no interfering substance.

(*c*) Furthermore, in recovery tests, it was shown that a quantitative estimate was possible of the ascorbic acid or gluco-reductones, or both, added to various dehydrated vegetable products or other food preparations, already containing (i) ascorbic acid without the interfering substances produced on caramelization, or (ii) the interfering substances without the ascorbic acid, or (iii) both present simultaneously.

(*d*) Results of determination of ascorbic acid in caramelized dehydrated vegetables and other products by the continuous-flow method agreed well with those obtained by the formaldehyde method (Mapson, 1943*b*) and by biological assays.

5. The limit of error was low, the ascorbic acid or interfering substance, determined in recovery tests, ranging from about 97 to 103% of the theoretical value, expressed as a percentage of the total reducing content.

No significant amount of interfering substances was found in numerous fresh vegetables, in certain common fruit juices, or in vegetables cooked by the usual methods of boiling or steaming, or processed or dehydrated in absence of caramelization. Hence, for the usual run of fruit and vegetable materials it is confirmed that the ordinary indophenol titration is accurate, since it gives the same result as that obtained by the continuous-flow method. Only with plant products heated in the presence of alkali, or heated to exceptionally high temperatures as, for example, in the preparation of chip potatoes, are interfering substances encountered. In doubtful cases, or when new types of products are being examined, the flow method provides a rapid and convenient means of ascertaining whether or not certain interfering substances are present.

We are greatly indebted to Messrs B. J. Constable and A. Ward for their expert technical assistance, and to Dr A. J. P. Martin for a helpful discussion of the chemical dynamics and viscosity relations. One of us (L.W.M.) took part in this work as a member of the Food Investigation Staff of the Department of Scientific and Industrial Research working at the Dunn Nutritional Laboratory by arrangement with the Medical Research Council.

REFERENCES

Allen, R. J. L. & Mapson, L. W. (1944). *J. Soc. chem. Ind., Lond.*, **63**, 78.
Bessey, O. A. (1938). *J. biol. Chem.* **126**, 771.
Birch, T. W., Harris, L. J. & Ray, S. N. (1933). *Biochem. J.* **27**, 590.
Brightwell, S. & Mapson, L. W. (1946). Unpublished data.
Cruickshank, E. M., Harris, L. J. & Mapson, L. W. (1947). In the Press.
Eekelen, M. van & Emmerie, A. (1935). *Biochem. J.* **30**, 25.
Enders, C. (1943). *Biochem. Z.* **313**, 352.
Euler, H. von & Martius, C. (1933). *Liebigs Ann.* **505**, 73.
Franke, W. & Brathuhn, G. (1931). *Liebigs Ann.* **487**, 1.
Harris, L. J. (1933). *Nature, Lond.*, **132**, 27.
Harris, L. J. (1943). *Lancet*, **244**, 515.
Harris, L. J. & Mapson, L. W. (1944). *Biochem. J.* **38**, xxiv, xxv.
Harris, L. J., Mapson, L. W. & Wang, Y. L. (1942). *Biochem. J.* **36**, 183.
Harris, L. J. & Olliver, M. (1942). *Biochem. J.* **36**, 155.
Harris, L. J. & Ray, S. N. (1933*a*) *Biochem. J.* **27**, 303.
Harris, L. J. & Ray, S. N. (1933*b*). *Biochem. J.* **27**, 580.
Harris, L. J. & Ray, S. N. (1935). *Lancet*, **228**, 71.
Hartridge, H. & Roughton, F. J. W. (1923). *Proc. roy. Soc.* A, **104**, 395.
Hopkins, F. G. & Slater, B. R. (1935). *Biochem. J.* **29**, 2503.
Kertesz, Z. I. (1934). *J. biol. Chem.* **104**, 483.
Lavine, T. F. J. (1935). *J. biol. Chem.* **109**, 141.
Lugg, J. W. H. (1942). *Aust. J. exp. Biol.* **20**, 273.
Mapson, L. W. (1942). *Biochem. J.* **36**, 196.
Mapson, L. W. (1943*a*). *Nature, Lond.*, **152**, 13.
Mapson, L. W. (1943*b*). *J. Soc. chem. Ind., Lond.*, **62**, 223.
Mapson, L. W. (1946). Unpublished data.
Millikan, G. A. (1935). *Biochem. J.* **29**, 2817.
Mindlin, R. L. & Butler, A. M. (1937). *J. biol. Chem.* **122**, 673.
Nutrition Society (1945). *Nutrition Society, Standing Advisory Committee for Co-ordination of Methods of Survey: Report of Laboratory Panel.* Issued by Bureau of Nutrition Surveys, London, 1945.
Reichstein, T. & Oppenauer, R. (1933). *Helv. chim. Acta*, **16**, 988.
Snow, G. A. & Zilva, S. S. (1944). *Biochem. J.* **38**, 458.
Tomkins, R. G., Mapson, L. W., Allen, R. J. L., Wager, H. G. & Barker, J. (1944). *J. Soc. chem. Ind., Lond.*, **63**, 225.
Vokes, F., Organ, J. G., Duncan, J. & Jacoby, F. C. (1943*a*). *Nature, Lond.*, **152**, 14.
Vokes, F., Organ, J. G., Duncan, J. & Jacoby, F. C. (1943*b*). *Biochem. J.* **37**, 695.

Lancet 264, 1021-24 (1953)

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EFFECT OF A.C.T.H. AND CORTISONE ON VITAMIN-C METABOLISM AND ON THE WEIGHT AND COMPOSITION OF THE LIVER

IN GUINEAPIGS AND OTHER SPECIES

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THE investigations recorded here began in 1950 in an attempt to throw new light on the puzzling problem of the apparent interrelation between adrenal function and vitamin-C metabolism.

Presumptive evidence for the existence of this interrelation is afforded by such facts as the following: (1) the silver-staining properties of the adrenal cortex (Szent-Györgyi 1928) attributable to vitamin C, which is present in remarkably high concentrations both in the cortex and in the medulla (Harris and Ray 1932, 1933); and (2) the action of adrenocorticotropic hormone (A.C.T.H.) in bringing about a transient fall in the concentration of the vitamin within the adrenal (Sayers et al. 1944).

Our original object in these experiments was to ascertain whether the repeated administration of large doses of A.C.T.H. to guineapigs would lead to so great a loss of vitamin C from their adrenals as to increase the effective requirement for the vitamin, and thereby to hasten the onset of scurvy in guineapigs kept on diets deficient in it. Although the answer to this question was substantially negative, some unexpected observations were made—particularly the fact that, in the guineapigs given these repeated injections of A.C.T.H. there resulted a remarkable and continuous increase in the weight of the liver. As we have published hitherto only brief summaries of our conclusions (Bland et al. 1952, Hughes et al. 1952, Harris 1953), it seems desirable to give some typical numerical examples of our actual data; and we are also taking the opportunity, at the same time, to present some of our later results.

EXPERIMENTAL

Unless otherwise stated, the findings given below relate to experiments in which young guineapigs weighing 300–350 g. had a course of intramuscular injections of A.C.T.H. (2 mg. of Armour standard L.A.I.A., four times daily). The standard "basal diet" consisted of Sussex ground oats 650 parts, bran 144, dried egg-yolk 80, dried brewers' yeast 108, and salt mixture 18, supplemented with 25 mg. of ascorbic acid daily (omitted for experiments with scurvy), 4 drops of halibut-liver oil weekly, and water ad lib. In other tests a "natural" diet was used instead, consisting of Sussex ground oats 1000 parts, and bran 220, supplemented with 15 g. of cabbage per animal per day, water and hay ad lib., and roots when available. None of our guineapigs was subjected to hypophysectomy. Rats, when used, were kept on the standard "synthetic" diet used in this laboratory, and rabbits on the above-mentioned "natural" diet, with cabbage ad lib.

RESULTS

(1) *Effect of A.C.T.H. on Vitamin-C Metabolism*

Contrary to our expectations, it was found that the vitamin-C economy of the body as a whole was not appreciably changed. Although the concentrations of ascorbic acid in the liver and adrenals of the guineapigs were diminished, the total quantities of ascorbic acid in these two organs (both of which were enlarged as a consequence of the treatment) were not substantially affected (fig. 1). The administration of A.C.T.H. did not hasten the onset of scurvy in guineapigs on the deficient diet; nor did it increase the rate of depletion of ascorbic acid from the liver or the adrenals during the development of deficiency.

(2) *Effect of A.C.T.H. on A.A.: D.H.A.A. Equilibrium in the Adrenals*

After a single injection of A.C.T.H. no substantial change was found in the relative proportion of dehydroascorbic acid to ascorbic acid present in the adrenals of guineapigs, although the concentration of ascorbic acid was temporarily depressed.

(3) *Liver Weights*

In the young guineapigs given the standard course of injections of A.C.T.H. (see above) a remarkable increase was seen in the weight of the liver, which was already apparent after only two days and continued thereafter day by day—e.g., up to ten days—when the increase had reached a value of about 50%, compared with the controls (fig. 2). This increase in weight has now been observed repeatedly in nine series of tests extending for over twenty months and involving over 350 animals, and is statistically highly significant ($P = < 0{\cdot}01$).

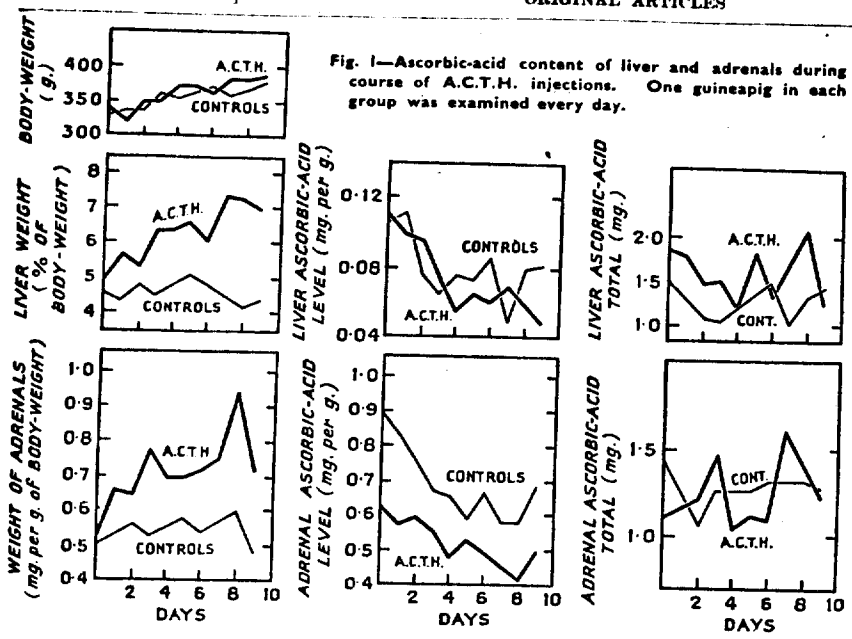

Fig. 1—Ascorbic-acid content of liver and adrenals during course of A.C.T.H. injections. One guineapig in each group was examined every day.

In adult guineapigs A.C.T.H. had a similar effect to that in young growing ones in causing a great increase in the liver weight (av. = 39%, after 10 days, statistically highly significant).

(4) *Liver Weights : Dose-response Curves*

Dose-response curves have been constructed, relating the increases in liver weight observed to the size of the dose of A.C.T.H. administered (fig. 3). It seems likely that our findings may have possible uses in biological assay, as in characterising the action of different hormones —e.g., the various A.C.T.H. fractions (see also below under *Cortisone*).

(5) *Composition and Structure of Enlarged Liver*

Analyses were made of the fat, protein ("Kjeldahl-nitrogen"), carbohydrate, and water contents of the enlarged liver, and the composition of the "increment" was calculated by difference (fig. 4). It will be seen that during the first day or so the increased weight of the liver was due essentially to extra glycogen and water but thereafter there was also an appreciable addition of protein. Histological examination by Dr. C. P. Cherry, at the Strangeways Research Laboratory, showed that there was an increase in the size of the liver cells, which was attributable to an increased volume of the cytoplasm and was equivalent in magnitude to the increase observed in the weight of the whole liver.

Under the conditions of our experiments the A.C.T.H. caused a diminution in the percentage of fat in the liver and not a rise (as might perhaps have been expected from previous reports relating to other species).

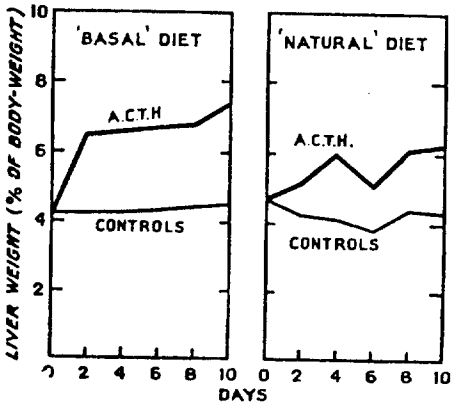

Fig. 2—Weight of liver (average of group) during a course of A.C.T.H. injections.

There was little effect on the concentration of sulphydryl compounds (including glutathione) in the liver.

(6) *Long-term Injections*

When the injections of A.C.T.H. were continued for longer periods, up to forty days, the general effect on the chemical composition of the liver was similar—i.e., a further increase in the amounts of water, protein, and glycogen (absolute weights) in the liver, compared with the controls (or, in terms of percentages, an increase in glycogen, but some decrease in the protein and fat, and little change in the water).

(7) *Result of Discontinuing the Treatment*

When, after ten days, the injections of A.C.T.H. were discontinued, there followed a sharp fall in the weight of the liver, with the result that two days thereafter it weighed no more than the control (and, from three to thirty days subsequently, was about 10% smaller). The glycogen in the liver decreased to within the normal limits, of the controls, within about a couple of days, and the protein some days later.

(8) *Effect of Cortisone on Liver Weight and Composition*

In young guineapigs cortisone had an effect similar to that of A.C.T.H. in increasing the weight of the liver, but to an even greater degree. For example, after ten days' treatment with intramuscular cortisone acetate 7·5 mg. per day in two doses, there was an average increase in weight of 50%; and with 15 mg. per day (see fig. 5) an average increase of 72% after four days, or of 123% after ten days ($P = \leqslant 0{\cdot}01$).

The effect of cortisone on the proximate chemical composition was similar to that observed with A.C.T.H. (fig. 4).

Incidentally, the injections of cortisone and A.C.T.H. had the effects, as expected, of diminishing and of enlarging, respectively, the weights of the adrenals (cf. figs. 1 and 5).

(9) *Effect of Cortisone on Weights of Other Organs*

In the young guineapigs given the repeated injections of cortisone there was no significant change in the average weights of the pancreas, lungs, heart, thymus, or spleen. There was, however, an increase in the average weight of the kidneys, amounting to 8% after 7·5 mg. of cortisone per day for 10 days, or of 20% after 15 mg. per day, these figures being statistically "significant" ($P = 0{\cdot}05$) and "highly significant" ($P = \leqslant 0{\cdot}01$) respectively. A possible effect on the weights of the testes seemed less consistent, and needs further study (33% increase in weight after 7·5 mg. of cortisone acetate per day, with $P = 0{\cdot}05$—i.e., "significant"—but no significant effect after 15 mg. of cortisone acetate per day).

(10) *Species Differences*

In young rats, contrasted with guineapigs, A.C.T.H. had no important effect on liver weight, and cortisone caused some loss in the weight (absolute) of the liver, in place of a rise. (At the higher levels of dosage of cortisone, with the consequent decline in body-weight, the liver of the rats lost somewhat less weight than did the body as a whole, and there was thus some increase in the relative weight of the liver, although not in its absolute weight.)

Again, in chicks and mice given A.C.T.H. no important changes were observed in liver weight.

With rabbits, on the other hand, the effect of A.C.T.H. on the liver was similar to that in guineapigs, the increase in weight being very pronounced (48% increase, after ten days, on 24 mg. of A.C.T.H. daily, in 3 doses per day), and statistically "highly significant" (P = 0·01).

COMMENTS

A.C.T.H. and Vitamin-C Metabolism

Our findings on A.C.T.H. in relation to vitamin-C requirements have been essentially negative. Previous published reports have been somewhat conflicting, some observers—e.g., Hyman et al. (1950), Eisenstein and Shank (1951), and Long (1947)—reporting that A.C.T.H. may apparently "spare" vitamin C, and others—e.g., Clayton and Prunty (1951)—noting an absence of any such effect.

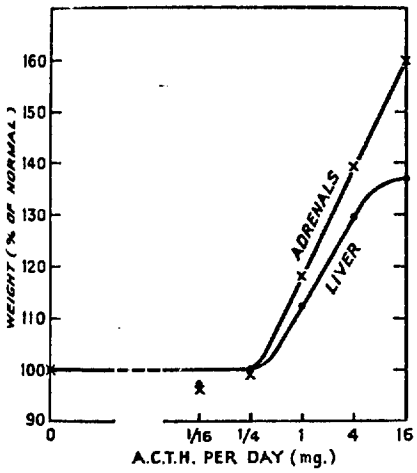

Fig. 3—Dose-response curve, showing increase in weights of liver and adrenals (after 10 days) depending on amount of A.C.T.H. administered daily (log. scale).

There have been indications also (Stewart et al. 1953, Cornforth and Long 1952, Loxton and Le Vay 1953) that both A.C.T.H. and cortisone may affect the equilibrium between ascorbic acid and dehydroascorbic acid in the blood. Under the conditions of our own tests we found no change in A.A. : D.H.A.A. ratio, so far as the adrenals were concerned, following on the injections of A.C.T.H.

Liver Enlargement

It is remarkable that the massive increase in liver weight in guineapigs after A.C.T.H. does not seem to have been noted by previous investigators. The explanation perhaps is that the guineapig has not been the animal most usually employed in research on A.C.T.H. Also, other unusual features in our experiments were the prolonged course of injections and the absence of hypophysectomy.

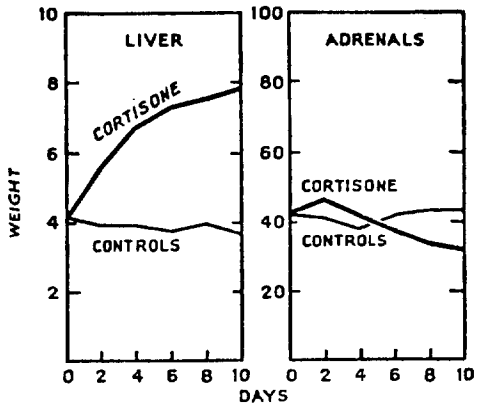

Fig. 5—Weights of liver and adrenals (average of group) during a course of cortisone injections. (Liver weights in g. per 100g. body-weight; adrenal weights in mg. per 100g. body-weight.)

It seems clear from our results that this action of A.C.T.H. on the liver is to be attributed largely if not entirely to its influence in stimulating the production of cortisone. In terms of proximate components, the enlargement is associated with an increase in the absolute amounts of glycogen, water, and protein—i.e., Kjeldahl-nitrogen. The effect of cortisone in stimulating glycogen production is, of course, already well known. The more novel feature in our results is what we may term the "intracellular œdema." The fact that there is some increase in the protein in the liver, but that it is not in full proportion to the total increase in weight of the liver, can perhaps be most easily interpreted as representing the result of an increased passage of lymph into the liver. We cannot, however, rule out the possibility that some gradual building up of extra protein may take place as the functional response of the liver to the increased activity put upon it, under the stimulus of the cortisone. More detailed analyses of the various nitrogenous fractions, in place of the simple Kjeldahl-nitrogen determinations here recorded, are obviously needed to throw further light on such questions.

Species Differences

The cause of the species difference in the liver enlargement is uncertain. Guineapigs, together with primates, are unique, it is true, in being the only species known to need vitamin C in their diet, other species being able to synthesise it. However, the general negative trend

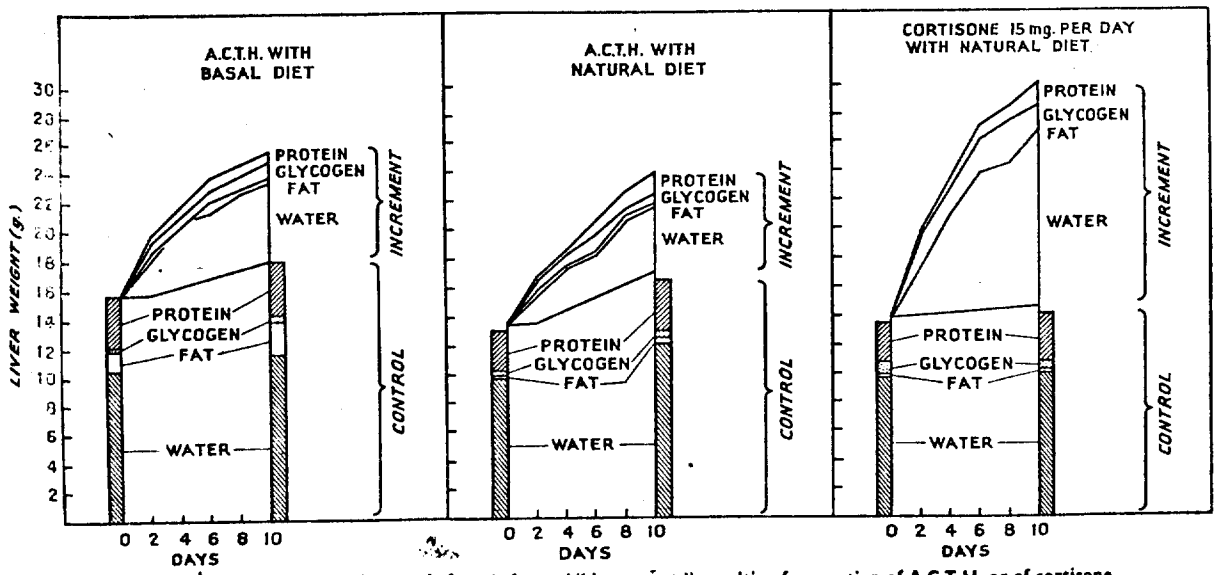

Fig. 4—Composition of normal liver, and of newly formed "increment," resulting from action of A.C.T.H. or of cortisone.

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of our results on vitamin C, together with the additional fact that rabbits (not known to need vitamin C) behave like guineapigs argue against any explanation based on the difference of species in their vitamin-C metabolism. Nor can a species difference in regard to the release of cortisone after A.C.T.H. be held responsible, for in rats both A.C.T.H. and cortisone were equally ineffective in producing any liver enlargement. The possibility remains that guineapigs and rabbits are more liable than are other species to develop œdematous changes after A.C.T.H. (the addition of water being, as we have seen, an essential feature of the liver enlargement in our experiments); but, if that is so, it does not seem to have been hitherto reported.

In any event this spectacular effect on the liver seems of sufficient interest to warrant further study, in view of both the many side-effects produced by these hormones and the importance of the liver as the seat of so many diverse chemical activities.

REFERENCES

Bland, M. N., Constable, B. J., Harris, L. J., Hughes, R. E. (1952) *Biochem. J.* **51**, xxxv P; Résumés des Communications, IIe Congrès International de Biochimie. Paris; p. 325.

Clayton, B. E., Prunty, F. T. G. (1951) *Brit. med. J.* ii, 927.

Cornforth, J. W., Long, D. A. (1952) *Lancet*, i, 950.

Eisenstein, A. B., Shank, R. E. (1951) *Proc. Soc. exp. Biol., N.Y.* **78**, 619.

Harris, L. J. (1953) *Proc. Nutr. Soc.* **12**, 142.

— Ray, S. N. (1932) *Biochem. J.* **26**, 2067.

— — (1933) *Ibid*, **27**, 303, 2006.

Hughes, R. E., Harris, L. J., Constable, B. J., Bland, M. N. (1952) *Ibid*, **51**, xxxv P.

Hyman, G. A., Ragan, C., Turner, J. C. (1950) *Proc. Soc. exp. Biol., N.Y.* **75**, 470.

Long, C. N. H. (1947) *Fed. Proc.* **6**, 461.

Loxton, G. E., Le Vay, D. (1953) *Nature, Lond.* **171**, 524.

Sayers, G., Sayers, M. A., Lewis, H. K., Long, C. N. H. (1944) *Proc. Soc. exp. Biol., N.Y.* **55**, 238.

Stewart, C. P., Horn, D. B., Robson, J. S. (1953) *Biochem. J.* **53**, 254.

Szent-Györgyi, A. (1928) *Ibid*, **22**, 1387.

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Microchem. J. 16(4), 645-651

MICROCHEMICAL JOURNAL 16, 645–651 (1971)

Determination of Ascorbic Acid and Differential Detection of the Vitamin and Reducing Sugars

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Received July 1, 1971

Determination of vitamin C by chloramine-T(*11*) suffers from lack of selectivity and sensitivity. Roe and Keuther's (*16*) method is laborious and time consuming as it requires a prior careful oxidation for the total determination of ascorbic acid and dehydroascorbic acid. The indophenol–xylene extraction (*15*) method is sensitive of pH. The method proposed by Schmall and co-workers (*17, 18*) using 4-methoxy-2-nitroaniline is not sensitive for lower amounts of vitamin C. The determination cannot be made in the presence of *p*-aminobenzoic acid or cysteine. The *N*-bromosuccinimide titration method (*2*) does not work when sodium sulfite, sulfide, or thiourea are present in the assay solution. The procedure has been modified using 1,2-naphthoquinone-4-sulfonate (3) but the interference by the above-mentioned compounds cannot be eliminated. Similarly the colorimetric procedure with 3,4-dinitrobenzoic acid (*20*) requires solvent extraction and the investigators do not mention the effect of interfering substances generally present in the determination of vitamin C. The potentiometric determination with iodine(*1*) cannot be employed when iodine-oxidizable substances are present in the solution. Ganguly (*8*) developed a method for the determination of vitamin C by titration with iodine and chloramine-T but does not mention the behavior of extraneous substances. The potassium ferricyanide method (*19*) is not only sensitive to pH but ferrous ions also interfere seriously in the determination of ascorbic acid. All methods known previously have a lack of specificity (*7*).

During systematic investigation of color reactions for determination of gold, it was observed that chloroauric acid solution can be used as a reagent for visual detection of ascorbic acid (*4*) and reducing sugars individually, as well as in presence of each other. Ascorbic acid produces a blue-violet coloration with chloroauric acid in aqueous solution while alkaline solution of reducing sugars gives pink-violet coloration.

The production of blue-violet coloration has further been developed into a colorimetric procedure for the determination of vitamin C. The present method is sensitive, simple, and rapid, and can be adopted for routine analysis. The visual limit of detection is 2 μg/ml; and the reaction obeys Beer's law between 20 to 200 μg/ml of ascorbic acid. The method has not been reported previously in literature (*5, 6, 10, 12, 21, 22*).

EXPERIMENTAL METHODS

EQUIPMENT AND REAGENTS

All absorbance measurements were made with SP 1300 Unicam colorimeter.

An aqueous solution of chloroauric acid containing 200 μg/ml of gold.

Standard ascorbic acid solution is prepared by dissolving 125 mg of the vitamin to make 500 ml with distilled water.

Sodium hydroxide, 0.1 *N* aqueous solution.

PROCEDURE

Detection of ascorbic acid. Take 0.5 ml of neutral test solution and add to it 0.5 ml of aqueous chloroauric acid solution. Spontaneous appearance of blue to blue-violet color indicates the presence of ascorbic acid. Sometimes, a blue colloidal suspension appears which is due to large amount of ascorbic acid in the test solution.

Detection of reducing sugars. Take 1 ml of test solution and make it alkaline by the addition of 0.2 ml of 2% sodium hydroxide. Add 0.5 ml of chloroauric acid solution and let it stand for 3 minutes. Appearance of pink to pink-violet coloration indicates the presence of reducing sugar.

Detection in presence of each other. To 0.5 ml of test solution, add 0.5 ml of chloroauric acid solution; spontaneous appearance of blue or violet color indicates presence of ascorbic acid. Add more of chloroauric acid solution until color intensity becomes constant, which indicates that all the ascorbic acid present in the solution has reacted completely. Add 0.2 ml of 2% sodium hydroxide to make the solution alkaline, followed by a few drops of chloroauric acid solution, and let it
tion of the solutions indicates the presence of reducing sugar in the test
tion of the solutions indicates the presence of reducing sugar in the test
sample. The visual limits of detection of reducing sugars and ascorbic acid are given in Table 1.

Determination of ascorbic acid. To 1.0 ml of the neutral test solution containing 20–200 μg of vitamin C (devoid of sugars), add 1.0 ml of the color producing reagent, chloroauric acid solution, diluted to 5 ml

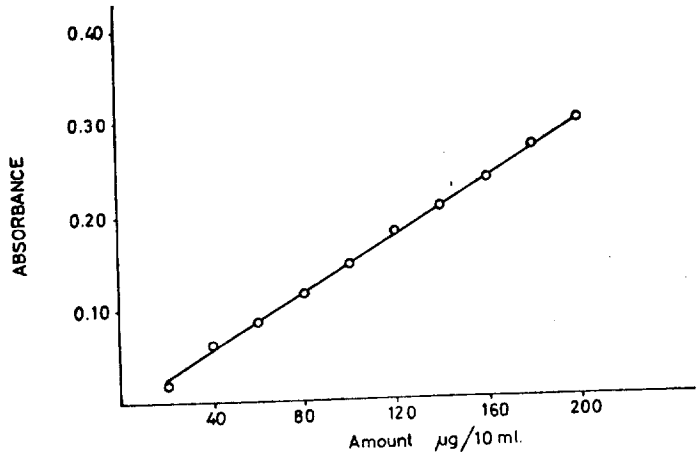

FIG. 1. Typical calibration curve for determining ascorbic acid.

with distilled water, and add 0.6 ml of 0.1 *N* sodium hydroxide. Shake well and make final volume to exactly 10 ml. Keep for 20 minutes at room temperature (30°). Measure the absorbance of violet-blue color with SP 1300 colorimeter using 1 cm cells and filter No.3. A calibration curve (Fig. 1) is prepared by taking different amounts of ascorbic acid and following the above procedure. The visual limit of detection is 2 μg/ml; and the reaction obeys Beer's law.

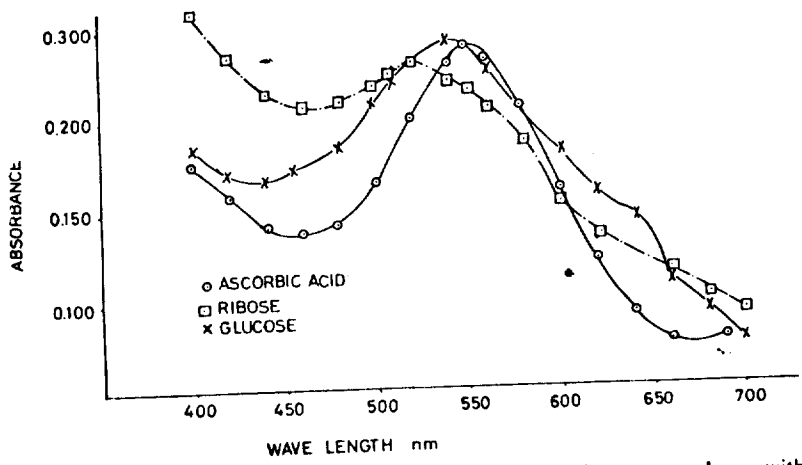

FIG. 2. Characteristics of ascorbic acid, ribose, and glucose complexes with chloroauric acid.

RESULTS AND DISCUSSION

Differential detection of ascorbic acid and reducing sugars is based on visual color affect. The sensitivities of reducing sugars and ascorbic acid are given in Table 1. The colors in Table 1 are those obtained with pure samples of sugars and ascorbic acid. Deviation from the standard colors in presence of other substances when exceeding the tolerable limits has been observed (cf. Table 2). Any change in color can lead to erroneous results. Thus, development of color in presence of other compounds, which are expected to be present along with ascorbic acid or reducing sugars has been studied in detail. These include vitamins, amino acids, and inorganic salts. The results are given in Table 2. In differential detection, it has been established that vitamin C can be detected in neutral solutions even in presence of 1500 μg of sugars. Detection of ascorbic acid and sugars in acidic media is not possible. Certain compounds such as vitamins B_1, B_2, and B_6, tryptophan and β-alanine interfere in the detection of sugars when their amount exceeds 5 μg/ml.

The effects of time, temperature, reagent concentration, and pH on the color development of ascorbic acid with chloroauric acid have been studied. Minimum time required for complete color development is 15 minutes and the best temperature range is 20–40°. The rate of reaction becomes slow when carried below 20° and colloidal suspension appears on increasing the temperature (above 40°). The minimum amount of

TABLE 1

SENSITIVITIES OF SUGARS AND VITAMIN C

Compound	Color [a]	Sensitivity (mg/ml)	Time for color development (minutes) [b]
D(+)-Glucose	Blue violet	0.30	3
D(−)-Fructose	Blue violet	1.00	3
D(+)-Xylose	Blue violet	0.10	1
D(+)-Galactose	Blue violet	0.30	3
L(+)-Rhamnose	Blue violet	1.00	3
D(−)-Ribose	Pink violet	0.20	2
L(−)-Sorbose	Blue violet	0.50	3
L-Arabinose	Blue violet	0.30	2
Sucrose	Blue violet	20.00	4
Ascorbic acid	Pink violet	0.01	Spontaneous

[a] Color may change with amount and presence of other compounds. These colors have been recorded with pure form of compounds and at the sensitivity limits.

[b] Time for development of color may be shorter with the higher amounts of these compounds.

color producing reagent which reacts completely with 200 μg of ascorbic acid is 140 μg but when the amount of the reagent exceeds 280 μg, a decrease in color intensity is observed. The color reaction is carried out between pH 8 to 10.

In the previous method (*9*) for the determination of ascorbic acid with 2,3,5-triphenyltetrazolium chloride, a very fine colloidal suspension due to the formation of red formazan (*13, 14*) was observed; but, in the present case, there is no such colloidal suspension formation and the solution remains clear. The reaction obeys Beer's law between 10 to 200 μg/ml of ascorbic acid. The characteristics of chloroauric acid complexes with ascorbic acid, ribose, and glucose are shown in Fig. 2.

TABLE 2

MAXIMUM AMOUNTS OF SOME OF VITAMINS, AMINO ACIDS, AND INORGANIC SALTS WHICH DO NOT INTERFERE IN THE DETERMINATION OF ASCORBIC ACID

Compound	Maximum amount not interfering with vitamin C, (μg/ml)	Color when higher amounts are present
Vitamins		
Thiamine HCl	5	Blue
Riboflavin [a]	23	Blue
Pyridoxine HCl	50	Blue
Biotin	20	Blue
Folic acid [a]	10	Blue
Choline chloride	200	Pink
Cyanocobalamin [a]	8	Pink
Amino acids		
L-Aspartic acid	50	Violet
DL-Ornithine HCl	5	Blue
DL-Serine	25	Violet
DL-Threonine	80	Violet
L-Asparagine	70	Violet
DL-Lysine HCl	123	Violet blue
L-Hydroxyproline	70	Violet blue
DL-Valine	40	Violet pink
Tyrosine	60	Violet blue
Glutamic acid	10	Violet blue
Salts		
Cupric chloride	31	—
Ferrous sulfate	50	—
Manganese chloride	10	Masking effect

[a] Original color of the compound interferes when the amount exceeds the tolerable limit.

The amount of vitamin C from an unknown solution can be directly found with the help of following expression:

$$A = V \cdot d \cdot 69,$$

where, A = amount of vitamin C (μg) in volume V; V = volume of the solution having absorbance d, after complex formation with gold: d = absorbance of the solution.

SUMMARY

A new spot test for the detection of reducing sugars and vitamin C, when present individually or together, has been developed. Chloroauric acid solution has also been used for the quantitative determination of vitamin C. The reaction obey Beer's law in the range of 20–200 μg and the visual limit of identification in 2 μg/ml. The amounts of different compounds which do not interfere in the determination are reported.

REFERENCES

1. Adams, J., Acker, M., and Frediani, H. A., Iodometric titrimetric determination of ascorbic acid. *J. Amer. Pharm. Ass., Sci. Ed.* **36**, 170–171 (1947).
2. Barakat, M. Z., El-Wahab, M. F. A., and El-Sadr, M. M., Action of *N*-bromosuccinimide on ascorbic acid, *Anal. Chem.* **27**, 536–540 (1955).
3. Barakat, M. Z., Shehab, S. K., and El-Sadr, M. M., New titrimetric method for the determination of ascorbic acid. *Analyst (London)* **80**, 828–833 (1955).
4. Emmerie, A., Reducing properties of Ascorbic acid; a color reaction with selenous acid and with gold chloride. *Acta Brevia Neer. Physiol. Pharmacol. Microbiol.* **4**, 141–145 (1934).
5. Feigl, F., "Spot Tests." Vol. 1, Elsevier, New York, 1964.
6. Feigl, F., "Spot Tests," Vol. 2, Elsevier, New York, 1966.
7. Freed, M., "Methods of Vitamin Assay," 3rd ed., p. 290. Wiley, (Interscience), New York, 1966.
8. Ganguly, S. K., Iodometric estimation of vitamin C in pharmaceutical preparations. *Indian J. Pharm.* **10**, 3 (1948).
9. Hashmi, M. H., Adil, A. S., Viegas, A., and Ahamd, I., Microdetermination of ascorbic acid and tryptophan by colorimetry. *Mikrochim. Acta* **1970**. 457–462.
10. Jacobson, C. A., "Encyclopaedia of Chemical Reactions," Vol. 3, Reinhold New York, 1949.
11. Leonhardt, H., and Moeser, W., Chlorometric determination of *l*-ascorbic acid. *Z. Anal. Chem.* **122**, 3–6 (1941).
12. Meites, L., "Hand-book of Analytical Chemistry," pp. 6–53. McGraw-Hill, London 1963.
13. Mork, H. B., Backer, L. M., Pinkel, D., and Papa, L. J., 2,3,5-Tripenyl-2H-tetrazolium chloride as a reagent for the determination of sugar mixtures by a differential reaction-rate technique. *Talanta* **12**, 27–34 (1965).
14. Noller, C. R., "Chemistry of Organic Compounds," 2nd ed., p. 638. Saunders, Philadelphia, 1957.

15. Robinson, W. B., and Stotz, E., The indophenol-xylene extraction method for ascorbic acid and modification for interfering substances. *J. Biol. Chem.* **160**, 217–225 (1945).
16. Roe, J. H., and Kuether, C. A., 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 (1943).
17. Schmall, M., Pifer, C. W., and Wollish, E. G., Determination of ascorbic acid. *Anal. Chem.* **25**, 1486–1490 (1953).
18. Schmall, M., Pifer, C. W., and Wollish, E. G., Colorimetric determination of ascorbic acid. *Anal. Chem.* **26**, 1521–1522 (1954).
19. Shimomura, S., Determination of vitamin C by potassium ferricyanide. *J. Pharm. Soc. Jap.* **81**, 1415–1418 (1961).
20. Takemoto, T., Daigo, K., and Takai, T., Colorimetric determination of ascorbic acid with 3,4-dinitrobenzoic acid. *J. Pharm. Soc. Jap.* **75**, 1025 (1955).
21. Welcher, F. J., "Organic Analytical Reagents." Vol. 1, Van Nostrand, New York, 1955.
22. Welcher, F. J., "Organic Analytical Reagents." Vol. 2, Van Nostrand, New York, 1955.

Mikrochimica Acta [Wien] 1970, 457—462
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Microdetermination of Ascorbic Acid and Tryptophan by Colorimetry

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With 2 Figures

(Received September 26, 1969)

During a systematic study of the color reactions, it was found that an aqueous solution of 2,3,5-triphenyltetrazolium chloride reacts with ascorbic acid in alkaline medium and gives beautiful pink color. Similarly hexamine reacts with tryptophan to give yellow color which is specific to tryptophan. Reference to the literature[1–4] indicates that these color reactions have not been reported previously for the determination of ascorbic acid and tryptophan respectively.

The color reaction of ascorbic acid provides the basis of a new colorimetric method for the determination of vitamin C from pharmaceutical products. The visual limit of ascorbic acid is 5 μg per ml. The maximum tolerable amount of reducing sugars[5] and other organic compounds which do not interfere is reported and the usefulness of the method is demonstrated by the determination of vitamin C from pharmaceutical preparations.

The reaction of hexamine with tryptophan provides the basis of a new method for the colorimetric determination of tryptophan in small quantities. The visual limit of identification is 5 μg per ml. The maximum tolerable amount of other amino acids and vitamins which do not interfere with the determination are reported.

Experimental

Reagents

All reagents were of analytical grade or comparable purity.

Aqueous solution of 2,3,5-triphenyltetrazolium chloride, 0.5% by weight, was used as color producing reagent for ascorbic acid. Hexamine, 1% solution in 80% alcohol was prepared.

Apparatus

All absorbance measurements were made with Unicam SP 1300 colorimeter using 1-cm cells.

Procedure

Ascorbic Acid. To 1 ml of the test solution containing 50 to 250 μg of ascorbic acid, add 1 ml of 2,3,5-triphenyltetrazolium chloride and 1.5 ml of 0.2 *M* sodium hydroxide with vigorous shaking. The volume of the

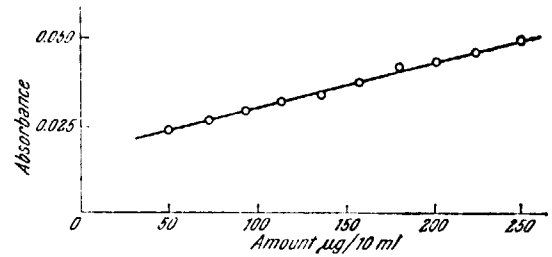

Fig. 1. Typical Standard line for determination of ascorbic acid

solution is made 10 ml with distilled water immediately after the addition of alkali. The solution is kept at 25° in the dark for 30 minutes before the absorbance of pink color is measured with Unicam SP 1300 colorimeter using 1-cm cells and filter No. 4. The experiment is repeated by taking

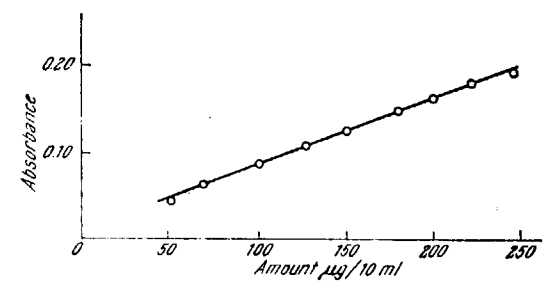

Fig. 2. Typical Standard line for determination of Tryptophan

different amounts of ascorbic acid and a calibration curve is prepared. A typical curve is shown in Fig. 1.

Tryptophan. Add 0.3 ml of hexamine reagent to 1 ml of test solution containing 50 to 250 μg of tryptophan followed by 1.5 ml of conc. hydrochloric acid. Shake well and place in a water-bath previously set at 94° for about 110 minutes. Cool the reactants and dilute to 10 ml with distilled water. Measure the absorbance of the yellow color thus produced with Unicam SP 1300 colorimeter using 1-cm cells and filter No. 1.

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Prepare a calibration curve by this procedure. The reaction obeys *Beer*'s law. A typical calibration curve is shown in Fig. 2.

Results and Discussion

For the determination of ascorbic acid, the effect of time, temperature and excess reagent on the development of color intensity was studied and it was found that color intensity increases with time and becomes constant after 10 minutes between 15 to 30°. The concentration of the color producing reagent between 5 to 25 mg per 10 ml does not effect the color intensity and is suitable for use during determination. However, if the concentration of the color producing reagent is less than 5 mg/10 ml, the color intensity is decreased and inaccurate results are obtained in the above procedure. The color intensity is also decreased if the temperature is increased beyond 30°. All absorbance measurements were taken at appropriate time, temperature and reagent concentration where the color intensity remains constant. Since the determinations are made at high alkali concentration, the effect of p_H cannot be determined. However, the color intensity remains constant if the amount of alkali is not varied throughout the experiment. For accurate results, it is

Table I. Quantitative Assessment of Tolerable Amounts of Different Compounds*

Compound	Maximum amount not interfering (μg/10 ml)
Glucose	70
Fructose	50
Sucrose	5000
Mannose	150
Xylose	70
Glactose	100
Lactose	100
Riboflavin**	5
Cyanocobalamine**	7
Folic acid**	1000

* Formaldehyde, acetaldehyde, benzaldehyde, thiamine, pantothenic acid, biotin and pyridoxine do not interfere with the determination.

** The interference is due to own color of the compound and not because of any reaction with color producing reagent.

Table II. Determination of Ascorbic Acid from Pharmaceutical Preparations

Ascorbic acid from	Amount present μg/10 ml	Amount found μg/10 ml
Pure	50	50
	100	98
	150	148
Polyvit*	100	95
	150	150
Celin**	50	48
	100	100

* Each 5 ml of Polyvit syrup (from P. D. H. Laboratories, Lahore, West Pakistan) contains vitamins A 6000 I. U., D 1000 I. U., B_1 4 mg, B_2 2 mg, B_6 1 mg, B_{12} 0.0003 mg, panthenol 5 mg, nicotinamide 20 mg, C 75 mg, *l*-lysine 200 mg.

** Each tablet of Celin (from Glaxo Laboratories, Ltd., Karachi, West Pakistan) contains 100 mg ascorbic acid.

necessary that the contents of the test solution may be diluted with distilled water immediately after the addition of alkali and this solution may not be exposed to sunlight or UV light.

Sugars also give pink color with 2,3,5-triphenyltetrazolium chloride. However, ascorbic acid can be determined in presence of sugars provided their amount do not exceed the maximum tolerable amount reported in Table I. The results for the determination of ascorbic acid from pure compounds, multivitamin syrup and tablets are given in Table II which indicates that ascorbic acid can be determined accurately from commercially available pharmaceutical preparations.

Unlike iodate, bromate[6], isonicotinic acid hydrazide[7] and metal ions[8,9], ascorbic acid does not give pink color with 2,3,5-triphenyl-

Table III. Determination of Tryptophan*

Amount added (μg/10 ml)	Amount found (μg/10 ml)
50	50
70	68
100	100
150	150

* The experiments were repeated with the addition of different amounts of various amino acids and vitamins. The determinations are not affected provided the amount of added amino acids and vitamins do not exceed the maximum tolerable limit (*cf.* Table IV).

Table IV. Quantitative Assessment of Tolerable Amounts of Different Amino Acids and Vitamins in Tryptophan Determination

Compound	Maximum amount not interfering mg/10 ml
Amino Acids	
L-Cystine	10
β-Alanine	10
Glycine	10
DL-Serine	10
L-Threonine	10
DL-Valine	10
L-Methionine	10
DL-Phenylalanine	10
L-Leucine	10
L (−) Tyrosine	10
DL-Asparagine	10
Glutamine	10
L-Aspartic acid	10
L (+) Glutamic acid	5
DL-Ornithine	10
L-Arginine	10
Hydroxy Proline	10
Vitamins	
Pantothenic acid	1
Biotin	1
Pyridoxine	1

* Riboflavin, cyanocobalamine and folic acid interfere because of their own color.

tetrazolium chloride in acid medium. In alkaline medium, the only serious interferences are expected from sugars[5] which themselves form pink colored formazan[10]. However, the experimental conditions under which ascorbic acid is determined, a reasonable amount of different sugars do not interfere (*cf.* Table I) and hence, the method can be used for the determination

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of ascorbic acid in presence of sugars. The mechanism of the color reaction is the same as with sugars[5]. Pink-colored formazan[5,10] is formed having maximum absorption at 480 nm.

For the determination of tryptophan, the effect of time, temperature and reagent concentration on color intensity was studied. The color intensity increases with time and temperature and becomes constant after 110 minutes at 94°. The color intensity also remains constant if the amount of hexamine lies between 0.6 to 3.75 mg per 10 ml otherwise it is decreased. Hence, for the determination of tryptophan, all experiments were performed under these set of conditions. Since the procedure prescribes high concentration of HCl, it is not possible to record the effect of p_H on color intensity. However, the color intensity is not affected if the concentration of HCl is not varied during determination.

The results for the determination of tryptophan are given in Table III, which indicates that it can be determined accurately with the present procedure. The maximum tolerable amount of other amino acids and vitamins which do not interfere with the determination are given in Table IV. In analyzing tryptophan, the amount of other amino acids and vitamins present in the test solution must not exceed the maximum tolerable amount (*cf.* Table IV). The color reaction is specific for tryptophan. The mechanism of the color reaction is not clear.

Summary

Microdetermination of Ascorbic Acid and Tryptophan by Colorimetry

Ascorbic acid reacts with 2,3,5-triphenyltetrazolium chloride in alkaline medium giving pink color having 5 μg/ml as visual limit of identification. This color reaction has been utilized for the colorimetric determination of ascorbic acid from pharmaceutical preparations in minute quantities. The maximum tolerable amount of other vitamins, sugars and organic compounds during determination of ascorbic acid is reported.

Tryptophan reacts with hexamine to give yellow color having 5 μg/ml as limit of identification. The color reaction provides the basis of a new method for colorimetric determination of tryptophan. The maximum tolerable amount of various amino acids and vitamins is reported. The mechanism of the color reaction is not clear.

Zusammenfassung

Ascorbinsäure reagiert mit 2,3,5-Triphenyltetrazoliumchlorid in alkalischem Milieu unter Rosafärbung. Erfassungsgrenze: 5 μg/ml. Diese Farbreaktion wurde zur kolorimetrischen Bestimmung kleiner Mengen Ascorbinsäure in pharmazeutischen Präparaten verwendet. Die tolerierbare Höchstmenge an anderen Vitaminen, Zuckern und organischen Verbindungen wurde

angegeben. Tryptophan reagiert mit Hexamin unter Gelbfärbung. Erfassungsgrenze: 5 μg/ml. Die Farbreaktion dient als neue Methode zur kolorimetrischen Tryptophanbestimmung. Die nicht störenden Höchstmengen an anderen Aminosäuren und Vitaminen sind angegeben. Der Reaktionsmechanismus ist nicht geklärt.

References

1 *F. Feigl*, Spot Tests in Organic Analysis. New York: Elsevier. 1966.

2 *F. J. Welcher*, Organic Analytical Reagents, Vol. II to IV. New York: Van Nostrand. 1955.

3 *F. D. Snell* and *C. T. Snell*, Colorimetric Methods of Analysis, Vol. II. New York: Van Nostrand. 1951.

4 *L. Meites*, Handbook of Analytical Chemistry. London: McGraw Hill Book Co. 1963. p. 6–53.

5 *H. B. Mork*, *L. M. Backer*, *D. Pinkel*, and *L. J. Papa*, Talanta **12**, 27 (1965).

6 *M. H. Hashmi*, *H. Ahmad*, *A. Rashid*, and *F. Azim*, Analyt. Chemistry **36**, 2471 (1964).

7 *M. H. Hashmi*, *A. Rashid*, and *F. Azim*, Mikrochim. Acta [Wien] **1966**, 513.

8 *M. H. Hashmi*, *A. Rashid*, *H. Ahmad*, *A. A. Ayaz*, and *F. Azam*, Analyt. Chemistry **37**, 1027 (1965).

9 *M. H. Hashmi* and *A. Rashid*, Analyt. Chemistry **38**, 1423 (1966).

10 *C. R. Noller*, Chemistry of Organic Compounds, 2nd Ed. Philadelphia: W. B. Saunders. 1957. p. 638.

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Analyst, 81, 376-377

AN IMPROVED SPOT TEST FOR THE DETECTION OF ASCORBIC ACID IN FLOUR

WITH the recent introduction of ascorbic acid as a commercial flour improver, the need has arisen for a rapid routine test for its detection in bread flours. Several tests have been examined for specificity and sensitivity in the range 10 to 20 p.p.m. of ascorbic acid, and a method applicable in the presence of other flour additives has been devised.

Hitherto, the detection of small quantities of ascorbic acid in foodstuffs and the like has been achieved by the qualitative application of Tillman's 2:6-dichlorophenolindophenol reagent. Treatment of a flour sample with a dilute aqueous solution of this reagent reveals the presence of ascorbic acid as light spots on a blue or purplish background. The test may be misleading if the particles of ascorbic acid are very small or if the flour contains active benzoyl peroxide. Comparatively rapid deterioration of the reagent solution is a further disadvantage.

EXPERIMENTAL

Many of the known colour reactions of ascorbic acid were briefly investigated, but the majority of these were rejected owing to lack of sensitivity or simplicity of application. The most promising results were obtained with Tauber's ferric - ferricyanide reagent.[1] In accordance with the original details of the method, adequate response was shown at very low levels of ascorbic acid, a positive test being indicated by the formation of bright blue spots on a yellow background. The smallest particles of ascorbic acid were identifiable with this reagent.

In the examination of a number of commercial flours, however, it was found that blue spots often appeared when it was known that ascorbic acid treatment had not been used. The spots appeared more slowly than those characteristic of ascorbic acid, and showed greater uniformity of shape and size. These results were attributed to the presence of reduced iron added to the flour in the form of enrichment mix.

Modification of Tauber's reagent to reduce the total acidity and thus lower the tendency for ferrous iron to pass into solution successfully eliminated this interference. The modified reagent and procedure as described below was capable of detecting 10 p.p.m. or less of ascorbic acid, and was unaffected by all the commonly used improvers and enrichment materials.

METHOD

REAGENTS—

Modified Tauber reagent—Dissolve 1 g of ferrous sulphate in 50 ml of water and add 10 ml of 85 per cent. phosphoric acid. Heat to boiling, and oxidise by adding a 1 per cent. solution of potassium permanganate until a faint pink colour persists. Cool, and add 20 per cent. sodium hydroxide solution until a permanent turibidity is produced. Clear the solution by cautious addition of 10 per cent. sulphuric acid, and then make up to 100 ml and filter. Add 0·5 g of potassium ferricyanide dissolved in 100 ml of water and mix.

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The mixed reagent should be pure yellow in colour and remains active so long as no change in colour occurs. In this laboratory the reagent has been stored for periods up to 3 months without loss of sensitivity.

PROCEDURE—

Smooth a sample of the flour on a white tile or Pekar slab and wet it by quickly immersing the tile or slab in water and draining. Treat the wet surface with sufficient of the modified Tauber reagent to cover completely and leave for 1 to 2 minutes. The presence of ascorbic acid is indicated by the appearance of bright blue spots or flecks. The level of treatment may be approximated by comparison with flours containing known amounts of ascorbic acid.

It is important that the moistened test sample should be examined for the formation of spots in the time specified, as, in exceptional cases, a slow response may occur as a result of treatment with reduced iron. Such a response is unlikely in the absence of excessive chlorine treatment, which may render the iron particles more reactive, but, should the necessity arise, closer examination of the spots by means of a hand lens will reveal their origin. Spots due to ascorbic acid are irregular in shape and size, whereas those due to reduced iron appear as blue points surrounded by a diffuse ring.

I thank the Directors of Novadel Ltd. for permission to publish this Note.

REFERENCE

1. Tauber, H., *Mikrochem. Mikrochim. Acta*, 1935, **17**, 111.

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K. J. HAYDEN
January 5th, 1956

J. Clin. Endocrinol. 15: 1257-1261 1955

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LACK OF EFFECT OF ASCORBIC ACID ON HYDROCORTISONE METABOLISM IN MAN*

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THE work of Bacchus has indicated that in the rat massive doses of ascorbic acid slow the metabolic degradation of administered cortisone, and this inhibition of breakdown prolongs the biologic actions of the cortisone (1–6). Because of the obvious clinical importance, an investigation was undertaken to determine if this effect of ascorbic acid also occurred in man.

EXPERIMENTAL PROCEDURE

Five young men in the age range of 19 to 22 years, who were conscientious objectors to military service, voluntarily acted as experimental subjects. All were in good health at the time of the study.

The metabolism of orally administered hydrocortisone was followed by three groups of determinations: plasma hydrocortisone, by the technique of Sweat *et al.* (7, 8); urinary 17-hydroxycorticoids, by the technique of Reddy *et al.* (9); and urinary 17-ketosteroids, by the techniques of Drekter *et al.* (10) and Vestergaard (11).

The subjects were awakened to void urine at 7:00 A.M. on the morning of the experiment; the sample was discarded. At 8:00 A.M. the subject again voided; this specimen was collected, and blood was drawn into test tubes containing oxalate for determination of the plasma hydrocortisone level. Then the subject was given 50 mg. of hydrocortisone orally. Blood and urine specimens were again collected at 9:00 A.M., noon, 4:00 P.M. and at 8:00 A.M. the following morning. The subjects ate no breakfast on the day of the experiment, but had lunch and dinner. They were kept in bed until 4:00 P.M. and were permitted light activity from then until bedtime.

Five to nine days later the experimental procedure was repeated, but at this time the subjects received ascorbic acid in addition to the hydrocortisone. The ascorbic acid was given on the following schedule: At bedtime the night before the experiment 1 Gm. of ascorbic acid orally; at 8:00 A.M. on the morning of the experiment 2.5 Gm. intravenously and 2.5 Gm.

Received for publication January 12, 1955.

* The opinions expressed in this paper are those of the authors and do not necessarily represent the official views of any governmental agency.

intramuscularly; and at 9:00 A.M. 1 Gm. orally. The total ascorbic acid administered was thus 7 Gm. or 84 to 104 mg. per Kg. of body weight. The plasma and urine specimens were frozen until analyses were performed. Analyses of samples for the control day and the day on which ascorbic acid was given were performed simultaneously in order to minimize possible effects of day-to-day variations in analytical techniques.

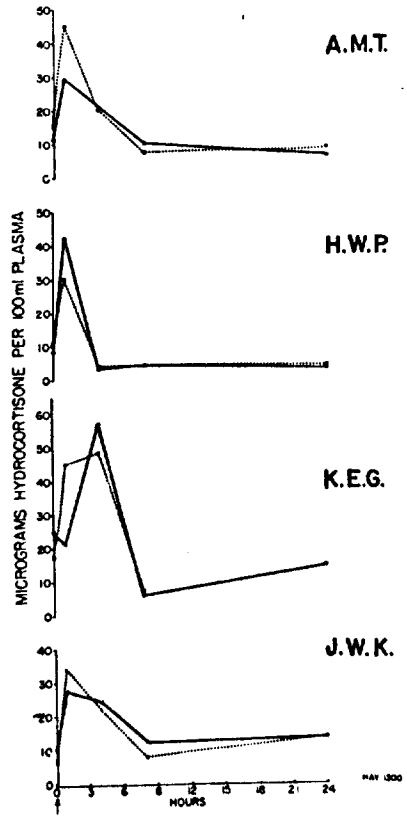

FIG. 1. The level of plasma hydrocortisone expressed in μg. per 100 ml. of plasma. Arrow indicates time hydrocortisone was administered. Values on day hydrocortisone alone was given are represented by a solid line. Values on day ascorbic acid and hydrocortisone were given are represented by a dotted line.

RESULTS

The blood concentration of hydrocortisone rose to a high level within one hour after oral administration of hydrocortisone, and the level returned to normal within eight hours. This is in agreement with the work of Richards and Sweat (12). The changes in plasma hydrocortisone levels on the control day and the day ascorbic acid was given are indistinguish-

able. There was no prolongation of elevated plasma levels, as has been reported in rats, and the height of the curve did not appear to differ significantly from that of the control when ascorbic acid was given. The results of plasma hydrocortisone determinations are given in Figure 1.

The urinary excretion patterns of 17-hydroxycorticoids and 17-ketosteroids did not change significantly when ascorbic acid was given to the subjects. The 17-hydroxycorticoid excretion rate increased shortly after

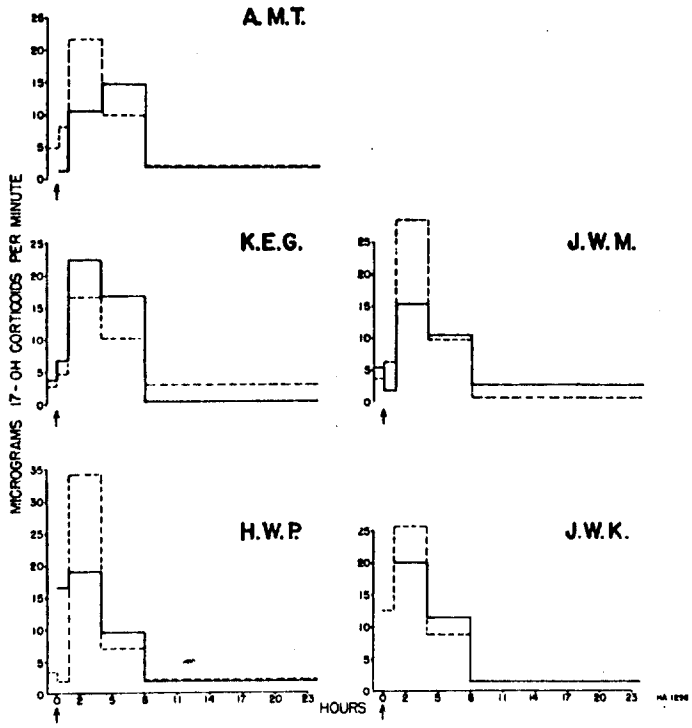

FIG. 2. The rate of urinary excretion of 17-hydroxycorticoids expressed in μg. per minute. Arrow indicates the time hydrocortisone was administered. Values on day hydrocortisone alone was given are represented a by solid line. Values on day ascorbic acid and hydrocortisone were given are represented by a broken line.

administration of hydrocortisone. However, the regular diurnal rhythm of 17-ketosteroid excretion did not seem to be affected by the small dose of hydrocortisone. This is in agreement with the findings of others (13). The excretion of 17-hydroxycorticoids and 17-ketosteroids is shown graphically in Figures 2 and 3.

DISCUSSION

These experiments failed to demonstrate any effect of ascorbic acid on hydrocortisone metabolism in man.

It should be pointed out that there are several differences between the experiments reported here and the experiments of Bacchus, who has reported an effect of ascorbic acid on adrenal steroid hormone metabolism. The first difference concerns species variation; the experiments of Bacchus were performed on rats, and the authors' experiments were performed on men. Second, in the rat studies the ascorbic acid was given intraperitoneally; in these experiments it was given orally, intravenously and intramuscularly. Third, cortisone was used in rats; hydrocortisone was used in man. Fourth, the cortisone was given to the rats subcutaneously; the

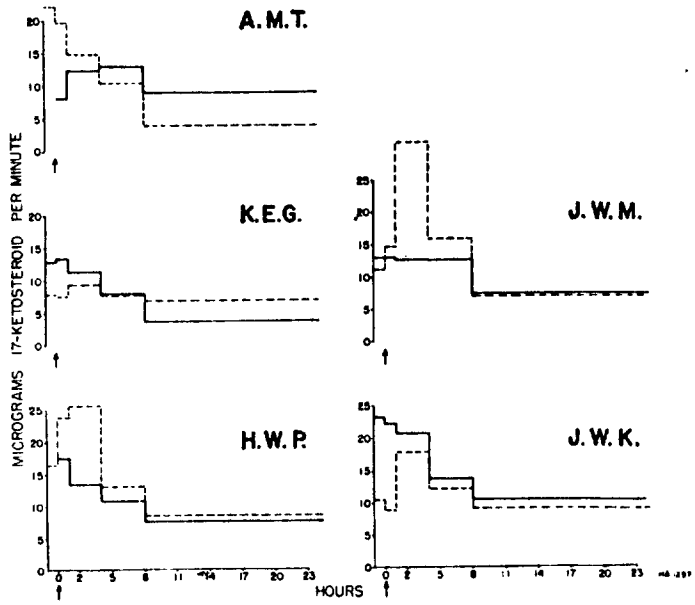

FIG. 3. The rate of urinary excretion of 17-ketosteroids expressed in μg. per minute. Arrow indicates the time hydrocortisone was administered. Values on day hydrocortisone alone was given are represented by a solid line. Values on day ascorbic acid and hydrocortisone were given are represented by a broken line.

hydrocortisone was given orally to the human subjects. Fifth, the dose of steroid was 20 mg. per Kg. of body weight in the rat, but approximately 0.7 mg. per Kg. body weight in man.

Any one of these factors could account for the disparity of the results of the two studies. Which particular variable is the critical one is not known at this time.

SUMMARY AND CONCLUSIONS

Under the conditions of these experiments, massive doses of ascorbic acid given to human subjects failed to affect significantly the metabolism

of a small dose of orally administered hydrocortisone, as measured by changes in plasma hydrocortisone levels and urinary 17-hydroxycorticoid and 17-ketosteroid excretion.

Acknowledgments

We wish to thank the volunteer experimental subjects, The Colorado State Selective Service Organization and Bishop E. M. Yost of the First Mennonite Church of Denver, Colorado, whose cooperation made this study possible.

We also wish to acknowledge generous gifts of ascorbic acid from the William S. Merrell Company, Cincinnati, Ohio, and hydrocortisone from Merck and Company, Rahway, New Jersey.

REFERENCES

1. Bacchus, H.: Effect of ascorbic acid, potassium chloride flooding, and cortisone on the course of formaldehyde-irritation arthritis, *Endocrinology* **49**: 789, 1951.
2. Bacchus, H., and Toompas, C. A.: The influence of ascorbic acid pre-treatment on the leukocyte response of rats exposed to sudden stress, *Science* **113**: 269, 1951.
3. Bacchus, H.; Altszuler, N., and Heiffer, M. H.: Prolongation of effects of adrenal cortical secretion by ascorbic acid: proposed mechanism of action, *Proc. Soc. Exper. Biol. & Med.* **80**: 88, 1952.
4. Bacchus, H.; Altszuler, N., and Heiffer, M. H.: Effect of ascorbic acid on 17-ketosteroid excretion, *J. Clin. Endocrinol. & Metab.* **12**: 908, 1952.
5. Bacchus, H., and Heiffer, M. H.: Urinary corticosteroids and 17-ketosteroids in adrenalectomized female rats treated with cortisone and ascorbic acid, *Am. J. Physiol.* **173**: 33, 1953.
6. Bacchus, H.: Maintenance of blood 17-hydroxycorticosteroids following ascorbic acid treatment, *Endocrinology* **54**: 402, 1954.
7. Sweat, M. L.; Abbott, W. E.; Jefferies, W. McK., and Bliss, E. L.: Adrenocortical steroids in human peripheral and adrenal venous blood as determined by fluorescence, *Fed. Proc.* **12**: 141, 1953.
8. Sweat, M. L.: Sulfuric acid-induced fluorescence of corticosteroids, *Analyt. Chem.* **54**: 402, 1954.
9. Reddy, W. J.; Jenkins, D., and Thorn, G. W.: Estimation of 17-hydroxycorticoids in urine, *Metabolism* **1**: 519, 1952.
10. Drekter, I. J.; Heisler, A.; Scism, G. R.; Stern, S.; Pearson, S., and McGavack, T. H.: The determination of urinary steroids. I. The preparation of pigment-free extracts and a simplified procedure for the estimation of total 17-ketosteroids, *J. Clin. Endocrinol. & Metab.* **12**: 55, 1952.
11. Vestergaard, P.: Rapid micro-modification of the Zimmermann/Callow procedure for the determination of 17-ketosteroids in urine, *Acta endocrinol.* **8**: 193, 1951.
12. Richards, J. B., and Sweat, M. L.: Peripheral blood concentrations of steroids in man after oral administration of 17-hydroxycorticosterone, *Proc. Soc. Exper. Biol. & Med.* **84**: 125, 1953.
13. Thorn, G. W.; Jenkins, D.; Laidlaw, J. C.; Goetz, F. C.; Dingman, J. F.; Arons, W. L.; Streeten, D. H. P., and McCracken, B. H.: Pharmacologic aspects of adrenocortical steroids and ACTH in man, *New England J. Med.* **248**: 232, 323, 414, 588, 632, 1953.

J. Clin. Invest. 17: 671-676 1938

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REQUIREMENTS FOR VITAMIN C IN MAN

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(Received for publication June 2, 1938)

Tests for requirements for vitamin C give varying results according to the criteria which are established; for example, there are the widely different requirements (*i*) for complete saturation and (*ii*) for protection against scurvy. In guinea pigs, doses of ascorbic acid which assure normal growth curves and freedom from scurvy are not sufficient to protect against the early pathological changes caused by deficiency of this vitamin (1, 2, 3, 4). It has been shown also that these animals are protected against scurvy by very small doses of vitamin C although their tissues contain scarcely detectable amounts of this vitamin (5, 6). In man, also, the daily dose of ascorbic acid adequate to prevent scurvy is far less than that required to maintain saturation of the body. In this paper, the latter amounts, *viz.*, the doses used by saturated subjects under normal and pathological conditions, have been investigated.

If a previously saturated subject is resaturated after having omitted all vitamin C for a certain length of time, the dose of ascorbic acid required to produce saturation corresponds to the quantity used from the body stores during the vitamin C-free period. Consequently, this amount when divided by the number of days the experiment lasted, can be considered as the daily expenditure (7, 8). Saturation, as referred to in this paper, is defined as the condition when considerable portions of a moderate test dose of ascorbic acid appear in the urine. Under these circumstances the tissues contain maximal quantities of ascorbic acid and, in man, the concentration in whole blood is 14 to 15 mgm. per liter. Unless maximal amounts have been stored, the body retains administered ascorbic acid avidly; beyond this point of saturation, however, any excess of ascorbic acid is rapidly wasted into the urine, a fact on which the saturation test is based (9, 10, 11). As stated before, the estimation of the daily requirements of vitamin C depends necessarily on the criteria applied.

[1] Honorary Fellow.

HEALTHY SUBJECTS

At one extreme, about 0.4 mgm. per kgm. daily is sufficient to protect against abnormal capillary permeability resulting from deficiency of vitamin C (12, 13, 14). At the other extreme, more than 0.8 mgm. per kgm. is metabolized daily by a subject saturated with ascorbic acid (7, 8). All variations are possible between these extremes. By his method, van Eekelen has demonstrated that the further removed from saturation the subject is, the smaller is the amount metabolized daily. The amounts required to produce saturation have been calculated as 0.4 mgm. per kgm. after 94 days, as about 0.55 mgm. per kgm. after 40 days, and as about 0.8 mgm. per kgm. after 27 days of vitamin C deprivation (7, 8). Probably even more than 0.8 mgm. per kgm. would be metabolized at complete saturation levels. That for the maintenance of a lower level of vitamin C reserves less is required is shown in another way by the following experiment. A subject (78 kgm.) partly depleted as the result of a diet devoid of vitamin C, was supplied with about 0.5 mgm. per kgm. (40 mgm.) daily *per os* for 16 days. The concentration of ascorbic acid in whole blood had not changed during this period (5.4 mgm. per liter on February 28, 5.5 mgm. per liter on March 16). In whole blood (56), an ascorbic acid concentration of 14 to 15 mgm. per liter indicates saturation; about or less than 2 mgm. per liter have been observed in scurvy. This small intake of about 0.5 mgm. per kgm. prevented further decline in the blood level. That it fell far short of causing saturation is demonstrated by the failure of a test dose of 750 mgm. of ascorbic acid, taken as a single dose *per os*, to cause any increase in the urinary excretion. While from this observation 0.5 mgm. per kgm. can not be considered to cover maximal demands and to produce saturation in a depleted organism, it appears that this dose just maintains a blood level of about 5 to 6 mgm. per liter, at which level no clinical manifestations of scurvy have as yet been observed.

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There is experimental evidence that 0.4 mgm. per kgm. daily represents the minimal requirement only and does not meet maximal demands. A subject was saturated and thereafter supplied daily with 0.33 mgm. per kgm. for 32 days (15), and on another occasion with 0.5 mgm. per kgm. for the longer period of 53 days (16). In both instances a total of 16 mgm. per kgm. was necessary for resaturation. The blood content had fallen from the saturation level of 14 to 15 mgm. per liter to 7.2 mgm. and 6.4 mgm. per liter respectively. Under both these experimental conditions, requirements calculated according to the method described in principle above also amount to 0.82 mgm. per kgm. (17).

These maximal requirements of 0.8 mgm. per kgm. daily may represent somewhat more than the optimum because there is no clinical evidence that a smaller supply, *e.g.*, 0.6 mgm. per kgm. has any deleterious effect. Furthermore, it has already been shown that the amount of ascorbic acid used by the body decreases to some extent when the body stores diminish.

Table I summarizes the results of determining maximal requirements in normal adults (8, 17, 18, 19), including one subject (J) with scurvy (19). Results agreeing closely to 0.83 to 0.84 mgm. per kgm. were obtained by calculating the weights for the experimental subjects from a weight-height-age table (20). As can be seen, maximal requirements in man are correlated with weight; this has been observed also for minimal requirements in man (14) and for guinea pigs (4). It has been suggested that requirements are comparatively larger in children than in adults (21, 22, 23); they are not larger in young guinea pigs than in old ones (4). O'Hara and Hauck (24) investigated the storage of vitamin C in 4 normal adults; their data (kindly supplemented by a personal communication) also indicates that the optimum requirement is considerably greater than the amount of vitamin C necessary to prevent scurvy. Thirty mgm. of ascorbic acid have been postulated as the daily minimum, 50 mgm. as the daily optimum for the German population (25). There is some evidence that requirement for vitamin C varies with the total metabolism (26, 27, 28, 29), but it is apparently immaterial whether the calories are supplied by protein or carbohydrate (18).

TABLE I

Vitamin C requirements of normal subjects

Subject	Age	Weight	Daily requirements of ascorbic acid	
	years	*kilos*	*mgm.*	*mgm. per kgm.*
v. E.	30	90	63	0.70
v. W.	38	72	56	0.77
J.	18	53	44	0.83
El.	25	68	53	0.78
			52	0.76
H.	37	75	63	0.84
			63	0.84

From the foregoing, it follows that at least 0.8 mgm. per kgm. of ascorbic acid is used daily by a saturated subject. Smaller doses, *e.g.*, 0.5 mgm. per kgm. or even less, are sufficient to protect against scurvy. But, even if it is true that smaller doses may assure good health under optimal conditions, it would seem useful to supply the maximum requirements as a factor of safety against altered circumstances which may increase requirements.

DISEASED SUBJECTS

An abnormally high supply seems to be needed in many diseases (30, 31, 32, 33, 34, 35), but this does not appear to be specific for any one disease. So far, the knowledge concerning ascorbic acid requirement in disease is very incomplete; the methods applied to the problem have not always been adequate (36). Data obtained by the method of van Eekelen on a small group of patients are presented in Table II. From the table it follows that requirements are unusually high in cases of tuberculosis (37, 38). This agrees with observations by Heise and Martin (39) who, by a different method, found that 55 to 138 mgm. of ascorbic acid daily were required by a group of 44 tuberculous patients. These authors and others (40, 41, 42) also observed that the stores of vitamin C in the tuberculous organism are very insufficient. Fever has been considered as an important factor causing exhaustion of body stores (33, 35, 43); this may be referable to the increased total metabolism during fever. On the other hand, it appears from observations of Patients N and v.D. that requirements can be increased in spite of normal body temperature. The

TABLE II

Vitamin C requirements of diseased subjects

Subject	Diagnosis	Weight	Daily requirements of ascorbic acid	
		kilos	*mgm.*	*mgm. per kgm.*
K.....	Active pulmonary tuberculosis; high temperature	59	82	1.4
L.....	Active pulmonary tuberculosis; high temperature	55	139	2.5
V.*....	Active pulmonary tuberculosis; high temperature	50	110	2.2
J.*....	Active pulmonary tuberculosis; high temperature	44	93	2.1
N.....	Tuberculosis (spondylitis); normal temperature	72	86	1.2
E.....	Healed tuberculosis; normal temperature (pleurisy 7 months ago)	55	45	0.8
v. D.*.	Empyema following pneumonia; normal temperature	44	90	2.0
v. E.* .	Empyema following pneumonia; low grade fever	45	57	1.3
Th. ...	Osteosclerotic (?) anemia. Normal gastric acidity. B. M. R. −5; low grade fever	76	121	1.6
St.*...	Peptic ulcer	65	83	1.3
Bl.*...	Peptic ulcer	63	75	1.2
V. O...	Peptic ulcer	50	65	1.3
M.....	Peptic ulcer	69	83	1.2
R.....	Peptic ulcer	?	85	?

* While, as a rule, ascorbic acid was given by mouth, these patients received it subcutaneously in order to exclude the possibility that faulty absorption might only simulate increased requirements.

observation of normal requirements in a case (E.) of healed tuberculous pleurisy without any symptoms of activity agrees with results of saturation tests from which Abbasy *et al.* (34) concluded that body stores are normal in cases of quiescent surgical tuberculosis, while in active cases they are depleted. Increased requirements have been demonstrated also in tuberculous guinea pigs; kept on a diet devoid of vitamin C, they develop scurvy earlier than a healthy control group (44, 45). A report from South Africa similarly shows that the incidence of scurvy among the natives rises with the morbidity from tuberculosis (46, 47). These observations indicate that *tuberculosis predisposes to scurvy by increasing the requirements for vitamin C.* Amounts that will meet normal demands become inadequate.

It has been suggested before that the amounts required to maintain complete saturation may be even higher than those estimated by the method of van Eekelen employed for the present study. This suggestion appeared to be substantiated by the following observation: when a patient (J) was placed on his calculated daily amount for 2 weeks, the blood content decreased from 13.4 mgm. per 1000 cc. of whole blood to 8.7, at which level at least 600 mgm. of ascorbic acid would have been needed for resaturation. Abnormally increased requirements are not specific for tuberculosis as follows from determinations in other diseases (Patients v.D., v.E., and Th.).

Requirements have been found to be increased to some extent in patients with peptic ulcer, in whom similar experimental data were obtained by either oral or subcutaneous administration of the vitamin (Table II). Saturation tests have revealed that deficiency of vitamin C is rather common in this group of patients (32, 48, 49, 50, 51); but in only a few cases has manifest scurvy been described (35, 48, 49, 52).

Depletion was demonstrated also by measuring ascorbic acid in whole blood of hospitalized patients (Table III) who had been treated by the Sippy régime.

TABLE III

Milligrams of ascorbic acid per 1000 cc. of whole blood

Subject	Peptic ulcer	Subject	Active tuberculosis
G.....	2.3	v. W.....	2.7
B.....	1.8	J.....	5.7
H.....	1.8	v. E.....	6.7
St.....	1.8	N.....	9.4
Z.....	1.8	L.....	3.0
R.....	1.8	Br.....	3.1
L.....	2.7	P.....	3.3
Go.....	1.8	v. Z.....	3.2
Bl.....	3.0	Vi.....	5.7
D.....	1.7	Je.....	4.5
Mi.....	3.1	T.*.....	1.8
Wi.....	2.5	v. E.....	3.8

* This patient had manifest symptoms of scurvy which were promptly influenced by ascorbic acid.

Table III also presents, for purposes of comparison, the ascorbic acid content in whole blood of a number of patients suffering from tuberculosis, whose requirements have been found to be greater than those of patients with peptic ulcer. The values observed in the blood of tuberculous patients are low in spite of large amounts of orange juice taken for a few weeks previous to the determination of the vitamin in blood. The low concentration of ascorbic acid in the blood

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of patients with peptic ulcer, on the average even lower than in cases of tuberculosis, is owing chiefly to the dietary treatment, which obviously provides an insufficient supply of the anti-ascorbic vitamin. In agreement with investigations in other countries (53, 54) pasteurized milk was found to contain between 0.4 and 1.0 mgm. per 100 cc. Since milk constitutes the main source of vitamin C in the diet commonly prescribed, the daily intake during the first and second week of Sippy treatment, for instance, amounts to no more than 12 to 15 mgm. of ascorbic acid.

As has been stated before, about 2 mgm. or less of ascorbic acid per 1000 cc. of whole blood are observed in scurvy. Yet, in spite of levels lower than 2 mgm. per liter, none of these patients with peptic ulcers had any symptoms of scurvy, nor was capillary fragility increased. The freedom from scurvy may be due to the fact that they were hospitalized and had almost complete rest. Exercise has been found to increase requirements and to exhaust the stores of vitamin C in rats (55). In this connection it is interesting to note that the only patient with scurvy—among the group with a blood level of less than 2 mgm.—had entered the hospital, after several days of activity, considerably too fatiguing for his condition.

Recently, requirements in two cases of peptic ulcer have been studied in a different way. After the concentration of ascorbic acid in his whole blood (56) had been measured, one patient who could not take food by mouth received 100 mgm. daily intravenously, another 60 mgm. *per os* plus about 12 mgm. daily with his diet. After 6 days, repeated analyses of blood showed that in the first patient 100 mgm. daily had not maintained the initial blood level, which had decreased from 9.3 mgm. per liter to 7.0 mgm. per liter. The second patient had only a slight decrease (14.5 to 13.5 mgm. per liter). His daily intake of about 75 mgm. probably just covered his requirements. Studied similarly, a patient with infectious mononucleosis with slight elevation of body temperature showed approximately normal requirements; the initial blood level of 14.0 mgm. per liter rose to 15.3 after receiving daily for 6 days 1.2 mgm. per kgm. of body weight. This method does not permit an exact quantitative evaluation of daily requirements. Since it distinguishes between normal and distinctly increased requirements in a fairly simple way, it is proposed as a clinical method.

The amount of ascorbic acid needed for saturation of the subject to be studied is estimated from the result of a preliminary analysis of whole blood (56). With a concentration of 8 mgm. per liter of whole blood about 1000 mgm. of ascorbic acid should be necessary, with 4 mgm. per liter, about 2000 mgm. (57). The amount presumably needed is supplied in single doses of 250 to 300 mgm. Then, after an interval of about 12 hours, the concentration of ascorbic acid in whole blood is determined. It should be about 10 to 12 mgm. This initial saturation, necessary because maximum requirements are used only by a subject nearly saturated, is followed by a daily supply of the estimated normal requirements, 0.8 mgm. per kgm. or even more when larger requirements are assumed, for one week. Another analysis of blood on the 8th day demonstrates whether the daily supply has been more than (rising concentration in blood), less than (decrease in blood), or just equal to the amount metabolized (no change in blood).

In this investigation, requirements in healthy subjects have been estimated from the effects of ascorbic acid administered *per os*. It has been claimed that smaller doses, injected intravenously (58) or subcutaneously (59) are sufficient. In a few diseased subjects, in whom the parenteral route has been chosen for certain reasons, the estimated requirements are about the same as when the vitamin is given by mouth.

SUMMARY

At least 0.8 mgm. of ascorbic acid per kgm. of body weight is used daily by healthy subjects saturated with this vitamin. Smaller amounts maintain a lower concentration in the body stores. To protect against scurvy, 0.4 mgm. per kgm. or even less per day appear to be sufficient.

Abnormally high requirements are observed in patients with active tuberculosis, but are not specific for this disease.

Requirements have been found to be increased to some extent in patients with peptic ulcer.

A comparatively simple method for rough estimation of daily requirements is proposed.

BIBLIOGRAPHY

1. Giroud, A., Ratsimamanga, R., Baratte, A., and Sylva, F., Réactions des animaux carençables à des doses croissantes d'acide ascorbique. Compt. rend. Soc. de Biol., 1935, 120, 701.
2. Giroud, A., Leblond, C. P., and Ratsimamanga, R., The vitamin C requirement of the guinea-pig. Yale J. Biol and Med., 1937, 9, 573.
3. Zilva, S. S., The ascorbic acid content of the intestine of the guinea pig. Biochem. J., 1935, 29, 100.

   The effect of incomplete diets on the concentration of ascorbic acid in the organs of the rat. Ibid., 1936, 30, 857.

   Vitamin C requirements of the guinea pig. Ibid., 1936, 30, 1419.
4. Dann, M., and Cowgill, G. R., The vitamin C requirement of the guinea pig. J. Nutrition, 1935, 9, 507.
5. Fox, F. W., and Levy, L. F., Experiments confirming the antiscorbutic activity of dehydroascorbic acid and a study of its storage and that of ascorbic acid by the guinea pig at different levels of intake. Biochem. J., 1936, 30, 211.
6. Bessey, O. A., and King, C. G., The distribution of vitamin C in plant and animal tissues and its determination. J. Biol. Chem., 1933, 103, 687.
7. van Eekelen, M., Over opname, verbruik en uitscheidung van vitamine C door de mens. Thesis, 1936, Drukkerij Fa. Schotanus and Jens, Utrecht.
8. van Eekelen, M., On the amount of ascorbic acid in blood and urine. The daily human requirements for ascorbic acid. Biochem. J., 1936, 30, 2291.
9. Harris, L. L., Ray, S. N., and Ward, A., The excretion of vitamin C in human urine and its dependence on the dietary intake. Biochem. J., 1933, 27, 2011.
10. van Eekelen, M., Emmerie, A., Josephy, B., and Wolff, L. K., Investigations about vitamin C. Acta brev. Neerland., 1933, 3, 168.
11. Johnson, S. W., and Zilva, S. S., The urinary excretion of ascorbic and dehydroascorbic acids in man. Biochem. J., 1934, 28, 1393.
12. Göthlin, G. F., Methode zur Bestimmung der Festigkeit der Haut-capillaren und zu indirekter Beurteilung des individuellen C-vitamin standards. Klin. Wchnschr., 1932, 11, 1469.
13. Göthlin, G. F., Human daily requirements of dietary ascorbic acid.. Nature, 1934, 134, 569.
14. Göthlin, G. F., Frisell, E., and Rundqvist, N., Experimental determinations of the indispensable requirements of vitamin C (ascorbic acid) of the physically healthy adult. Acta Med. Scandinav., 1937, 92, 1.
15. Heinemann, M., Dietary influences on the amount of ascorbic acid and other reducing substances in urine. Acta brev. Neerland., 1936, 6, 67.
16. Heinemann, M., On the urinary excretion of thiosulphate and ascorbic acid during different diets. Acta brev. Neerland., 1936, 6, 141.
17. Heinemann, M., On the relation between diet and urinary output of thiosulphate (and ascorbic acid). II. Human requirements for vitamin C. Biochem. J., 1936, 30, 2299.
18. Heinemann, M., On human requirements for vitamin C under different conditions. Acta brev. Neerland., 1936, 6, 144.
19. van Wersch, H. J., Determinations of the daily requirements for ascorbic acid of man. Acta brev. Neerland., 1936, 6, 86.
20. van Eekelen, M., Heinemann, M., and van Wersch, H. J., On the daily requirements for ascorbic acid. Acta brev. Neerland., 1936, 6, 107.
21. Szen-Györgi, A., Die medizinische Bedeutung des Vitamins C. Deutsche med. Wchnschr., 1934, 60, 556.
22. Widenbauer, F., Ascorbinsäurestudien an Säuglingen. Klin. Wchnschr., 1936, 15, 815.
23. Neuweiler, W., Vitamin C-Stoffwechsel bei Neugeborenen. Ztschr. f. Vitaminforch., 1937, 6, 75.
24. O'Hara, P. H., and Hauck, H. M., Storage of vitamin C by normal adults following a period of low intake. J. Nutrition, 1936, 12, 413.
25. Wachholder, K., Über die Vitamin C-Bilanz des Menschen. IV. Einschränkung des Bedarfes bei mangelhafter Zufuhr und deren ernahrungsphysiologische Bedeutung. Klin. Wchnschr., 1938, 17, 5.
26. Mosonyi, J., Skorbutische Stoffwechselstörung und Hyperthyreoidismus. Klin. Wchnschr., 1936, 15, 24.
27. Svirbely, J. L., The effect of desiccated thyroid, α-dinitrophenol and cortical hormone extract on the vitamin C content of some organs of the guinea pig fed graded doses of ascorbic acid. J. Biol. Chem., 1935, 111, 147.
28. Kreitmair, H., Beiträge zur Pharmakologie der Ascorbinsäure (Vitamin C). Arch. f. exper. Path. u. Pharmakol., 1934, 176, 326.
29. Widenbauer, F., Der Vitamin C Haushalt des Menschen unter verschiedenen Verhältnissen. Klin. Wchnschr., 1937, 16, 600.
30. Mettier, S. R., Minot, G. R., and Townsend, W. C., Scurvy in adults. Especially the effect of food rich in vitamin C on blood formation. J. A. M. A., 1930, 95, 1089.
31. Schroeder, H., Die Ausscheidung der Ascorbinsäure im gesunden und kranken Organismus. Klin. Wchnschr., 1935, 14, 484.
32. Harris, L. J., Abbasy, M. A., Yudkin, J., and Kelly, S., Vitamins in human nutrition. Vitamin C reserves of subjects of the voluntary hospital class. Lancet, 1936, 1, 1488.
33. Bullowa, J. G. M., Rothstein, I. A., Ratish, H. D., and Harde, E., Cevitamic acid excretion in pneumonias and some other pathological conditions. Proc. Soc. Exper. Biol. and Med., 1936, 34, 1.

3500

34. Abbasy, M. A., Hill, N. G., and Harris, L. J., Vitamin C and juvenile rheumatism with some observations on the vitamin C reserves in surgical tuberculosis. Lancet, 1936, 2, 1413.
35. Wright, J. S., The present status of clinical use of cevitamic acid (ascorbic acid) (crystalline vitamin C). Am. J. M. Sc., 1936, 192, 719.
36. van Eekelen, M., and Heinemann, M., Critical remarks on the determination of urinary excretion of ascorbic acid. J. Clin. Invest., 1938, 17, 293.
37. Heinemann, M., Ascorbic acid requirements in human tuberculosis. Acta brev. Neerland., 1937, 7, 48.
38. Heinemann, M., Metabolism of ascorbic acid in human tuberculosis. Acta brev. Neerland., 1937, 7, 113.
39. Heise, F. H., and Martin, G. J., Ascorbic acid metabolism in tuberculosis. Proc. Soc. Exper. Biol. and Med., 1936, 34, 642.
40. Burckhardt, J. L., and Weiser, F., Ueber Vitamin C Ausscheidung bei tuberkulösen Kindern. Schweiz. med. Wchnschr., 1936, 66, 832.
41. Hasselbach, F., Vitamin C und Lungertuberkulose. Ztschr. f. Tuberk., 1936, 75, 336.
42. Deggeller, O., Onderzoekingen over het gehalte aan vitamine-C van het bloed. Thesis, 1936, Drukkerij Fa. Schotanus and Jens, Utrecht.
43. Abbasy, M. A., Harris, L. J., Ray, S. N., and Marrack, J. R., Diagnosis of vitamin C subnutrition by urine analysis. Quantitative data—experiments on control subjects. Lancet, 1935, 2, 1399.
44. Mouriquand, G., Rochaix, A., and Michel, P., Action réciproque du terrain scorbutique et de l'infection expérimentale par une tuberculose virulente. Compt. rend. Soc. de biol., 1924, 91, 205.
45. Heymann, B., Versuche an Meerschweinchen über die Beziehungen zwischen Skorbut und chronischer Tuberkulose. Klin. Wchnschr., 1926, 5, 59.
46. Delf, E. M., Studies in experimental scurvy—with special reference to the antiscorbutic properties of some South African foodstuffs. Publications of the South African Institute for Medical Research, 1921, 14, Johannesburg.
47. Tuberculosis Research Committee, Ibid., 1932, 30, Johannesburg.
48. Davidson, P. B., The development of deficiency disease during therapeutic diets. J. A. M. A., 1928, 90, 1014.
49. Archer, H. E., and Graham, G., The subscurvy state in relation to gastric and duodenal ulcer. Lancet, 1936, 2, 364.
50. Portnoy, B., and Wilkinson, J. F., Vitamin C deficiency in peptic ulceration and haematemesis. Brit. M. J., 1938, 1, 554.
51. Bourne, G., Vitamin C deficiency in peptic ulceration estimated by the capillary resistance test. Brit. M. J., 1938, 1, 560.
52. Platt, R., Scurvy as the result of dietetic treatment. Lancet, 1936, 2, 366.
53. van Wijngaarden, J. C., Carotine, vitamin A en vitamine C in koemelk. Thesis, 1935, Drukkerij P. den Boer, Utrecht.
54. Whitnah, C. H., and Riddell, W. H., Milk as a source of vitamin C. Science, 1936, 83, 162.
55. van Eekelen, M., and Kooy, R., The ascorbic acid content of suprarenal glands and livers from normal and fatigued rats. Acta brev. Neerland., 1933, 3, 169.
56. Emmerie, A., and van Eekelen, M., The chemical determination of vitamin C with removal of interfering reducing and coloured substances. Biochem. J., 1934, 28, 1153.
57. van Eekelen, M., Emmerie, A., and Wolff, L. K., Ueber die Diagnostik der Hypovitaminosen A und C durch die Bestimmung dieser Vitamine im Blut. Ztschr. f. Vitaminforsch., 1937, 6, 151.
58. Schultzer, P., On the saturation of a scurvy patient with small doses of ascorbic acid. Consideration of the daily human requirements. Biochem. J., 1937, 31, 1934.
59. Hou, H. C., Comparison of oral and subcutaneous administration of protective doses of ascorbic acid (vitamin C). Proc. Soc. Exper. Biol. and Med., 1935, 32, 1391.

Br. J. Derm. (1969) **81**, 226.

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THE EFFECTS OF ADMINISTRATION OF ASCORBIC ACID IN EXPERIMENTALLY INDUCED MILIARIA AND HYPOHIDROSIS IN VOLUNTEERS

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SUMMARY.—A dose of ascorbic acid 1 G. daily restores normal sweating in 1 week, in areas of skin in which hypohidrosis and prickly heat have been induced by polythene occlusion for 48 hr.

REISS (1943) showed that tropical suppurative syringadenitis responded to large doses of ascorbic acid. Hindson (1968) showed that miliaria rubra can be prevented and its duration shortened by daily administration of ascorbic acid in a dose of 15 mg./Kg. body weight. Griffin *et al.* (1967) showed that miliaria and hypohidrosis could be produced by polythene occlusion of skin for 48 hr. Orentreich *et al.* (1966) showed that hypohidrosis, following polythene occlusion of small areas of skin for 1 week, is detectable for 3 weeks following unwrapping.

It was thought probable that in the tropical climate of Malaya, where the experiment was conducted, using a modification of Griffin's technique, miliaria and hypohidrosis could be induced by polythene occlusion of 1 arm only for 48 hr., and this was done in 2 groups of subjects, one taking ascorbic acid 1 G daily, and the other a placebo.

METHOD

A polythene sleeve was fitted to 1 arm of each of 36 apparently healthy caucasoid servicemen, aged from 19 to 37, from above the wrist to just below the axilla and secured with adhesive plaster at each end.

The men returned to normal outside duty during the day and slept in tents at environmental temperature for the duration of the experiment, but did not take vigorous exercise.

Half were given ascorbic acid 1 G. (Redoxon Effervescent Roche Pharmaceuticals Ltd.) and the other half a similar effervescent tablet manufactured by Messers Roche, which contained the effervescent ingredients but no ascorbic acid, administration beginning on the day of wrapping and continued daily throughout the experiment. The allocation of subjects to ascorbic acid and placebo was made by another medical officer and was unknown to the investigators during the period of the experiment.

All subjects were also taking Paludrine (proguanil hydrochloride) 0·1 G. daily for malaria prophylaxis and the ascorbic acid was given simultaneously at the daily Paludrine parade.

All subjects were unwrapped at 48 hr., examined and tested on Day 1 (24 hr. after unwrapping) and Day 7.

Abrasion caused by pressure from the adhesive tape at either end of the sleeve was ignored, and the degree of miliaria was assessed 4 in. above and below the ante cubital fossa, according to the classification of Griffin *et al.* (1967):—

Grade 0: No lesions. Grade I: No erythema and scattered papules visible only on close examination. Grade II: Patches of erythema with concomitant areas of papules.

Accepted for publication August 30th, 1968.

Grade III: Diffuse papular eruptions with unbroken erythematous background. Grade IV: Grade III with vesicles and non follicular pustules.

A template 8 in. by 2 in. was applied along both arms with its centre along the centre of the ante cubital fossa.

Bromphenol blue powder, which is pink in the anhydrous state and in the presence of sweat turns orange to blue depending on its pH, was then applied over the area with a camel hair brush. The subject then took 2 min. vigorous exercise to induce sweating, after which the degree of sweating in the wrapped arm was compared with that in the unwrapped arm and hypohidrosis recorded as follows.

Grade 0: No hypohidrosis in wrapped arm; both arms sweating equally. Grade I: Slight hypohidrosis in wrapped arm compared to unwrapped arm. Grade II: Marked hypohidrosis in wrapped arm compared to unwrapped arm.

Hypohidrosis was assessed independantly by both investigators.

During the period of the experiment, the mean ambient temperature measured hourly was 82·4° F. (dry bulb) with a range from 75° F. to 86 F. The mean relative humidity was 76·5%, with a range from 61% to 86%.

RESULTS

		Day 1		Day 7	
		Ascorbic acid	Placebo	Ascorbic acid	Placebo
Miliaria	Grade 0	3	2	16	12
	Grade I	9	2	1	6
	Grade II	3	11	1	0
	Grade III	3	3	0	0
Hypohidrosis	Grade 0	3	1	16	1
	Grade I	11	4	2	7
	Grade II	4	13	0	10

Prickly heat was evident in 16 of the control patients and 15 of the ascorbic acid patients on Day 1, 24 hr. after unwrapping.

Hypohidrosis was detected in 17 of the control group and 15 of the ascorbic acid group, at the same time.

However, when, subjects were retested at 1 week, although 12 of the placebo group had no clinical prickly heat, bromphenol blue testing showed that 17 of this group still had evidence of hypohidrosis, of whom 10 still had marked hypohidrosis, whereas only slight hypohidrosis could be detected in 2 of the 18 subjects of the ascorbic acid group.

We wish to thank Lt-Col D. R. D. Riddick R.A.M.C. Officer Commanding, Major A. J. Shaw, R.A.M.C., Second in Command and officers and men of No. 8 Casualty Clearing Station R.A.M.C., for organising the double blind trial and for volunteering themselves. And also to Dr. N. T. Pollitt and Messers Roche (Pharmaceuticals) Ltd., for supplies of Ascorbic acid, and for defraying the cost of the colour plate.

REFERENCES

Griffin, T. B., Maibach, H. I. and Sulzberger, M. B. (1967) Miliaria and Anhidrosis. II. The Relationships between Miliaria and Anhidrosis in Man. *J. invest. Derm.*, **49**, 379.

Hindson, T. C. (1968) Ascorbic Acid for Prickly Heat. *Lancet*, i, 1347.

Orentreich, N., Berger, R. A., and Auerbach, R. (1966) Anhidrotic Effects of Adhesive Tapes and Occlusive Film. *Archs Derm.*, **94**, 709.

Reiss, F. (1943) Syringadenitis Suppurativa Tropicalis (a Complication of Lichen Tropicus). Histologic Appearance and Etiologic Considerations Particularly as to a Possible Relationship of Ascorbic Acid and Carbohydrate Metabolism. *J. Lab. Clin. Med.* **28**, 1082.

Brit. J. Pharmacol. 40: 740-746 1970

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Br. J. Pharmac. (1970), 40, 740-746.

Ascorbic acid deficiency and pituitary adrenocortical activity in the guinea-pig

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Summary

1. Guinea-pigs kept on a diet deficient in ascorbic acid lost weight and became moribund in about 24 days.

2. The adrenal ascorbic acid concentration fell rapidly during the first 2 weeks, and the plasma corticosteroid concentration and 17-oxogenic steroid excretion rose sharply in the third week of ascorbic acid deficiency.

3. Both histamine and corticotrophin increased the plasma corticosteroid concentration when injected during the second week but failed to change the pre-existing high concentration of the steroid in the third week of ascorbic acid deficiency.

4. The observations confirm that ascorbic acid is not involved in corticoidogenesis and that scurvy is a severe stress which increases adrenocortical activity to such an extent that the rate of synthesis of corticosteroids is incapable of matching the rate of their release.

Introduction

Ascorbic acid, normally present in adrenal glands in high concentrations, is usually depleted by exposure to stress or by the injection of corticotrophin (ACTH). Although this fall in adrenal ascorbic acid concentration is associated with increased adrenocortical activity, the precise function of the vitamin in the synthesis and release of corticosteroids is not understood. The concentration of ascorbic acid in the adrenal glands of the guinea-pig can be reduced rapidly to scarcely detectable levels by diets deficient in ascorbic acid (Oesterling & Long, 1951) and this paper describes the results of experiments in which pituitary adrenocortical activity was studied in guinea-pigs made scorbutic in this way.

Methods

Animals. One hundred and forty female Dunkin Hartley guinea-pigs (Beecham Research Laboratories), weighing between 250 and 350 g, were used. They were fed on rat cubes (Diet 41B, Lane-Petter & Dyer, 1952) which contain no vitamin C, and tap water *ad lib.* For 10 days the diet was supplemented with greenfood and only those animals which grew satisfactorily were used. Greenfood was then withdrawn and the animals were divided into test and control groups. The test group received no ascorbic acid and the controls the same diet with ascorbic acid (0·2 mg/ml) in the drinking water. This concentration was selected after preliminary experiments on fluid intake to ensure that every animal received approximately 10 mg

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ascorbic acid/day. Blood and urine samples and adrenal glands were collected at various times during the course of the experiment and, in some cases, 2 h after the injection of corticotrophin or exposure to stress (injection of histamine).

Blood samples. The animals were anaesthetized with 25% (w/v) urethane solution (0·5 ml/100 g body weight) and blood was collected from a large mesenteric artery into 10 ml glass syringes. The samples were collected within 2 min of removal of the animals from their cages, centrifuged within 1 h of collection, and the plasma was stored at −12° C. Corticosteroid concentrations were measured by the method of Zenker & Bernstein (1958) and the results expressed as cortisol and corticosterone calculated as described by Stockham (1963).

Adrenal glands. Adrenal glands were removed immediately after the withdrawal of the blood samples, dissected free from peri-adrenal fat and connective tissue and weighed on a torsion balance. Left adrenals were ground with the aid of sand and a glass rod in 4% (w/v) trichloracetic acid solution and their ascorbic acid contents were determined by the method of Roe & Kuether (1943). Right adrenals were ground similarly in 10% (v/v) ethanol for the determination of cortisol and corticosterone.

Urine. Twenty-four hour urine samples were collected using conventional metabolism cages, and frozen until analysis for 17-oxo and 17-oxogenic steroids as described by Besch & Barry (1964).

Drugs. Histamine acid phosphate 2·5 mg/ml and corticotrophin (Cortrophin, Organon) 5 i.u./ml in normal saline were injected intraperitoneally and subcutaneously, respectively, in volumes of 0·1 ml/100 g body weight.

Results

The growth rate of the guinea-pigs is shown in Fig. 1. The test animals continued to grow well until the thirteenth day on the ascorbic acid deficient diet, when they

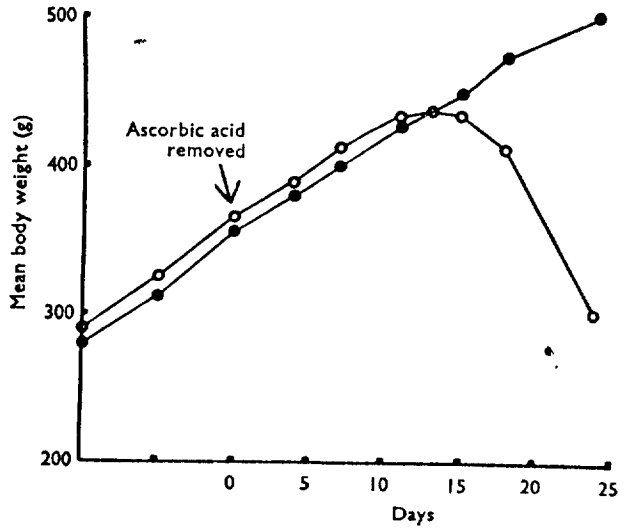

FIG. 1. Growth rate in guinea-pigs on a diet deficient in ascorbic acid. Controls ●---●; tests ○——○.

began to lose weight markedly. After 24 days they were moribund. Control animals grew normally. The adrenal ascorbic acid concentration fell rapidly during the first week of vitamin C deficiency and then more slowly. After 2 weeks the adrenals were almost completely depleted of the vitamin and by the third week there was none present. The adrenal ascorbic acid content in the control animals did not alter (Fig. 2). The ascorbic acid deficiency caused adrenal hypertrophy and at the end of the experiment the adrenal weights of the test animals were significantly ($P<0.05$) greater than those of the controls (Table 1).

The plasma cortisol and corticosterone concentrations in the test animals did not differ from the controls for the first 17 days despite the almost complete disappearance of ascorbic acid from the adrenal glands. Subsequently, they rose very rapidly and by the end of the experiment were almost 10 times those of the controls in which the plasma corticosteroid concentrations remained unchanged (Fig. 3).

Adrenal cortisol and corticosterone concentrations in the test animals remained constant for the first 17 days (Fig. 4), after which they increased considerably, reached a maximum on the twentieth day and then fell sharply. By the end of the experiment the adrenal corticosteroid concentrations were well below their initial values.

TABLE 1. *Effect of ascorbic acid deficiency on adrenal weight in the guinea-pig*

Day of experiment	Mean adrenal weight (mg ± standard error)		Mean greatest body weight (g)		Ratio of adrenal weight (mg) to greatest body weight (g)	
	Control	Test	Control	Test	Control	Test
0	111·6 ± 7·0		366		0·305	
7		126·3 ± 6·6		383		0·329
17	162·3 ± 12·6	165·3 ± 14·0	447	472	0·362	0·344
24	206·5 ± 12·6	265·6 ± 18·1	526	477	0·392	0·557

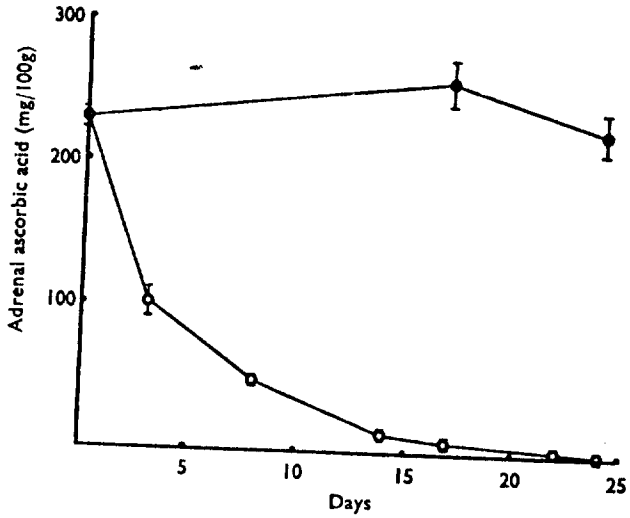

FIG. 2. Adrenal ascorbic acid concentrations in guinea-pigs on a diet deficient in vitamin C. Controls ●——●; tests ○——○. Vertical bars indicate standard errors.

Ascorbic acid deficiency caused an increase in 17-oxo steroid excretion which reached a peak on the fourteenth day and remained elevated for the next 7 days. In contrast, the excretion of 17-oxogenic steroids fell during the first 2 weeks of ascorbic acid deficiency and then increased considerably (Fig. 5).

Control animals responded to the injection of corticotrophin or histamine with a considerable rise in the plasma cortisol concentration (Fig. 6). The responses to

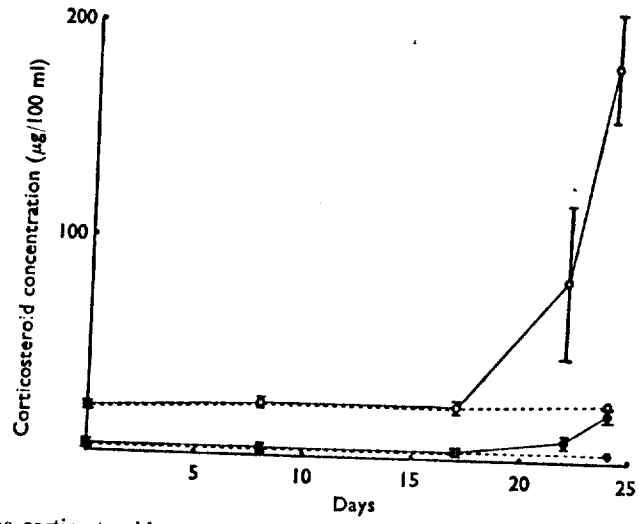

FIG. 3. Plasma corticosteroid concentrations in guinea-pigs on a diet deficient in ascorbic acid. ○—○, Cortisol; ●——●, corticosterone concentrations. The dotted lines show control values and the vertical bars the standard errors.

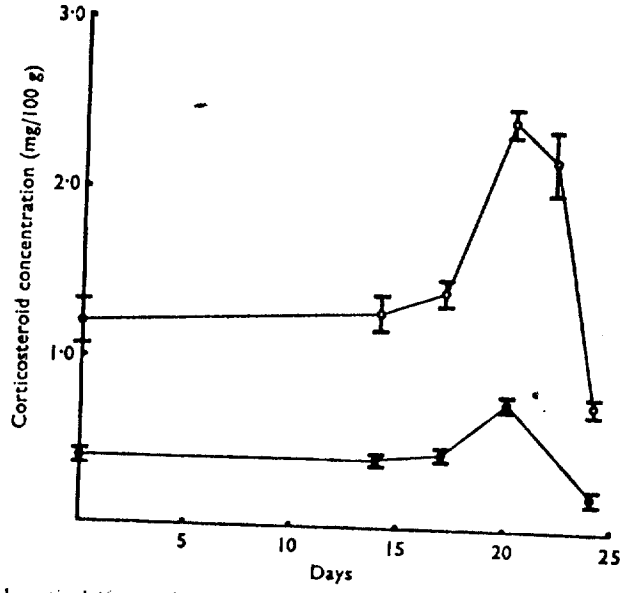

FIG. 4. Adrenal cortisol (○—○) and corticosterone (●——●) concentrations in guinea-pigs on a diet deficient in ascorbic acid. The vertical bars indicate the standard errors.

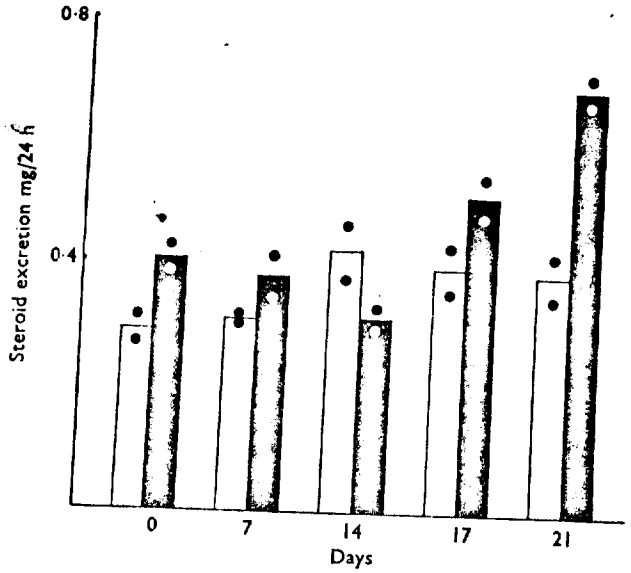

FIG. 5. Urinary 17-oxo (□), and 17-oxogenic (■) steroids excreted in 24 h by guinea-pigs on a diet deficient in ascorbic acid. The dots indicate individual values.

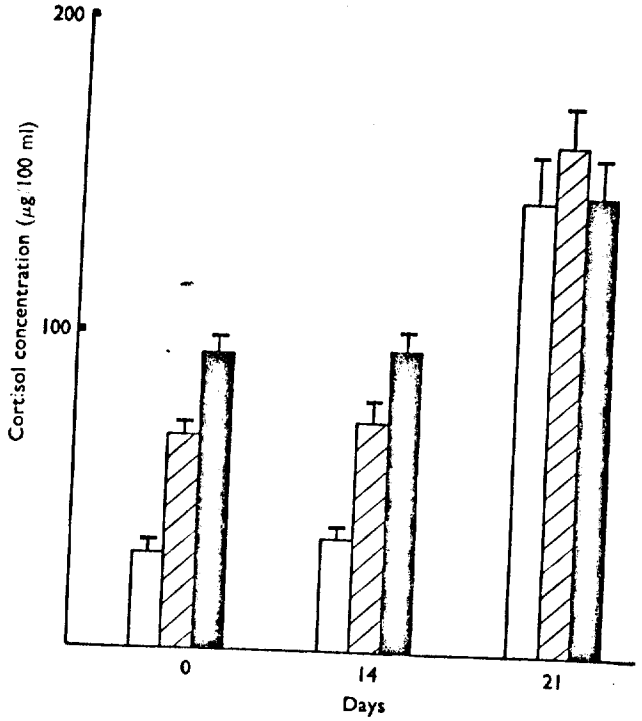

FIG. 6. Plasma cortisol concentrations 2 h after intraperitoneal injection of 0·1 ml/100 g saline (□), 250 μg/100 g histamine (▨), and subcutaneous injection of 0·5 i.u./100 g ACTH (■), in guinea-pigs on a diet deficient in ascorbic acid. The vertical bars indicate the standard errors.

these stimuli were not different after 2 weeks on the diet deficient in ascorbic acid. However, after 3 weeks of ascorbic acid deficiency, the pre-existing high plasma corticoid concentrations were not increased further by the administration of either histamine or corticotrophin.

Discussion

Although it is more than 40 years since the presence of ascorbic acid in adrenal glands was first demonstrated by Szent-Gyorgyi (1928), the functional importance of the vitamin in corticosteroid synthesis and release is still not known.

Our work does not support the view (Giroud, Martinet & Bellon, 1941 ; Lockwood & Hartman, 1933 ; Lowenstein & Zwemer, 1946) that ascorbic acid is necessary for the synthesis of corticosteroids. Guinea-pigs with virtually no ascorbic acid in their adrenal glands responded to stress or the injection of corticotrophin with normal changes in plasma corticosteroid concentration. The remarkably high blood levels of cortisol and corticosterone which occurred in the later stages of scurvy also suggest that corticoidogenesis can proceed in the absence of ascorbic acid. However, the elevated plasma corticosteroid concentrations in the scorbutic animals need not necessarily have been due to increased adrenocortical secretion but to impaired steroid metabolism and excretion. Such a possibility is not likely because ascorbic acid deficiency causes no change in the clearance or conjugation of exogenous cortisol (Jones, Peric-Golia & Eik-Nes, 1958) and there is a parallelism between the plasma cortisol concentration and 17-oxogenic steroid excretion in the second and third week of ascorbic acid deficiency. The initial drop in 17-oxogenic steroid excretion is not easy to explain, but a similar fall followed by a rapid rise was also seen in ascorbic acid deficient guinea-pigs by Prunty, Clayton, McSwiney & Mills (1955). However, we were unable to confirm their demonstration of a fall in 17-oxo steroid excretion in the second week of vitamin C deficiency, by which time 17-oxo steroid excretion in our animals had reached its peak.

Again our findings are not in accord with the possibility that ascorbic acid exerts an inhibitory effect on corticoidogenesis as has often been suggested (Hayano, Saba, Dorfman & Hechter, 1956 ; Jones *et al.*, 1958 ; Kitabchi, 1967). Kapanowski (1969) observed that animals with ascorbic acid deficiency show augmented adrenocortical responses to corticotrophin, but we were unable to confirm his work. Furthermore, although marked changes in plasma corticosteroid concentrations and urinary 17-oxogenic steroid excretion occurred in our scorbutic guinea-pigs, the considerable time lag between the fall in adrenal ascorbic acid and the increase in adrenocortical activity makes involvement of ascorbic acid in the inhibition of adrenal corticosteroidogenesis unlikely.

It appears that ascorbic acid is not essential for the elaboration of corticosteroids (Eisenstein & Shank, 1951) and that the increased adrenocortical activity in the terminal stages of scurvy is caused by the severe stress imposed by vitamin C deficiency (Howard & Cater, 1959 ; Oesterling & Long, 1951). A drop in adrenal corticosteroid concentration occurs in the final stages of ascorbic acid deficiency, possibly because the rate of synthesis of the corticosteroids is incapable of matching the rate of their release. The inability of stress or ACTH to provide a further increase in the already very high plasma cortisol concentration also suggests that the adrenal glands are under maximal stimulation by endogenous ACTH. Direct

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estimation of circulating corticotrophin in scorbutic animals may provide more information on the role of ascorbic acid in pituitary-adrenocortical physiology.

The work was financed by a generous grant from Beecham Research Laboratories to whom we are very grateful. Our thanks are also due to Mr. E. Vowles for invaluable technical assistance.

REFERENCES

BISCH, P. K. & BARRY, R. D. (1964). In: *Steroid Determinations*, pp. 28–31; 41–45. Ohio: American Medical Technologists.

EISENSTEIN, A. B. & SHANK, R. E. (1951). Relationship of ascorbic acid to secretion of adrenocortical hormones in guinea-pigs. *Proc. Soc. exp. Biol. Med.*, **78**, 619–624.

GIROUD, A., MARTINET, M. & BELLON, M. T. (1941). Dépendance de la fonction cortico-surrénale, chez l'homme, vis-a-vis de l'acide ascorbique: Variations de l'élimination urinaire de l'homme corticale. *C. r. Séanc. Soc. Biol.*, **135**, 514–516.

HAYANO, M., SABA, N., DORFMAN, R. I. & HECHTER, O. (1956). Some aspects of the biogenesis of adrenal steroid hormones. *Recent Prog. Horm. Res.*, **12**, 79–123.

HOWARD, A. N. & CATER, D. B. (1959). The adrenal cortex and the effect of ACTH and cortisone in scorbutic guinea-pigs. *J. Endocr.*, **18**, 175–185.

JONES, R. S., PERIC-GOLIA, L. & EIK-NES, K. (1958). Ascorbic acid deficiency and adrenocortical function in the guinea-pig. *Endocrinology*, **63**, 659–668.

KAPANOWSKI, L. (1969). Evaluation of adrenal function in experimental vitamin C deficiency. *Acta. med. pol.*, **10**, 119–136.

KITABCHI, A. E. (1967). Ascorbic acid in steroidogenesis. *Nature, Lond.*, **215**, 1385–1386.

LANE-PETTER, W. & DYER, F. J. (1952). *Technical note No. 7: Compressed Diets.* London: Laboratory Animals Bureau.

LOCKWOOD, J. E. & HARTMAN, F. A. (1933). Relation of adrenal cortex to vitamins A, B_1 & C. *Endocrinology*, **17**, 501–521.

LOWENSTEIN, B. E. & ZWEMER, R. L. (1946). The isolation of a new active steroid from the adrenal cortex. *Endocrinology*, **39**, 63–64.

OESTERLING, J. & LONG, C. N. H. (1951). Adrenal cholesteral in the scorbutic guinea-pig. *Science, N.Y.*, **113**, 241–242.

PRUNTY, M. A., CLAYTON, B. E., MCSWINEY, R. R. & MILLS, I. H. (1955). Studies of the interrelationship between the adrenal cortex and ascorbic acid metabolism. *Ciba Fdn Colloq. Endocr.*, **8**, 324–339.

ROE, J. H. & KUETHER, C. A. (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.

STOCKHAM, M. A. (1963). The assay of cortisol and corticosterone in human plasma. *J. Endocr.* **26**, IV–V.

SZENT-GYORGYI, A. J. (1928). Observations of the function of peroxidase systems and the chemistry of the adrenal cortex: Description of a new carbohydrate derivative. *Biochem. J.*, **22**, 1387–1409.

ZENKER, N. & BERNSTEIN, D. E. (1958). The estimation of small amounts of corticosterone in rat plasma. *J. biol. Chem.*, **231**, 695–701.

(*Received August* 19, 1970)

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N. Engl. J. Med. 285: 635-638, 1971,

ASCORBIC ACID AND TOXICITY

To the Editor: No one will argue with the general belief in medicine that the only completely safe chemical is one that is also completely inert. It is doubtful if any such chemical exists. A discussion of toxicity that ignores efficacy is a futile exercise. We seldom hesitate to use extremely toxic chemicals such as many of the modern psychochemicals because the alternative of not using that drug may have even more serious consequences for the patient. Patients will run the risk of minor and even major toxic complications as long as they believe that their physician knows what he is doing and will minimize or protect them against serious side effects. If, therefore, it were generally believed that ascorbic acid in megadoses did reduce the frequency of the common cold, there would be no question that it would be the treatment of choice since it is recognized that ascorbic acid is less toxic even in megadoses than any other currently recommended treatment for cough or cold. The AMA Drug Evaluations, 1971 (first edition) states, "There is no evidence that large doses are harmful."

I have used megadoses of ascorbic acid, 3 to 30 g per day, since 1953 on perhaps over 1000 patients. Most of them were schizophrenic, but patients with other diagnoses also were given ascorbic acid. During this long period, I have not seen one case of kidney-stone formation, of miscarriage, of excessive dehydration or of any other serious toxicity. The most common side effect is diarrhea. If it does occur, one merely reduces the dose by 1 or 2 g. I routinely use ascorbic acid as a laxative on the few psychiatric patients who are constipated, especially elderly people.

The main question remains whether in spite of its remarkable relative safety, ascorbic acid is efficacious. It is my impression, gained over years of practice from the use of megadoses of ascorbic acid, that it does reduce the frequency of colds.

There really is no point in continuing theoretical arguments. Someone will have to get down to the dirty business of running large-scale controlled trials that can resolve the issue both of toxicity and efficacy. We should beware of falling into the trap of accepting the flimsiest evidence if it supports a popular view and rejecting good evidence if it goes counter to it.

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Food Technol. 3, 100-10 (1949)

Further Studies on Frozen Fruit and Vegetable Purees

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This article surveys regulations and standards pertaining to the canned baby-food industry, reports studies of several methods of preparing frozen fruit and vegetable purees, discusses danger of contamination by pathogenic organisms, includes results of a preliminary survey of acceptability to hospital patients of frozen as compared with canned purees.

Introduction

The suggestion of freezing purees for use as baby foods, in certain restricted diets for adults, and in other ways has created considerable interest during the past several years. The investigation in this laboratory on this problem was initiated in about 1940 by M. A. Joslyn and has been continued by the present authors and others. Reports of some phases have already been published by Hohl *(12)* and by Hohl and Smith *(13)*. Many uses for frozen purees other than those mentioned above have been suggested (see Paul, *18*, and Joslyn and Hohl, *14*).

In considering the possibilities of freezing purees several problems are involved. Those upon which data are presented in this paper are the following: What are the chief advantages of frozen over canned purees? What assurance does the consumer have of the microbiological safety of the product? How are the vitamins and other nutrients retained? What would be the best methods of processing, thawing, utilizing and serving?

Standards

A survey was made of all possible sources of regulations and standards used as guides in the manufacture of canned baby foods. The results of this survey may be summarized with the statement that baby foods are manufactured under the general code of the Federal Pure Food, Drug, and Cosmetic Act (Chapter IV, Revision No. 2, February 15, 1946). Private communications with the U. S. Department of Agriculture Production and Marketing Administration, the Food and Drug Administration, and several nationally known canners' associations, and baby food canners, all indicated that the manufacture of purees was governed not only by the "letter" but also by the "spirit of the law." Most processors set themselves rigid and high specifications in the production of baby foods and are alert to possible improvements in their procedures. The administration of the regulations is reasonable. If the processor is using sound, wholesome raw material, under sanitary conditions without adulteration, and is striving for high quality, the requirements of the law are considered to be fulfilled.

So far the only standards established for purees are those for tomato puree, which consist of maximum tolerances for mold filaments and worm fragments, total solids, and adulteration.

The general pediatric literature was searched for data concerning the salt and sugar requirements of infants. The consensus of opinion seems to be that salt may be included in vegetable purees, mainly to increase palatability, since milk supplies all the requirements for salt. Sweetening agents may be added to fruits to increase both their nutritive value and palatability. Infant foods should not, however, be very sweet in order not to encourage an excessive taste for sweets in the child.

Sucrose is usually considered to be suitable, although lextrose, maltose, lactose, or dextrin mixtures are considered advisable in some cases.

Analytical Methods Used in These Studies

Total solids, alcohol-insoluble solids, alcohol-soluble solids, and total acids were determined by the Association of Official Agricultural Chemists *(2)* methods. Vacuum was measured by puncturing the sealed cans with a U. S. Gauge Co. combination vacuum and pressure gauge. pH was measured with a Beckman glass electrode. Flavor and aroma were judged subjectively by several judges. Color was matched with the plates in Maerz and Paul *(16)*, and descriptive names were added as a general guide. Consistency was measured in a Bostwick consistometer, and was recorded as the distance (in centimeters) of flow which occurred within 10 seconds at room temperature (70° ± 5° F.). Sugars were estimated by refractometer and were also sometimes determined chemically by Hassid's ceric sulfate method *(11)*. Sodium chloride was determined by titration with silver nitrate using the procedure described by Vaughn et al. *(28)*. Following essentially the method of Bessey and King *(7)*, ascorbic acid was determined by direct titration of a metaphosphoric acid extract. Thiamin was determined by the fluorometric procedure of Conner and Straub *(9)* and riboflavin by Mackinney and Sugihara's *(15)* modification of the Conner and Straub combined method for these two vitamins. Vitamin A, or more accurately, total carotinoid, was determined by the following procedure outlined by Mackinney of this laboratory. A 5- to 50-gram sample was extracted by grinding with sand and acetone in a mortar until colorless, using successive portions of acetone; 25 cc. of petroleum ether were then added to the acetone extract with a separatory funnel, the contents gently shaken, washed with water (using a scrubbing technique developed by Dr. Mackinney) and the aqueous layer discarded. This was repeated. If chlorophyll was present the extract was at first prepared with alkaline methyl alcohol to saponify the chlorophyll, which was then removed by shaking with water. A suitable aliquot was chromatographed using a firmly packed column of MgO and Supercel (1:1 ratio by weight). The photoelectric determination of carotene was essentially that of A. O. A. C. *(2)*, sections 36.10 and 36.15, using the Evelyn colorimeter.

Comparative Analyses of Canned and Frozen Purees

A selection was made of representative samples of commercially canned and experimentally frozen fruit and vegetable purees prepared in a variety of ways. These were analyzed chemically and compared for general appearance, odor, palatability and consistency.

The results of these analyses are summarized in Tables 1 and 2. A study of these tables shows that for fruits the flavor and aroma of frozen puree are not always superior to those of canned puree. In vegetable purees the difference between canned and frozen aroma and flavor is so great that the relative merits of the two types become a matter of individual taste. In color, the results varied so greatly with brand, and probably with variety of fruit, that no general comparison could be made between canned and frozen samples in this regard. In green vegetables, the color of frozen purees is far more natural than the yellow-green which is typical of all canned green vegetables. The pH and total acidities showed no consistent differences between standard canned samples and our experimentally frozen ones. Sugar (and consequently also total solids) content of our frozen samples was higher than that of the canned, indicating that the 5 + 1 ratio which we used

TABLE 1

Summary of Observations on Flavor, Aroma, Color and Consistency of Experimentally Frozen and Commercially Canned Baby Foods

Sample	No. of Cans	Aroma	Flavor	Color (Maerz and Paul)	Consistency, flow in cm. in 10 secs.
Canned Apple Sauce	4	Good	Good	Variable	4.1
Frozen Apple Sauce	4	Fair	Fair	Variable	2.2
Canned Apple and Prune	1	Fair	Good	8 L 10 (Dark brown)	5.5
Frozen Apple and Prune					
Canned Apricot and Peach	1	Good	Good	10 L 10 (Orange peel)	7.3
Frozen Apricot and Peach	1	Fair	Good	9 L 9 (Lighter Orange	11.5
Frozen Apricot and Apple	1	Fair	Good	10 L 8 (Florida Gold)	5.0
Frozen Nectarine and Plum	1	Good	Good	11 J 7 (Greenish Apricot)	7.8
Canned Peach	4	Good	Good	Variable	9.4
Frozen Peach	2	Fair	Fair	Variable	13.5
Canned Peach, Pear, Apricot	1	Stale	Good	10 L 8 (Florida Gold)	7.5
Vegetable Soup	4	Good	Fair	Variable	1.5
Canned Beets	2	None	Fair	Good	2.5
Frozen Beets	1	Good	Good	Good	0
Canned Carrots	1	Good	Good	9 L 10 (Marigold)	4.5
Frozen Carrots	1	Good	Good	2 A 12	0
Canned Beans (green)	1	Good	Flat	21 L 1 (Yellow-green)	4.5
Canned Peas	4	Good	Fair	Varied (Yellow-green)	0.6
Frozen Peas	1	Heavy	Starchy	18 K 7 (Bright spring green)	0
Canned Spinach	4	Good	Good	15 L 5 (Olive green)	2.0
Frozen Spinach	2	Strong	Immature	Excellent	very runny

TABLE 2

Summary of Chemical Analyses of Experimentally Frozen and Commercially Canned Baby Foods

Sample	No. of Samples	Total Solids, percent	Alcohol Soluble Solids, percent	Alcohol Insoluble Solids, percent	Total[1] Acid	Ascorbic Acid, Mg./100g.	pH	Total Soluble Solids by Refractometer, percent	Salt (NaCl), percent
Canned Apple Sauce	4	15.4	....	2.8	0.38	0	3.5	14.2	....
Frozen Apple Sauce	4	25.3	20.1	2.6	0.38	0	3.4	22.8	....
Canned Apple and Prune	1	26.6	....	4.3	0.69	0	3.7	....	....
Canned Apricot and Peach	1	8.4	....	3.3	0.80	2	4.0	....	....
Frozen Apricot and Peach	1	24.3	22.8	2.1	0.61	33.1	4.2	23.7	....
Frozen Apricot and Apple	1	25.0	21.6	3.5	0.63	0	4.0	23.7	....
Frozen Nectarine and Plum	1	30.2	25.4	2.5	0.76	16.0	3.9	29.2	....
Canned Peach	4	15.7	....	2.0	0.48	0	3.7	14.8	....
Frozen Peach	2	21.3	19.6	1.7	0.45	15.7	4.1	21.0	....
Canned Peach, Pear, Apricot	1	19.1	....	3.2	0.61	2	3.9	....	....
Vegetable Soup	4	12.3	....	7.7	0.18	1.8	5.1 Ave.	....	....
Canned Beets	2	16.5	....	....	0.42	0	4.8	....	0.72
Frozen Beets	1	16.4	....	4.2	0.18	trace	5.9	....	0
Canned Carrots	1	8.9	....	3.1	0.13	4.5	4.9	....	0
Frozen Carrots	1	16.1	....	4.5	0.24	0	6.0	....	0
Canned Beans (green)	1	8.5	....	4.6	0.29	1	5.2	....	0.72
Canned Peas	4	15.2	....	11.6	0.19	7.6	5.8	....	0.48
Frozen Peas	1	21.6	....	15.7	0.32	15.8	6.9	....	....
Canned Spinach	4	8.8	....	5.0	0.15	19.9	5.2	....	0.68
Frozen Spinach	2	8.3	....	5.1	0.20	13.2	6.5	....	0

[1] Total acid is expressed as grams of citric (for peach, nectarine and plum, vegetable soup, beets, and peas) or as grams malic acid (for apples, carrots, spinach, and green beans) per 100 grams of sample.

for many of these samples was higher than that usually used in commercial practice. Ascorbic acid was notably higher in most frozen than in canned fruit and vegetable purees. The most striking difference between the frozen and canned purees was in their consistency. All the frozen samples (except apple sauce, carrots and beets) were much more fluid than similar canned samples even though the total solids contents of the former were usually higher. In such vegetables as spinach and asparagus, the purees tended to separate into a liquid and a solid phase after freezing and thawing. This phenomenon of change in consistency as a result of freezing and thawing of purees is being studied further. It is one of the most troublesome problems still to be solved and is probably related to the problem of texture breakdown in vegetables and fruits frozen in the usual manner. Salt content of commercially canned vegetable purees varied from 0 to 0.92 percent and obviously the quantity added was determined by the individual preference of the manufacturer.

Studies on Methods of Preparation of Purees for Freezing

A selection of well known varieties of peaches and a few numbered seedling varieties, all collected from the University's experimental orchards at Winters, California, at optimum eating maturity, were made into purees by the following procedure: The whole fruit was steamed for one minute, skins were slipped off and bruises trimmed away, fruits were halved and pitted, steamed for four minutes, air-cooled quickly and then pureed in an American Utensil Co. Sep-ro-sieve, using a 0.03 inch diameter screen. The pulp was run through the machine two or three times in order to extract a maximum of solids. Sugar was added at the rate of ten percent by weight and the purees were filled into glass jars, frozen and stored at 0° F. The above procedure made purees of excellent fresh flavor and keeping qualities. This amount of heating did not impart a cooked flavor to the resulting purees, but it did prevent enzymatic oxidative changes during storage. Apricots, nectarines, plums, and fresh prunes prepared in this same general manner, except that the skins were not removed before passing the fruit through the puree machine, also all made excellent products. If blanching was omitted the fruits tended to become oxidized in flavor, and brown on the surfaces.

Apple and pear purees were best prepared by paring, blanching or precooking (for apple sauce) before being passed through the Sep-ro-sieve. Dried prunes were also used for the preparation of purees. The best procedure for this fruit was to soak, and cook the fruit in the usual manner (without added sugar), pit and puree (including the cooking water) and freeze. If prunes of good initial quality are used, such a product is excellent, but it has no marked advantage over a similar canned product. Fresh prunes give a puree of better flavor and higher vitamin content than the dried prunes. However, if commercial production of frozen purees for baby foods ever becomes a reality, the manufacturers have all expressed the belief that a complete line of frozen baby foods would be highly desirable. Successful purees have been prepared in the laboratory from several varieties of peaches, nectarines, apricots, pears, and apples, as well as from figs, guavas, persimmons, and other fruits. For baby food purposes, fruit blends may offer certain advantages such as cutting down the natural tartness or improving the consistency of certain fruits. Prune,

apple or pear purees often have a thick consistency while nectarines, peaches, apricots, etc., are too thin. Thus pear and Fellenberg (Italian) prune, plum and nectarine, apricot and apple, or plum and a bland apple, or figs and more acid fruits made excellent blends.

A series of experiments were made on the effect of various antioxidants, individually or in combination, upon the quality and particularly upon the ascorbic

TABLE 3

Effect of Sulfur, Citric Acid and Blanching Upon Retention of Added Ascorbic Acid in Apricot Purees

	Treatment of Original Sample			Percent of Original Ascorbic Acid Left After			pH	Total Acid (as Grams Malic per 100 g.)	Color, Maerz and Paul
	Initial Ascorbic Acid Added (mg./100 g.)	Added SO_2 (p.p.m.)	Added Citric Acid (percent)	1 mo.	6 mos.	1 yr.			After 1 yr.'s storage
Raw	0	0	0	....	....	....	4.0	0.79	2 A 12
Raw	25	0	0	72	48	28	4.0	0.80	2 A 12
Raw	50	0	0	72	70	70	4.0	0.80	2 A 12
Raw	75	0	0	80	79	....	4.0	0.80	2 A 12
Raw	100	0	0	69	71	....	4.0	0.80	2 A 12
Raw	25	25	0	88	88	86	4.0	0.81	9 L 10
Raw	100	25	0	85	85	77	4.0	0.85	9 L 10
Raw	0	0	0.5	....	....	....	3.6	1.13	2 A 12
Raw	25	0	0.5	60	68	48	3.6	1.12	2 A 12
Raw	50	0	0.5	72	72	64	3.6	1.17	2 A 12
Raw	75	0	0.5	56	56	73	3.6	1.19	9 L 11
Raw	100	0	0.5	87	87	74	3.6	1.20	9 L 11
Raw	0	100	0	....	....	....	3.6	1.15	9 L 10
Blanched	25	0	0	56	56	56	3.9	0.72	9 L 11
Blanched	50	0	0	80	80	74	3.9	0.74	9 L 11
Blanched	75	0	0	76	74	77	3.9	0.74	9 L 11
Blanched	100	0	0	83	80	82	3.9	0.74	9 L 11
Blanched	50	100	0	80	84	100	3.9	0.78	9 L 10

TABLE 4

Effect of Sulfur Dioxide, Citric Acid, and Blanching Upon Retention of Added Ascorbic Acid in Nectarine Purees

	Treatment of Original Sample			Percent of Original Amount Added		pH	Total Acid (as Grams Citric Acid per 100 g.)	Color, Maerz and Paul	
	Ascorbic Acid Added (mg./100 g.)	SO_2 Added (p.p.m.)	Citric Acid Added (percent)	Ascorbic Acid, 4 mos.	Ascorbic Acid, 1 yr.			3 mos. after freezing	14 mos. after freezing
Raw—Gower and New Boy	0	0	0	....	....	4.0	0.62	9 E 7	11 D 7
Raw—Gower and New Boy	50	0	0	100 +	88	3.98	0.67	9 E 7	11 D 7
Raw—Gower and New Boy	100	0	0	100 +	85	3.98	0.68	9 E 7	11 D 7
Raw—Gower and New Boy	50	25	0	100 +	100	3.88	0.68	11 B 8	11 G 7
Raw—Gower and New Boy	100	25	0	100 +	93	3.79	0.69	11 A 8	11 G 7
Raw—Stanwick	0	0	0	....	....	4.45	0.42	10 I 4	10 H 3
Blanched—Stanwick	0	0	0	....	....	4.60	0.39	10 H 4	10 E 2
Blanched—Stanwick	200	0	0	95	58	4.50	0.39	10 H 4	10 H 3
Raw—Stanwick	50	0	0	100 +	100	4.40	0.42	10 I 3	10 I 2
Raw—Stanwick	100	0	0	100 +	91	4.40	0.43	10 I 3	10 H 2
Raw—Stanwick	200	0	0	89	93	4.10	0.50	10 F 3	10 H 2
Raw—Stanwick	50	25	0	100 +	100	4.29	0.44	11 F 2	10 H 2
Raw—Stanwick	100	25	0	100 +	99	4.21	0.45	11 F 2	10 H 2
Raw—Stanwick	200	25	0	86	45	4.15	0.49	11 F 2	10 H 2
Raw—Stanwick	50	0	0.5	84	100	3.65	0.90	11 G 3	10 H 2
Raw—Stanwick	100	0	0.5	66	97	3.67	0.90	11 G 3	10 H 3
Raw—Stanwick	200	0	0.5	77	100 +	3.62	0.98	11 G 3	10 H 2
Raw—Yellow Seedling	0	0	0	....	....	4.09	0.40	10 L 9	9 L 8
Raw—Yellow Seedling	50	0	0	72	78	4.08	0.46	10 L 9	9 L 8
Raw—Yellow Seedling	100	0	0	81	100 +	4.02	0.46	10 L 9	10 L 7
Raw—Yellow Seedling	200	0	0	71	88	3.96	0.47	10 L 9	9 L 8
Raw—Yellow Seedling	50	25	0	100 +	92	4.08	0.45	10 L 8	9 L 7
Raw—Yellow Seedling	100	25	0	84	79	4.05	0.47	10 L 8	9 L 7
Raw—Yellow Seedling	200	25	0	100 +	91	4.00	0.49	10 L 8	9 L 7
Raw—Yellow Seedling	50	0	0.5	100 +	84	3.75	0.94	9 L 8	10 L 8
Raw—Yellow Seedling	100	0	0.5	98	88	3.70	0.94	10 L 9	10 L 8

TABLE 5

Effect of Sulfur Dioxide, Citric Acid, and Blanching Upon Retention of Added Ascorbic Acid in Peach Purees

	Treatment of Original Sample			Percent of Original Amount Added		pH	Total Acid (as Grams Citric/100 g.)	Color, Maerz and Paul	
	Ascorbic Acid Added (mg./100 g.)	SO_2 Added (p.p.m.)	Citric Acid Added (percent)	Ascorbic Acid, 4 mos.	Ascorbic Acid, 1 yr.			3 mos. after freezing	14 mos. after freezing
Raw	0	0	0	....	....	4.0	0.49	13 B 11	13 B 11
Raw	40	0	0	80	57	4.0	0.49	11 L 8	10 L 8
Raw	80	0	0	100 +	76	4.0	0.47	11 K 9	9 L 8
Raw	120	0	0	100 +	83	3.95	0.48	11 L 9	9 L 8
Raw	40	0	.96	75	70	3.35	1.39	11 L 9	10 L 8
Raw	40	25	0	97	80	3.70	0.45	9 L 8	10 L 8
Raw	80	25	0	100 +	90	3.67	0.48	9 L 8	10 L 8
Blanched	0	0	0	....	....	4.05	0.46	11 L 7	10 L 7
Blanched	40	0	.96	70	100	3.40	1.33	11 L 7	10 L 8
Blanched	40	0	0	100	100	4.00	0.50	11 L 8	10 L 8
Raw	100	0	0	91	64	3.95	0.53	10 K 9	10 L 8
Blanched	100	0	0	100	84	3.95	0.53	11 K 9	10 K 7

acid (vitamin C) retention of frozen apricot, nectarine and peach purees. Tables 3, 4, 5, summarize the results of these experiments. A study of the general trends in these three tables indicates that 50 p.p.m. or higher concentrations of ascorbic acid in purees are more completely retained during storage in the raw and blanched series. In general, the blanched purees retained the added ascorbic acid better than the unblanched. Both SO_2 and citric appeared to exert slight protective effect upon ascorbic-acid retention in these purees during storage. The results were rather irregular, however, and more data would be required to prove the point definitely. Some of the fruit purees showed the protective effect while others were almost completely lacking in such trends. In Tables 3, 4, and 5, the total acidities, pH values and colors (taken from Maerz and Paul) are also recorded. For apricot purees the colors were tabulated only for the year-old samples, since those recorded earlier showed no differences, all of them being 2A12. The nectarine and peach samples showed greater changes during storage. The samples having the greatest additions of ascorbic acid and those with supplements of SO_2 showed the least color change during storage.

During the 1946 season varieties shown in Table 6 were tested for their suitability for freezing both as dessert fruit and as purees. All of these purees, except the last one listed, were prepared by pitting and trimming the fruit, steaming the halves for four minutes, slipping off the skins and passing the peeled fruit through the Sep-ro-sieve two or three times. Sugar was added at the rate of ten percent by weight. It will be noted from the data in Table 6 that the varieties differed considerably in color, pH, acidity, ascorbic acid content, flavor, aroma and consistency. Those whose consistometer readings were above 11 or 12 centimeters of flow in 10 seconds were too thin for baby foods. All of these purees were thinner than any of the commercially canned samples tested. Therefore, in the following season a number of varieties of apricots, peaches and nectarines were prepared in several different ways in an attempt to achieve better consistency in the frozen and thawed puree. A portion of each variety was prepared just as described above. Another portion was washed, trimmed, pitted, halved, steamed for four minutes, dehydrated to half its original weight and then made into a puree. Also an apricot puree was made by still another method, namely that of dehydrating to half weight after preparing the puree. This procedure was later abandoned because of greater difficulty in handling. The results of these experiments and the chemical analyses of these samples are summarized in Tables 7 and 8. It may be noted that the consistencies of the purees prepared just as they had been prepared in the previous season were all higher than those prepared from the corresponding varieties in the previous year. This may have been caused in part by seasonal differences—the 1946-1947 winter having been an extraordinarily dry one. It may also be noted that the sweetened purees always were more fluid in consistency than the corresponding unsweetened ones.

A partially concentrated frozen puree may be desirable for the saving in storage space which it would entail. Water could then be added at the time of serving. However, experimentation showed that addition of water in equivalent volume to that removed during dehydration resulted in a much more fluid puree than that which had been made by making puree from the original undehydrated fruit. The following example of unsweetened Rio Oso Gem peach purees illustrates this point: That prepared directly from the blanched fresh peaches flowed 8 centimeters in ten seconds at room temperature. That prepared from the half-dehydrated fruit flowed 5.2 centimeters while the same puree diluted back by addition of an equal weight of water flowed 14.5 centimeters and that diluted with one part water to two of the concentrated puree flowed 9.2 centimeters in ten seconds.

In the preparation of vegetable purees several different procedures were tested. It soon became apparent that the American Utensil Co. juice extractor which served well for fruits was not suitable for the preparation of purees from such vegetables as asparagus, spinch, broccoli, snap beans, nor even carrots or beets. There was not enough pressure or mechanical action to separate the soft tissues from the more or less stringy vascular elements of these leaf, stem, or root tissues. A Rietz disintegrator was successfully used in preparing purees from these vegetables, but this machine permits considerable aeration during preparation of puree. The Langsencamp laboratory pulper was used for some of the experiments and it yielded an excellent product.

TABLE 6

Qualities of Several Varieties of Peach Purees

Variety	Aroma	Flavor	Color (Maerz and Paul)	Consistency (cms. in 10 secs.)	pH	Total Acidity (gms. citric per 100 g.)	Ascorbic Acid (mg./100 g.)
Early Crawford	good	good	10 L 8	11.3	3.9	0.49	3.5
Late Crawford	fair	tart	10 K 8	16.5	3.6	0.61	2
Elberta	fair	watery	11 L 8	24 +	3.8	0.37	0
Early Elberta	heavy	over-ripe	10 L 9	18.0	4.0	0.50	3
Fay Elberta	good	watery	10 K 8	24 +	3.8	0.39	0
July Elberta	fair	too sweet	10 L 8	16.5	3.8	0.44	4
J. H. Hale	fair	good	11 C 10	16.0	3.8	0.43	0
White Hale	good	slightly flat	11 C 4	14.5	3.7	0.54	1
Lovell	fair	good	11 L 7	11.5	3.7	0.56	3
Muir	fair	low in acidity	10 L 7	14.0	4.4	0.33	3
Rio Oso Gem	good	good	11 E 11	17.5	3.8	0.47	0
St. John	good	sweetish	10 L 8	9.5	4.0	0.52	5
Seedling W 24-29	good	watery	10 L 10	15.5	3.8	0.49	3
Seedling W 24-35B	fair	tart	10 L 7	15.5	3.6	0.69	0
Sunbeam	good	watery	10 L 7	18.5	3.9	0.52	0
Raw Sunbeam (1945)	fair	watery	9 L 5	24 +	4.0	0.47	0

TABLE 7

Effect of Partial Dehydration of Fruits on Quality of Fruit Purees

Sample	Treatment	Aroma	Flavor	Color	Consistency (cm. of flow in 10 sec.)
Apricot Royal	fresh	fair	sharp	12 L 10	6.8
	blanched, dehydrated and pureed	very good	good	12 I 12	4.0
	blanched, pureed and dehydrated	good	fair	12 I 12	3.8
	blanched and pureed	good	tart	10 L 11	8
	blanched, dehydrated, pureed and 10% sugar	good	good	10 L 12	6
	blanched, pureed and 10% sugar	good	fair	10 L 12	8.4
Peaches Elberta	fresh	fair	sharp	11 K 8	10.0
	blanched, dehydrated and pureed	good	fair	11 L 11	6.3
	blanched, pureed and 10% sugar	good	tart	11 K 10	11.3
J. H. Hale	fresh	fair	flat	11 K 9	7.5
	blanched, dehydrated and pureed	good	fair	11 L 11	6.6
	blanched, pureed and 10% sugar	good	fair	11 K 10	9.0
Fay Elberta	fresh	fair	ripe	11 J 9	10.3
	blanched, dehydrated, pureed and 5% sugar	weak	fair	10 K 7	9.5
	blanched, pureed and 10% sugar	weak	sweet	10 K 6	17.0
	blanched, pureed and dehydrated	weak	sweet	10 K 7	9.5
Rio Oso Gem	fresh	fair	good	10 J 8	8.9
	blanched, pureed and 10% sugar	weak	good	10 K 9	10.5
	blanched and pureed	weak	sharp	10 K 8	10.5
	blanched, pureed, dehydrated and 10% sugar	weak	slightly sweet	11 L 9	6.5
	blanched, dehydrated and pureed	weak	slightly sharp	11 L 8	4.5
	blanched, dehydrated, pureed and packed with 5% sugar dry on top	weak	good	11 L 9	5.0
Nectarines Philip Seedling 24-6c	fresh	fair	good	11 J 9	6.5
	pureed and 10% sugar	fair	fair	11 J 10	5.8
	blanched, pureed and 10% sugar	good	fair	11 J 9	6.2
Humboldt	fresh	heavy	oxidized	11 L 9	3.0
	blanched, dehydrated and pureed	fair	oxidized	11 L 10	2.6
	blanched, pureed and 10% sugar	fair	oxidized	10 L 8	7.6
Giallo di Padova	blanched, pureed and 10% sugar	weak	sharp	10 L 5	8.2
Stanwick	blanched, dehydrated, pureed and 5% sugar	good	sweet	10 I 2	2.0
	blanched, dehydrated, pureed and packed with 5% sugar dry on top	good	sweet	10 H 2	2.2
	blanched, pureed and 5% sugar	weak	good	10 I 2	7.4

TABLE 8

Effect of Partial Dehydration of Fruits on Composition of Fruit Purees

Sample	Treatment	pH	Total Solids percent	Alcohol Soluble Solids percent	Water and Alcohol Insoluble percent	Total Acid*	Ascorbic Acid mg./100 g.	Carotene mg./100 g.	Sucrose by Refractometer percent
Apricot Royal	fresh	3.6	15.6	13.5	....	1.45	0	2000	14
	blanched, dehydrated and pureed	3.8	21.35	23.2	2.16	2.14	7.1	2143	19.4
	blanched, pureed and dehydrated	3.9	21.70	20.7	1.09	2.05	5.2	2287	21.4
	blanched and pureed	3.9	16.21	11.8	1.01	1.74	8.9	2036	14.6
	blanched, dehydrated, pureed and 10% sugar	3.6	25.31	22.9	1.03	1.57	7.5	2000	24.8
	blanched, pureed and 10% sugar	3.6	27.11	....	....	1.37	10.3	1700	22.6
Peaches Elberta	fresh	3.8	13.2	10.8	1.01	.46	0	255	12.3
	blanched, dehydrated and pureed	3.9	25.4	11.1	1.23	.61	1.7	290	24.5
	blanched, pureed and 10% sugar	3.9	21.9	10.0	1.12	.45	0	249	21.2
J. H. Hale	fresh	3.5	13.8	12.1	1.05	.73	0	150	12.7
	blanched, dehydrated and pureed	3.7	26.9	....	5.08	.91	0	173	25.3
	blanched, pureed and 10% sugar	3.7	21.9	11.9	1.10	.65	0	143	20.9
Fay Elberta	fresh	3.8	11.7	9.4	1.00	.55	4.9	1060	10.7
	blanched, dehydrated, pureed and 5% sugar	3.8	22.9	....	....	.71	....	....	22.2
	blanched, pureed and 10% sugar	3.8	20.3	....	....	.47	....	....	22.4
	blanched, pureed and dehydrated	3.8	31.0	....	....	.68	....	....	29.9
Rio Oso Gem	fresh	3.6	13.8	11.6	1.23	.78	6.0	1345	13.0
	blanched, pureed and 10% sugar	3.7	14.5	12.0	1.34	.61	5.5	1365	20.8
	blanched and pureed	3.7	....	....	....	.71	....	....	13.3
	blanched, pureed, dehydrated and 10% sugar	3.7	....	....	....	.87	....	....	25.9
	blanched, dehydrated and pureed	3.7	....	....	....	1.07	....	....	23.2
	blanched, dehydrated, pureed and packed with 5% sugar dry on top	3.7	....	....	....	.98	....	....	32.7
Nectarines Philp Seedling 24-6c	fresh	3.6	18.5	16.0	1.5	.88	15.2	425	17.6
	pureed and 10% sugar	3.6	18.1	15.5	1.01	.82	17.1	550	27.0
	blanched, pureed and 10% sugar	3.6	17.9	15.3	1.2	.86	14.3	500	27.2
Humboldt	fresh	4.1	22.3	18.1	2.2	.66	23.8	1560	21.2
	blanched, dehydrated and pureed	4.0	35.3	16.9	1.9	.79	16.4	1190	34.0
	blanched, pureed and 10% sugar	4.2	28.8	....	....	.56	....	....	27.5
Giallo di Padova	blanched, pureed and 10% sugar	3.7	27.8	....	....	.98	....	....	26.8
Stanwick	blanched, dehydrated, pureed and 5% sugar	4.2	37.1	....	....	.83	....	....	35.1
	blanched, dehydrated, pureed and packed with 5% sugar dry on top	4.2	37.0	....	....	.89	....	....	35.6
	blanched, pureed and 5% sugar	4.3	29.2	....	....	.54	....	....	28.0

* Expressed as grams malic per 100 grams for apricots and as grams citric for peaches and nectarines.

When a suitable puree machine had been chosen for a particular vegetable, comparisons were made among the following procedures for preparation for freezing:

(a) Prepare, blanch and cool the vegetable as for regular freezing, make puree, package and freeze.

(b) Prepare and precook the vegetable as for serving fresh, cool, make puree, package and freeze.

(c) Prepare, blanch and cool (as in *a*), make puree, heat to finish cooking and fill into scrupulously clean (sterile, if possible) containers, cool quickly and freeze.

(d) Partially dehydrate vegetables (to 50 percent moisture), make puree, package and freeze.

In general, the best quality, from the adult taster's point of view, was achieved by using either method *(a)* or *(c)*. Puree prepared by method *(a)* required some cooking after thawing and this may be a bit troublesome at times since purees "set" and scorch easily unless a double boiler is used and if a double boiler is used, it is difficult to accomplish the desired cooking. None of the samples prepared by method *(b)* were as acceptable as those prepared by the other methods. Method *(c)* was introduced in order to minimize the microbial contamination of the product. Most of the samples of green vegetables prepared in that way were better than those prepared by method *(b)*, because the green color and fresh flavor were better retained in that way. If frozen purees were prepared commercially in such a manner and were filled into sterile containers, they could be almost as safe, microbiologically, as the canned product. The partial dehydration method *(d)* was satisfactory for carrot puree, but for green vegetables it had a tendency to darken the color. Table 9 shows the results of such comparative processing methods with carrots.

A few vegetable purees were used to make a comparison of the following ways of preparing baby foods:

(a) Wash, prepare as for freezing, blanch, cool, make puree, package, freeze and store at 0° F.

(b) Prepare puree as for (a), package in sanitary cans, exhaust, seal heat process (8 oz. can, 30 minutes at 240° F.), cool and store at room temperature.

(c) Prepare puree as for (a), heat to finish cooking and fill into *clean* containers. Store at 0° F.

(d) Cook the fresh vegetable and press through a sieve as the housewife would do if she were preparing her own puree.

TABLE 9

Effect of Several Methods of Preparation on Quality and Composition of Carrot Purees

Method of Preparation	Aroma	Flavor	Color Maerz and Paul	Consistency flow in cms. in 10 secs.	Total Solids Percent	Alcohol Insoluble Solids Percent	Total Acid (as grams malic/ 100 g.	pH	Ascorbic Acid mg./100 g.
Blanched, pureed, frozen	Hay-like	Hay-like	11 L 12	0	10.75	3.10	.12	6.22	3.00
Precooked whole, pureed, frozen	Hay-like	Hay-like	11 L 10	0	9.5	....	.12	6.40	2.75
Precooked diced, pureed, frozen	Good	Good	10 L 10	0	12.6	3.76	.12	6.28	....
Blanch, puree, cook, and freeze	Somewhat hay-like	Hay-like	11 L 11	0	12.41	4.00	.13	6.10	2.60
Blanch, dehydrate, puree, and freeze	Hay-like	Hay-like	11 L 11	0	12.40	2.84	.13	6.0	4.29

TABLE 10

Effect of Method of Preparation Upon Quality of Vegetable Purees

Vegetable	Method of Preparation	Aroma	Flavor	Color Maerz and Paul	Consistency flow in cms. in 10 secs.
Snap beans	Raw fresh vegetable	strong	fair	14 L 2	5.5
	"Home cooked" and pureed	good	good	14 L 3	13
	Freshly prepared puree	fresh	half cooked	12 L 2	13
	Frozen puree	good	good	14 L 1	11.9
	Canned puree	canned	canned	13 L 7	11.3
Asparagus	Raw fresh vegetable	fair	slightly astringent	22 K 7	0.5
	Freshly prepared puree	good	good, flat	22 L 4	3.5
	Blanched, pureed, frozen	good	good, flat	22 L 5	5.8
	Canned puree	good	good	21 L 1	2.3
	Blanched, pureed, cooked, frozen	good	poor	22 L 5	5.5
	"Home cooked" and pureed	good	insipid	22 L 3	very fluid
Asparagus (after 6 mos. storage)	Blanched, pureed, frozen	strong	astringent	21 L 5	H_2O separated
	Canned puree	stale	good	12 L 3	1.5
	Blanched, pureed, cooked, frozen	strong	poor	21 L 3	H_2O separated

TABLE 11

Effect of Method of Preparation on Composition of Vegetable Purees

Vegetable	Method of Preparation	Total Solids percent	Alcohol Insoluble percent	pH	Total Acid gm. malic per 100 g.	Ascorbic Acid mg./100 g.	Carotene δ/100 g.	Riboflavin δ/g.	Thiamin δ/g.
Snap beans	Raw fresh vegetable	10.1	1.75	6.0	.38	9.6	120	0.66	0.61
	"Home cooked" and pureed	9.6	1.34	6.05	.31	4.3	120	0.94	0.39
	Freshly prepared puree	9.56	1.45	5.55	.43	9.0	160	1.22	0.30
	Frozen puree	9.39	1.52	5.45	.38	6.0	160	1.11	0.30
	Canned puree	9.50	1.71	5.32	.34	7.7	130	....	0.30
Asparagus	Raw fresh vegetable	7.51	....	6.3	....	25.5	334	1.48	0.52
	Freshly prepared puree	6.76	....	6.1	....	22.4	....	3.32	1.00
	Blanched, pureed, frozen	6.64	....	6.1	....	15.1	....	2.22	0.60
	Canned puree	6.89	....	5.4	....	15.9	....	1.85	0.70
	Blanched, pureed, cooked, frozen	6.98	....	6.1	....	12.6	....	2.58	0.48
	"Home cooked" and pureed	....	....	6.4	....	18.8	....	....	....
Asparagus (after 6 mos. storage)	Blanched, pureed, frozen	6.83	....	6.7	.15	11.3	280	1.17	0.54
	Canned puree	7.01	....	5.7	.23	16.1	380	1.23	0.35
	Blanched, pureed, cooked, frozen	7.11	....	6.6	.19	11.7	270	1.12	0.44

Analyses were made on these as well as on the original fresh material, and on the original puree prepared for *(a)*, *(b)* and *(c)* before it was frozen or further processed. Tables 10 and 11 summarize the results of these experiments on snap bean and asparagus purees. It will be noted that the riboflavin contents of both vegetables, thiamin of asparagus and the carotene of snap beans, were higher in the freshly prepared puree than in the original raw fresh vegetables. Possibly this may be explained by the fact that the vitamin-rich portions of the vegetables were the tender parts which remained in the puree while the fibrous conducting tissues which remained behind had little original vitamin content. Another observation of interest is that the processed purees (both frozen and canned) had a generally higher vitamin content than those prepared from similar raw material by ordinary home methods. (The general method followed to simulate home conditions was boiling the prepared vegetable in enough water to cover it in an open aluminum kettle just long enough to make it tender, pressing it through a stainless steel sieve, and adding back enough of the cooking water to give the desired consistency.)

When the frozen and canned purees of these experiments are compared, there is no striking apparent advantage for either method of processing with respect to vitamin content. However, the general quality of the precooked frozen puree *(c)* samples was markedly below that of the blanched frozen samples *(a)*. Bacterial counts were also made on these same samples by the methods and with media to be described in a subsequent section of this report. The original unprocessed puree was plated out and then the canned and frozen (both precooked and only blanched) were plated out after storage for five months. No significant change in total numbers of organisms occurred except that the heat-processed sample contained very few viable organisms. The precooked frozen sample gave an average count of 200 organisms per gram and the blanched pureed frozen sample gave an average of approximately 400 per gram, whereas the original sample had contained 150 per gram.

Bacteriological Problems and Keeping Qualities of Frozen Purees

In any discussions of plans for the commercial production of frozen purees for use in infant or invalid feeding, the question of survival of microorganisms in these products arises. As Tressler *(26)* pointed out, bacteria, as well as enzymes and vitamins, are a reliable index of quality in frozen foods. Very little data is available which could be applied specifically to this problem in purees. The early work reported by Magoon *(17)*, Berry *(3, 4, 5, 6)*, Wallace et al. *(29, 30)* and Tanner and Wallace *(25)* was concentrated mainly upon solving the problem of survival of microorganisms at low temperatures. All agreed that low temperatures killed many but not all of the molds, yeasts, and bacteria present in the food before it was frozen. Early studies (Haines, *10;* Prescott and Tanner, *21;* Straka and James, *23, 24;* and Prescott and Geer, *20*) also placed considerable stress upon the problem of survival of *Clostridium botulinum* in frozen foods, and if it survived, whether it might develop and produce toxin after thawing of the foods. All the investigators concluded that properly handled frozen vegetables posed no problem from this standpoint, but they all left doubt concerning what might happen if improper or careless handling should occur.

The problem has recently been reinvestigated thoroughly in a cooperative project of the Hooper Foundation of this University and the Western Regional Research Laboratory, U. S. Department of Agriculture. The conclusion has been drawn that there is no danger of *botulinus* toxin being present in frozen and thawed vegetables under any conditions. Acid producing bacteria generally develop first in frozen and thawed vegetables and these organisms have an inhibitory effect upon the growth and development of *Clostridium botulinum*.

Proctor and Phillips *(22)* made an extensive survey of the microorganism found by plating out samples of commercial frozen precooked foods. In meat and poultry products the predominant types were *Micrococcus* spp., in fish products *Flavobacter* spp., and in soups *Bacillus spp.* Many of the various products contained appreciable numbers of *Sarcina* and *Achromobacter* spp., *Staphylococcus* spp. were found in meat products, stews, poultry products and creamed products although in lower proportions than the other genera. Cooked foods experimentally inoculated with food poisoning strains of *Staphylococcus aureus*, alpha type *Streptococcus* and *Salmonella enteritidis* which were frozen and stored at 0° F., indicated that in some foods at least ten percent of the inoculated organisms survived such storage for six months. These types of bacterial pathogens in frozen precooked foods can increase in numbers after storage at defrosting temperatures of 30°-37° C. for six to eight hours. [See also Phillips and Proctor *(19)*].

While the results of these studies cannot be applied directly to frozen vegetable and other baby food purees, they are at least indicative of the possibilities and serve to emphasize the importance of using all possible means to control the entrance or development of such microorganisms.

Studies were made of the keeping quality of frozen purees after thawing and storing for a time both at room temperature (60°-75° F.) and in the household refrigerator (40°-42° F.) Vitamin C retention and bacterial plate counts on four kinds of agar were used as indices of keeping quality. The four agar media were eosine methylene blue (EMB), nutrient (N) made according to the standard formulae (American Public Health Association) *(1)*, yeast glucose (YG) and Wort (W) made as described by Vaughn *(27)*.

The vitamin assays and platings were made immediately after thawing, after storage at room temperature in the original container for one and two days and after storage in the refrigerator for one, two, three, and four days. No special precautions other than the usual care and cleanliness exercised in laboratory preparation of foods and cleaning of containers were taken either during preparation or storage of these samples. The platings and vitamin assays were discontinued after what was considered to be a reasonable maximum storage time after thawing, under the particular conditions of storage. These particular media were chosen for

platings because the combination would be likely to favor the growth of all types of organisms which might be found on these type of foods. Eosine methylene blue agar encourages the growth of lactose—utilizing Gram-negative organisms. In yeast glucose agar, lactic acid bacteria or yeasts (whichever is predominant in the population) are encouraged. Nutrient agar allows the development of aerobic spore formers, Gram-negative organisms, molds and yeasts to a slighter extent. Lactic acid bacteria are suppressed. Wort agar is particularly suitable for the growth of yeasts and molds. Tables 12 and 13 summarize the results of the platings of the samples held at these two temperatures after thawing. The organisms which were counted in these platings represent mainly or entirely the saprophytic organisms commonly present in vegetables and fruits. More data on these organisms as well as on the survival of the more dangerous pathogens or toxin producers would be desirable.

Table 14 gives a typical series of examples showing vitamin C retention of purees stored in a refrigerator for several days after thawing. It may be concluded from these results that this vitamin remains quite stable in these products after thawing except in some fruits, especially if they have been prepared without blanching.

TABLE 12

Summarized Results of Dilution Plate Counting of Purees Stored at Room Temperature (65-75° F.)

Kind of Puree	Storage time after thawing (days)	Numbers of organisms per gram			
		Medium			
		YG	N	W	EMB
Broccoli	0	2,200	2,200		675
Broccoli	1	11×10^6	16×10^6		12×10^6
Broccoli	2	200×10^6	155×10^6		167×10^6
Broccoli + Asparagus	0	1,900	1,900		440
Broccoli + Asparagus	1	49×10^6	16×10^6		23×10^6
Broccoli + Asparagus	2	540×10^6	275×10^6		130×10^6
Raw guava	0	20	18	7	
Raw guava	1	130	290	76	
Raw guava	2	700	405	55	
Steamed guava	0	8	20	7	
Steamed guava	1	985	1,045	640	
Steamed guava	2	105,000	56,000	81,000	
Persimmon	0	2,000	2,300	1,800	
Persimmon	1	3,000,000	3,950	3,000,000	
Persimmon	2	.....*	.....*	.....*	
Prune (dried)	0		3	0	
Prune (dried)	1	14	28	6	
Prune (dried)	2	72	206	10	

* Not plated because visibly fermenting.

TABLE 13

Summarized Results of Plate Counting of Purees Stored in the Refrigerator (40-42° F.)

Sample	Storage time after thawing (days)	Numbers of organisms per gram			
		Medium			
		YG	N	W	EMB
Broccoli	0	2,300	3,600		700
Broccoli	2	2,300	2,900		400
Broccoli	4	23,000	20,000		2,000
Broccoli + Asparagus	0	1,600	2,000		750
Broccoli + Asparagus	2	2,100	3,400		700
Broccoli + Asparagus	4	4,500	6,000		600
Raw guava	0	50	41	10	
Raw guava	2	185	155	120	
Raw guava	4	170	380	135	
Steamed guava	0	14	17	15	
Steamed guava	2	6	11	7	
Steamed guava	4	14	25	14	
Persimmon	0	1,800	2,200	1,800	
Persimmon	2	4,600	3,900	3,300	
Persimmon	4	70,000	80,000	470,000	
Prune (puree of dried)	0	4	12	2	
Prune (puree of dried)	2	3	13	2	
Prune (puree of dried)	4	12	19	9	

TABLE 14

Ascorbic Acid Retention in Frozen Purees Stored in a Household Refrigerator After Thawing

Sample and Treatment	Vit. C 0 hrs. after thawing mg./100 gm.	Vit. C 24 hrs. after thawing mg./100 gm.	Vit. C 72 hrs. after thawing mg./100 gm.	Percent retained during 72 hrs. at 40° F.
Broccoli Puree	56.6	56.4	54.7	96
Pea Puree	25.1	21.8	22.0	87
Peach + Apricot Puree	4.9	2.5	....	....
Peach + Apricot Puree (another sample)	2.8	1.8	1.2	43
Raw Muir Peach Puree	3.2	2.1	1.4	43
Peas + Pods Puree	24.7	20.8	17.2	69
Broccoli + Asparagus	44.8	39.8	38.7	86
Broccoli + Asparagus (another sample)	32.5	30.5	28.8	88
Blanched Peach + Nectarine Puree	11.4	....	10.5	92
Raw Peach + Nectarine Puree	6.4	3.1	2.2	34
Raw Guava Puree	179.0	169*	165	92
Steamed Guava Puree	173.0	172*	167	97

* 48 hrs.

Acceptability of Frozen Compared with Canned Purees

In order to test the relative acceptability to invalids of frozen and canned purees, some preliminary tests were made on samples of mixed green vegetables and vegetables plus beef as put up by a commercial baby food firm. The samples herein designated as "canned" were processed in a retort in the usual manner, while those designated as "frozen" were taken from the same cannery line after filling and sealing the cans but before heat processing. These samples were then "quick frozen" at —20° F., in a nearby commercial air-blast freezing tunnel. This procedure had the advantage of allowing uniformity of original material. Its disadvantage was that the frozen vegetables were precooked more than they would ordinarily be, so that the color of the green vegetables was not the bright green characteristic of the frozen green vegetable purees which we prepared on a laboratory scale, or which can also be prepared commercially. A sample of commercially frozen asparagus puree was also tested. Canned baby food manufacturers do not include asparagus puree among their selections, thus making it impossible for us to test a parallel canned sample. These samples were then distributed to two hospitals allowing fifty cans of each kind of food for each hospital. The dietitians who cooperated, heated and served the respective canned and frozen samples side by side to patients who were on soft diet and to a few members of the hospital staffs and kitchen personnel. The tasters were asked to answer the following questionnaire:

SCORE SHEET FOR TWO SAMPLES OF PUREES

(Check one) (a) (b)

1. I definitely like the sample
   Acceptable, but not outstanding
   I definitely dislike the sample
2. *Flavor*
   Good
   Fair. Not outstanding
   Poor
3. *Texture*
   Good firm feel
   Too thin and watery
   Too thick

4. *Color*
   I like the color
   Color is not objectionable
   I dislike the color

If the patients were very ill, the nurses or dietitian filled in the questionnaires. In some cases they were too ill to discriminate or remember what they had eaten. This probably accounted for the very large proportion of the tasters who expressed no preference. The data are summarized in Table 15. The interesting points brought forth from this study are: (1) There was a strong preference for the frozen samples of both types of puree, mainly on the basis of flavor. (2) Texture or consistency of the puree, which we consider to be one of the outstanding disadvantages of the frozen product, made little difference to these individuals. (3) The difference between the frozen and canned samples with respect to color was not of noticeable importance to them. However, as mentioned earlier, it was not as striking in these samples as it would have been if the frozen samples had received less heat treatment prior to freezing.

TABLE 15

Summary of results of comparative acceptability of frozen and canned purees in hospital tests

(All figures represent numbers of individual tasters)

	Mixed Green Vegetables	Vegetables + Beef
Preferred canned sample	9	11
Preferred frozen	32	41
Expressed no preference	19	33
Preferred flavor of canned sample	9	13
Preferred flavor of frozen sample	31	38
Expressed no preference for flavor	20	34
Preferred texture of canned sample	0	10
Preferred texture of frozen sample	12	17
Expressed no preference for texture	48	57
Preferred color of canned sample	2	4
Preferred color of frozen sample	6	13
Expressed no preference for color	52	67

Packaging

A problem which was not studied systematically by us, but which is of extreme importance to commercial production of such a product is that of packaging. Most of our experimental samples have been frozen in either tin cans, screw capped or crown capped glass jars, cellophane-lined paper cartons, or heavily waxed paper cartons with push-in lids (Nestrite type). These have all been satisfactory for experimental purposes and one or more might readily be adapted for commercial use. Small enough amounts for one or two servings for an infant might also be packaged individually in plastic sausage casings or in small cellophane containers about the size of ice cubes which might then be grouped into larger packages offering a variety of foods in a single dispensing unit.

Summary and Conclusions

A report of the work which has been completed to date on the experimental preparation and testing of frozen fruit and vegetable purees is given.

The results of a survey on regulations and standards pertaining to the canned baby food industry revealed that while there are no special regulations intended for this specific purpose, high standards are maintained as a result of consumer demand and industry competition. Commercially canned baby foods were chemically analyzed and the results were compared with analyses of experimentally frozen samples.

Several methods of preparing fruit and vegetable purees for freezing were tested and the results compared. The best results with fruit purees were obtained by blanching the fruit just long enough to inactivate the enzymes that catalyze darkening and then preparing the puree. For some fruits partial dehydration prior to pureeing was successful. In the preparation of vegetable purees, blanching as for regular freezing, followed by pureeing and freezing, yielded the best quality. Cooking had to be completed at the time of serving. This was also advantageous from the microbiological point of view. The use of several varieties of fruit, antioxidants added to fruit purees, and vitamin retention during storage were all included in the studies.

A survey of the literature indicated that there would be slight danger of pathogenic organisms surviving freezing storage. However, a direct study of this factor in relation to fruit and vegetable purees is needed. A few data were presented indicating that after thawing, these purees may be kept bacteriologically and nutritionally in good condition in a household refrigerator for two or three days. At room temperature they should not be held longer than one day after thawing.

A preliminary survey was made of acceptability to hospital patients of frozen compared with canned purees. There was a definite trend toward preference of the frozen for flavor and of the green vegetables for color. Additional data along these lines are required before definite conclusions can be drawn.

Further data on packaging, acceptability, bacteriology and some aspects of preparation, especially with a view toward improving consistency, are required. However, the general conclusion which might be drawn is that frozen purees should be capable of commercial production and consumer acceptance, in order to supplement and add variety to, rather than compete with, the familiar canned products, and to expand the market for fresh produce.

Acknowledgments

These investigations have been supported in part by special research grants from the Booth Fisheries Corporation and the Sugar Research Foundation. The authors also wish to express their appreciation to the following institutions, all of whom cooperated generously in making their facilities available: University of California Hospital, San Francisco; Highland Alameda County Hospital, Oakland; Clapp's Baby Food Division, American Home Foods Incorporated, San Jose; Hunt Brothers Canning Company, Hayward; Glacier Packing Company, Sanger; Juillard Frozen Foods, Watsonville; Pictsweet and Santa Clara Frosted Food Companies, both of Santa Clara; and Yosemite Growers' Cooperative, Merced. In addition, we thank the following graduate students for assistance in analyses and preparation of samples: Charles V. Finell, M. A. Jimenez, James Parsons, Robert Ramsey, and Margaret Belais Salmon.

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LITERATURE CITED

1. American Public Health Association. Standard methods for the examination of water and sewage. Ninth edition (1946).
2. Association of Official Agricultural Chemists. Methods of analysis. Sixth edition (1945).
3. Berry, James A. Bacteria question in cold packing. *Western Canner and Packer*, **23**, 17 (1932).
4. Berry, James A. Microbiology of frozen pack berries and vegetables. *Ice and Refrig.*, **84**, 204 (1933).
5. Berry, James A. Destruction and survival of microorganisms in frozen foods. *J. Bact.*, **26**, 459 (1933).
6. Berry, James A. Microbiology of frozen vegetables. *Western Canner and Packer*, **29**, 14 (1937).
7. Bessey, O. A., and King, C. G. The distribution of vitamin C in plant and animal tissues and its determination. *J. Biol. Chem.*, **103**, 687 (1933).
8. Conner, R. T., and Straub, G. J. Determination of thiamin by the thiochrome reaction. *Ind. and Eng. Chem., Anal. Ed.*, **13**, 380 (1941).
9. Conner, R. T., and Straub, G. J. Combined determination of riboflavin and thiamin in food products. *Ind. Eng. Chem., Anal. Ed.*, **13**, 385 (1941).
10. Haines, R. B. Bacteria in frozen vegetables. *Ice and Refrig.*, **93**, 199 (1937).
11. Hassid, W. Z. Determination of reducing sugars and sucrose in plant materials. *Ind. Eng. Chem., Anal. Ed.*, **8**, 138 (1936) and **9**, 228 (1937).
12. Hohl, Leonora A. Experiments prove value of freezing baby foods. *Quick Frozen Foods*, **7**, 30 (1944).
13. Hohl, Leonora A., and Smith, Mildred. Comparison of vitamin content and palatability of frozen, canned and dehydrated vegetable purees. *Fruit Prod. J.*, **24**, 54 (1944).
14. Joslyn, M. A., and Hohl, Leonora A. The commercial freezing of fruit products. California Agr. Expt. Sta. *Bull.* **703**, 108 pp. (1948).
15. Mackinney, G., and Sugihara, J. Riboflavin estimation in fruits and vegetables. *J. Am. Chem. Soc.*, **64**, 1980 (1942).
16. Maerz, A., and Paul, M. Rea. A dictionary of color. McGraw-Hill Book Co., Inc., New York, 207 pp. (1930).
17. Magoon, C. A. Microorganisms as affecting frozen foods. *Ind. Eng. Chem.*, **24**, 669 (1932).
18. Paul, Pauline. Fruit purees. Michigan Agr. Exp. Sta. Cir. **200**, 19 pp. (1946).
19. Phillips, A. W., Jr., and Proctor, B. E. Growth and survival of an experimentally inoculated *Staph. aureus* in frozen precooked foods. *J. Bact.*, **54**, 49 (1947).
20. Prescott, S. C., and Geer, L. P. Food poisoning organisms under refrigeration. *Refrig. Eng.*, **32**, 211 and 282 (1936).
21. Prescott, S. C., and Tanner, F. W. Microbiology in relation to food preservation. *Food Res.*, **3**, 189 (1938).
22. Proctor, B. E., and Phillips, A. W., Jr. Frozen precooked foods. *Am. J. Pub. Health*, **38** (No. 1, Part I), 44 (1948).
23. Straka, R. P., and James, L. H. Frozen vegetables. *Am. J. Pub. Health*, **23**, 700 (1932).
24. Straka, R. P., and James, L. H. Further studies on frozen vegetables. *J. Bact.*, **29**, 313 (1935).
25. Tanner, F. W., and Wallace, G. I. Effect of freezing on microorganisms in various menstra. *Proc. Soc. Exp. Biol Med.*, **29**, 32 (1932).
26. Tressler, D. K. Bacteria, enzymes, and vitamins—indices of quality in frozen vegetables. *Refrig. Eng.*, **36**, 319 (1938).
27. Vaughn, Reese H. The acetic acid bacteria. *Wallerstein Lab. Communications*, **5**, 5 (1942).
28. Vaughn, Reese H., Douglas, Howard C., and Gililland, J. Richard. Production of Spanish-type green olives. California Agr. Expt. Sta. *Bull.* **678**, 82 pp. (1943).
29. Wallace, G. I., and Tanner, F. W. Microbiology of frozen foods. I. Historical review and summary of results. *Fruit Prod. J.*, **13**, 52 and 109 (1934). II. Studies on frozen fruits and vegetables. *Ibid.*, **13**, 274 and 366, 377; and **14**, 145 (1935). III. Longevity of pure cultures of microorganisms frozen in various menstra. *Ibid.*, **14**, 235 (1935).
30. Wallace, G. I., and Park, S. E. Microbiology of frozen foods. Longevity of certain pathogenic bacteria in frozen cherries and in frozen cherry juice. *J. Infectious Diseases*, **52**, 146 (1933).
31. Wallace, G. I., and Park, S. E. Microbiology of frozen foods. V. The behavior of *Clostridium botulinum* in frozen fruits and vegetables. *J. Infectious Diseases*, **52**, 150 (1933).

Proc. Soc. Exp. Biol. Med. 78: 238-241 1951

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Megaloblastic Anemia in Pregnancy. Remission Following Combined Therapy with Ascorbic Acid and Vitamin B_{12}.* (19033)

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A recent report by Thompson and Ungley (1) has reviewed many of the clinical and hematologic features of megaloblastic anemia in pregnancy. These authors emphasize the lack of response to vit. B_{12}. Similar observations had previously been reported by Day, Hall and Pease(2). In this respect the megaloblastic anemia in pregnancy is similar to megaloblastic anemia experimentally produced in monkeys(3) and to the megaloblastic anemia in some infants(4). Remissions are regularly produced in these three as well as other megaloblastic anemias by folic acid therapy. The megaloblastic anemia in some infants and as experimentally produced in monkeys is related to a deficiency in ascorbic acid and can be prevented by a normal intake of this substance. May(3) has suggested that the ascorbic acid deficiency may interfere with the conversion of folic acid to the citrovorum factor as Nichol and Welch(5) have shown that ascorbic acid augments the conversion of folic acid to the citrovorum factor *in vitro*. It is entirely possible that megaloblastic anemia in some infants and that produced in monkeys results from a deficiency of the citrovorum factor which in turn is produced by altered folic acid metabolism resulting from ascorbic acid deficiency.

Observations on 5 patients with megalo-

* This study was carried out under a grant from Eli Lilly and Co.

1. Thompson, R. B., and Ungley, C. C., *Quart. J. Med.*, 1951, v20, 187.
2. Day, L. A., Hall, B. E., and Pease, G. L., *Mayo Clinic Staff Meetings Proc.*, 1949, v24, 7.
3. May, C. D., Nelson, E. W., Lowe, C. U., and Salmon, R. J., *Am. J. Dis. of Child.*, 1950, v80, 191.
4. May, C. D., Nelson, E. W., Aldrich, R. A., Salmon, R. J., and Lienke, R. I., *Am. J. Dis. of Child.*, 1949, v77, 127.
5. Nichol, C. A., and Welch, A. D., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 52.

blastic anemia in pregnancy indicate that a similar mechanism may hold for the pathogenesis of this anemia. Megaloblastic anemia in pregnancy is refractory to vit. B_{12}. Two of the cases to be reported confirm this finding. The pregnancy megaloblastic anemia may be related to ascorbic acid deficiency. A seasonal incidence was present in that all 5 cases appeared in April and May. Lund (6) has shown decreased plasma ascorbic acid values in normal pregnancy during winter and spring months when exogenous sources of ascorbic acid are apt to be low. In 3 of the patients on whom determinations for plasma ascorbic acid were run, decreased or zero values were obtained. To test the analogy further, 3 of the patients were treated with vit. B_{12} and ascorbic acid separately and then in combination. Combined therapy in each of the 3 patients produced a complete hematologic remission where separate therapy with ascorbic acid or vit. B_{12} was ineffective. A fourth patient was first seen after 10 days therapy with vitamin B_{12} during which time the anemia had become progressively more marked. This patient responded to combined folic acid and transfusion therapy. No therapy was given the fifth patient. A spontaneous remission followed delivery. The diagnosis of megaloblastic anemia was made in each case by bone marrow biopsy.

Case 1. D.H. Age 23 Para 2-0-0-2. The diagnosis of megaloblastic anemia was made 7 days before delivery. The hemoglobin was 8.8 g %, erythrocytes 2.66 million, hematocrit 23% and reticulocytes 1.2%. Free acid was present on gastric analysis. The plasma ascorbic acid level was 0.3 mg %. Daily administration of 500 mg of ascorbic acid by intramuscular injection produced a maximum reticulocyte response of 3.6% on the fifth day. The marrow was unchanged at the completion of this test period. While on ascorbic acid therapy the hemoglobin dropped to 6.2 g %, erythrocytes to 1.76 million and the hematocrit to 18%. A daily dose of 30 μg of vit. B_{12} was added to the ascorbic acid therapy and was followed by a maximum reticulocyte response of 25.4% 9 days after the onset of combined therapy. The marrow reverted to normoblastic development. Rapid clinical and hematologic improvement followed. On discharge from the hospital 27 days post partum the hemoglobin was 11.6 g %, erythrocytes 2.93 million and hematocrit 33%. Later checks showed a return to normal hematologic values.

Case 2. D.H. Age 24 Para 3-0-0-3 (Same patient as case 3). The patient was followed throughout this pregnancy. A diagnosis of megaloblastic anemia was made in the ninth month of pregnancy. Hemoglobin was 8.1 g %, erythrocytes 2.54 million, hematocrit 23% and reticulocytes 0.6%. After the initial workup, daily administration of 500 mg of ascorbic acid was started. A trial period of 4 days was allowed at the end of which time the marrow was still megaloblastic. No reticulocyte increase was observed. The hemoglobin had dropped to 6.4 g %, erythrocytes to 1.86 million and hematocrit to 20%. Thirty micrograms of intramuscular vitamin B_{12} was combined with the ascorbic acid and 9 days later a maximum reticulocyte response of 15.4% was noted. Marrow reversion to normoblastic pattern was noted after the institution of combined therapy. Clinical and hematologic response again was rapid. Twenty-eight days after delivery the hemoglobin was 11 g %, erythrocytes 3.72 million and hematocrit 37%.

Case 3. M.S. Age 18 Para 0-0-0-0. Megaloblastic anemia was diagnosed in the eighth month of pregnancy. Plasma ascorbic acid level was zero. Hemoglobin was 8.6 g %, erythrocytes 2.14 million, hematocrit 25% and reticulocytes 0.4%. Treatment with 30 μg of intramuscular vit. B_{12} over a 6-day period did not produce a reticulocyte response. At the end of this 6-day period the bone marrow was unchanged. The hemoglobin dropped to 7.6 g %. The combination of 300 mg of ascorbic acid with vit. B_{12} was followed in 4 days by an increase in reticulocytes to 13.4%. Seventy-two hours after the onset of combined therapy the bone marrow was normoblastic. Twenty days after admission and while still undelivered the hemoglobin was 10.4 g %, erythrocytes 2.67 million

6. Lund, C. J., and Kimble, M. S., *Am. J. Obstet. and Gynecol.*, 1943, v46, 635.

TABLE I. Summary of Data on 5 Cases of Megaloblastic Anemia in Pregnancy.

	Date	Comment	Hemoglobin, g %	Erythrocytes, million μ^3	Hematocrit, %	Reticulocytes, %
D.H.	2-28	Initial workup	8.8	2.66	23	1.2
	3-7	Day of delivery	7.4	1.75		1.3
	-8	Ascorbic acid therapy begun				
	-13		6.2	1.76	18	3.5
	-14	B_{12} therapy started				
	-23	Max retic response	7.5	2.11	24	25.4
	4-3	Discharge	11.6	2.93	33	3.4
D.H.	1-12	6th month of pregnancy	12.5	3.35	36	1.2
	4-12	Initial workup	8.1	2.54	23	.6
	-17	Ascorbic acid therapy begun				
	-18	Day of delivery				
	-20		7.1	1.86	20	1
	-21	B_{12} therapy started				
	-30	Max retic response	8.6	2.23	29	15.4
	5-16	Discharge	11.1	3.72	37	2.8
M.S.	5-9	8th month of pregnancy	8.6	2.14	25	.4
	-15	Vit. B_{12} therapy started	7.6			
	-21	Ascorbic acid therapy started				
	-25	Max retic response	10.7			13.4
	6-18	Day of delivery	13.5	3.05	37	1.8
I.M.	5-4	Admitted to hospital	5.1	1.41		
	-6	Crude liver and B_{12} started				
	-12	Transfusion 500 cc				
	-13	Day of delivery, folic acid	6.9	1.53	15	.3
	-21	Transfusion 500 cc	11	3.16		
S.K.	5-12	Day of delivery				
	-13	Initial workup	8.4	2.83	27.5	.2
	-23	Max retic response				5.5
	6-24		11.5	3.38	39	.6

and hematocrit 30%. At delivery 6 weeks after admission the hemoglobin was 13.5 g % and hematocrit 37%.

Case 4. I.M. Age 34 Para 1-0-2-1. Megaloblastic anemia was diagnosed on the day of delivery though the patient had been hospitalized and treated with vit. B_{12} and crude liver extract for 10 days prior to diagnosis. Following delivery an adequate response was obtained with folic acid and blood transfusions. The initial hemoglobin was 6.9 g %, erythrocytes 1.53 million, hematocrit 15% and reticulocytes 0.3%. Aside from the demonstration that vit. B_{12} administration had not prevented the appearance of this megaloblastic anemia, little information was obtained.

Case 5. S.K. Age 42 Para 7-2-2-9. The diagnosis of megaloblastic anemia was made on the day following delivery. The hemoglobin was 8.4 g %, erythrocytes 2.83 million, hematocrit 27.5% and reticulocytes 0.2%. There was free acid on gastric analysis. Plasma ascorbic acid value was zero. Inadvertently no therapy was given this patient but a spontaneous remission followed delivery. A maximum reticulocyte response of 5.4% appeared 11 days after delivery. When seen 43 days after delivery the hemoglobin was 11.5 g %.

The essential data are summarized in Table I. Other tests were made on these patients which are of some interest. In 4 patients on whom stool specimens were obtained before therapy there was no increase in urobilinogen excretion. Serum iron determinations made prior to therapy on 4 patients showed values of 318, 348, 365 and 268 γ %. The iron binding capacity of the serum was less than 50 γ % in the same 4 patients. Following combined therapy there was a sudden decrease in serum iron values to less than 50 γ % and increases in the iron binding capacity. Normal erythrocyte protoporphyrin values were obtained on all 5 patients prior to therapy. After therapy these values increased

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as the bone marrow reverted to normoblastic activity.

It is possible that ascorbic acid therapy alone over a long enough period of time would produce a remission. In Case 1 a suggestive increase in reticulocytes occurred but therapy was not continued long enough to warrant any conclusions. It is probable that megaloblastic anemia in pregnancy can be prevented by adequate ascorbic acid intake during pregnancy.

Summary. Five cases of megaloblastic anemia in pregnancy are reported. The relation of this anemia to a deficiency in ascorbic acid is suggested by the effectiveness of combined ascorbic acid and vit. B_{12} therapy where vit. B_{12} alone was ineffective. Two patients were refractory to adequate trial with vit. B_{12}. One of these patients later had a complete remission when ascorbic acid was added to the therapy. The other responded adequately to folic acid therapy and blood transfusions. Two patients were treated for short periods of time with ascorbic acid alone. A minimal response in reticulocytes was observed in one. Subsequent addition of vit. B_{12} to the therapy after saturation with ascorbic acid produced a complete hematologic remission. One patient had a spontaneous remission after delivery. In 3 patients combined therapy with ascorbic acid and vit. B_{12} produced a complete remission.

Received August 27, 1951. P.S.E.B.M., 1951, v78.

J. Lab. Clin. Med. 24: 1119-1127 1939

The Journal of Laboratory and Clinical Medicine

VOL. 24 AUGUST, 1939 No. 11

CLINICAL AND EXPERIMENTAL

THE EFFECT OF VITAMIN C ON LEAD POISONING

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IN a large industrial plant where the lead hazard was great due to fine metallic lead dust from filing down lead alloy seams, lead fumes from melting pots, and lead contacts from putty spray guns, there was an exceptional opportunity to diagnose and treat lead absorption and poisoning.

Four hundred men exposed to lead during their daily tenure at the plant were examined physically, and by laboratory methods. The latter consisted of monthly checks on the basophilic aggregation (B.A.), of the blood smear, according to McCord,[1] and on the degree of stippling, using Wright's stain. Records of these monthly tests were kept throughout the period of observation from April, 1937, until April 1, 1938. During the last three months of this period weekly tests were recorded on a cooperative group of 34, all of whom had symptoms and showed signs of lead poisoning. In addition to the above-mentioned tests, differentials were recorded on the Wright's smear, using the classification of Farley and others,[9] as used by Steinberg.* Urinalyses were not done due to technical difficulties at the factory.

During the period from April 1, 1937, to November 15, 1937, the gluconate, lactate, and diphosphate salts of calcium were given in the amount daily of from 80 to 100 grains to those who showed lead absorption by physical or laboratory signs. In addition, vitamin D (940 units) and vitamin A (9400 units) were given daily, using an accredited fish-liver oil.

The criterion used was a basophilic aggregation percentage of 1.5 or greater, abnormal stippling, or by request of the ill individual. Biweekly intravenous injections of 10 c.c. of 10 per cent calcium gluconate, together with daily oral therapy with calcium salts and vitamin D,[1] were given to

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Received for publication, November 14, 1938.

those whose basophilic aggregation registered higher (3.0 plus). Deleading with halides of potassium and ammonia were not done.

The period of intimate observation of the group of 34 lead poison patients upon whom we base this report began in November, 1937. It was then noted that, in spite of the intensive treatment with calcium and vitamin D, and the prophylactic measures taken by the plant management to reduce the lead hazard, many of the men failed by physical and laboratory standards to recuperate. In some, while there was a return to normal in percentage of basophilic aggregation and degree of stippling, there remained the complaint of ill-health, or abnormal cell morphology, or both.

Most frequent complaints were fatigue, anorexia, sleeplessness, or restless sleep; pains in the legs (gastrocnemius muscles) or in the forearms near the elbow; tenderness on deep pressure over the same areas, and irritability. Less frequently, complaint was made of trembling, constipation, sexual impotence, and joint pain. Observations on these patients consisted mainly of pyorrhea, spongy gums, and poor dentition in 75 per cent; tremor of varying degree, involving fingers and hand, usually bilaterally, and pallor of varying degree in 50 per cent; weakened extensibility at the wrist in about 10 per cent. One of the most striking signs was the surliness encountered in the majority of these subjects.

Ascorbic Acid Treatment.—One of us (E. J. A.) made the observation that the symptoms and signs exhibited by these men were similar to those found in subclinical scurvy. Ascorbic acid oral therapy was instituted in a group of 34 patients with chronic lead poisoning according to the following plan: Of 34 workmen (chronic patients) selected for this novel treatment, 17 were given ascorbic acid alone (at least two months after discontinuing the calcium salt injections), while an equal number continued the calcium therapy and at the same time took tablets of ascorbic acid.

Ascorbic Acid Without Calcium.—In general, the group of 17 patients made a marked gain in vigor, cheerfulness, color of skin, and blood picture. A week, or less than a week, after beginning the treatment of 100 mg. of ascorbic acid daily, most of the men enjoyed normal sleep, lost the irritability and nervousness so common with high calcium treatment of lead poisoning, enjoyed their food more, and no longer had tremors (if observed before). Several cases of leucopenia (probably due to a prolonged calcium therapy somewhat earlier) were cured by the ascorbic acid treatment.

Ascorbic Acid With Calcium.—The gain was less marked and rather irregular in the 17 patients given ascorbic acid with calcium. There was no gain in the number of mature neutrophiles in the blood—in strange contrast with the rapid gain when ascorbic acid alone was given.

In several instances alcohol nullified the good effects of calcium as well as of ascorbic acid, a complication that workmen exposed to lead hazards would do well to avoid.

CASE REPORTS

The detailed blood picture and the clinical observations for 3 workmen who received benefit from vitamin C in chronic lead absorption are outlined: The blood count was for 200 cells.

WORKMAN 1

CALCIUM GLUCONATE ALONE. LATER ASCORBIC ACID ALONE

DATE	B. A.* %	STIPPLING	RED BLOOD CELL MORPHOLOGY	MATURE NEUTROPHILES %	STABS %	JUVENILES %	MYELOCYTES %	LYMPHOCYTES %	EOSINOPHILES %
1937									
June	6	Strong							
Late June . . . Began biweekly intravenous injections of 10 c.c. 10 per cent calcium gluconate plus 30 grains calcium salts orally									
July	4.3	Strong							
Aug. 1	3.3	Strong							
. . . Discontinued calcium therapy. Plant closed Aug. 10 to Sept. 9. Lead hazard greatly reduced during September									
Oct.	3.0	Positive							
Nov.	2.1	Mild							
Feb.	1.4	Negative	Anisocytosis	24	2	0	2	68	4
Feb. 9 . . . Began daily doses of 100 mg. ascorbic acid									
Feb. 21	1.8	Strong		36	4	8	0	50	2
Feb. 28	1.5	Strong	Anisocytosis marked	49	0	2	0	55	2
Mar. 9	1.2	Mild	Normal	46	2	3	0	48	1
Mar. 16	1.0		Normal	66	2	4	0	18	6
Treatment discontinued									

*Basophilic aggregation.
Monocytes, none from Feb. 9 to March 16.
Basophiles, 0, 0, 1%, 0, 4% from Feb. 9 to March 16.

Clinically Workman 1 on February 9 showed definite symptoms of chronic lead poisoning with marked tremor, nervousness, irritability, sleeplessness. He was underweight, tired easily, and had a very sallow complexion. His blood picture was discouraging.

By March 16, after five weeks of ascorbic acid treatment, the tremor disappeared, and his complexion greatly improved. The man was very cheerful and no longer tired easily. The blood picture also greatly improved.

WORKMAN 2

CALCIUM SALT THERAPY. LATER ASCORBIC ACID ALONE

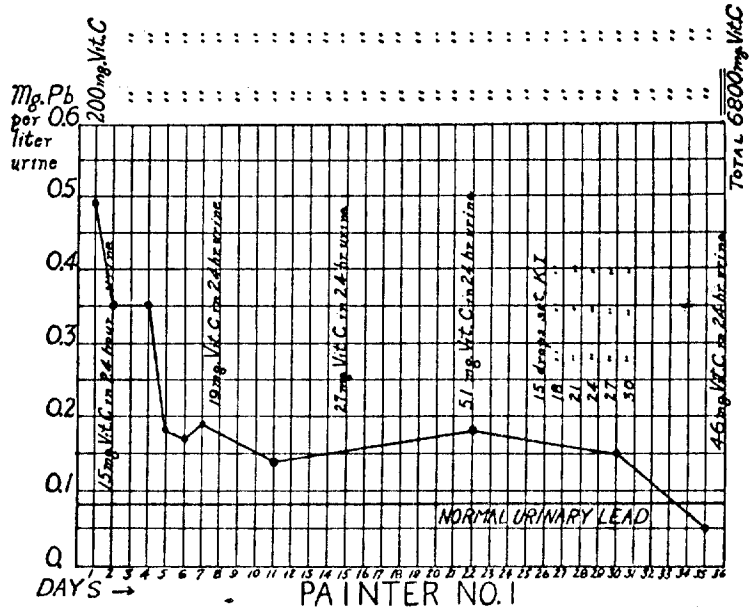
DATE	B. A.* %	STIPPLING	RED BLOOD CELL MORPHOLOGY	MATURE NEUTROPHILES %	STABS %	JUVENILES %	MYELOCYTES %	LYMPHOCYTES %	EOSINOPHILES %
1938 Feb. 9	3.4	Positive	Anisocytosis moderate	38	0	6	2	48 (?)	0
Feb. 10 . . . Began biweekly intravenous injections of calcium gluconate. Calcium lactate and Natola orally daily									
Feb. 21	1.4	Negative	Achromia, anisocytosis slight	34	2	4	0	44	0
Feb. 24	0.9	Negative	Anisocytosis moderate	49	2	6	0	39	3
Mar. 3	3.1	Strong	Anisocytosis, polychromasia, achromia moderate	40	2	8	0	44	4
Mar. 7	1.2	Negative	Anisocytosis moderate	40	2	4	4	48 (?)	0
Mar. 7 Began daily doses of 100 mg. ascorbic acid. Calcium discontinued									
Mar. 14	1.3	Negative	Normal	62	2	0	0	34	1

*Basophiles, 0, 6%, 1%, 0, 1%, 0 for the six dates.
Monocytes, 6%, 10%, 0, 2%, 0, 1% for the six dates.

Clinically Workman 2 showed symptoms of chronic lead poisoning, with moderate tremor, nervousness, irritability, sleeplessness, and pallor. He tired easily. One week after beginning the ascorbic acid treatment, tremor and pallor were gone. He slept well, did not tire, and was extremely cheerful and talkative.

Workman 3 displayed clinically an acute case of lead poisoning in June, 1937, with a basophilic aggregation of 14, strong stippling, etc. Calcium therapy from June to February, 1938, lowered his basophilic aggregation satisfactorily, but three weeks after discontinuing this treatment, it increased considerably with return of the symptoms of acute lead poisoning.

He was then given 100 mg. of ascorbic acid daily for sixteen days and his basophilic aggregation was again lowered to normal (as with calcium therapy), with no clinical symptoms of lead poisoning. The morphology of the red blood cells became nearly normal. The man looked well and felt fine.



House Painters.—The research was transferred to Oberlin to make a study of ordinary house painters. Here it was possible to make analytical determinations of lead and ascorbic acid in the urine. The dithizone method for urinary lead, as described by Ross and Lucas,[2] and improved, especially in the use of a calculation curve, by Miller[3] and his associates, was used.

For the determination of ascorbic acid in urine the titration against the dye, 2.6-dichlorophenolindophenol, as described by Harris and Ray,[4] or Hawk,[5] was used. We found it necessary to devise improved methods of collecting twenty-four-hour specimens of urine in order to prevent the usual considerable losses of vitamin C (ascorbic acid) by oxidation. The details are given in another paper. Tests with a number of healthy people convinced us that the common estimate of an average daily urinary excretion of 15 mg. of vitamin C is too low, due to this oxidation loss, and that 25 mg. to 35 mg. is the probable average for healthy persons.

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Of 14 painters observed clinically and by differential blood count, 2 were rated by clinical examination as chronic lead absorption patients, and the blood pictures added 5 more. Three of the 7 patients refused to cooperate further; hence we selected only 3 for study and treatment. Seven of the 14 picked up at random showed enough absorption to call for treatment or greater precautions.

CASE REPORTS

Case histories of 3 painters are here given. The final brief deleading was done by Dr. A. C. Siddall, who gave somewhat more potassium iodide than usual. In order to demonstrate more convincingly the value of a diet rich in vitamin C for men exposed to lead hazards, we determined the urinary lead and vitamin C of 2 more painters and gave them a brief deleading treatment. Furthermore, we determined the urinary vitamin C and lead of another painter who for many years had stressed fruits, vegetables, and milk in his diet.

Clinically the first painter and sprayer, who exhibited symptoms of chronic lead poisoning (physician's report) responded quickly to ascorbic acid treatment. After four days, he felt lazy and sleepy but not tired. Then he quickly changed to "feeling fine," ready for work, and fresh at the end of a hard day's work. At the same time there was a decided improvement in his blood picture. His urinary lead quickly fell to nearly normal, and his ascorbic acid excretion rose from 15 mg. per twenty-four hours to the very high value of 51 mg.

PAINTER 1

ASCORBIC ACID TREATMENT FOLLOWED BY DELEADING

DATE	B. A. %	STIPPLING	RED BLOOD CELL MORPHOLOGY	MATURE NEUTROPHILES %	STABS %	JUVENILES %	MYELOCYTES %	LYMPHOCYTES %	EOSINOPHILES %
1938 May 11	0.9	7,500	Anisocytosis and slight polychromasia	32	9	8	0	43	5
July 7	1.0	6,000	Anisocytosis and slight polychromasia	30	10	6	0	45	6
July 7	. . .	Began 200 mg. ascorbic acid daily							
July 19	2.2	8,000	Anisocytosis moderate	50	10	4	0	31	3
Aug. 1	. . .	Continued ascorbic acid and began deleading with potassium iodide							
Aug. 7	. . .	End of deleading							
Aug. 9	1.6	Negative	Normal	62	2	1	0	30	2
Aug. 10	. . .	End of ascorbic acid treatment							

Basophiles, 2% May 11, 2% July 7, 2% July 19, 1% Aug. 9.
Monocytes, 1% May 11, 1% July 7, 0 July 19, 2% Aug. 9.

Clinically Painter 2 was rated by one of us (E. J. A.) as showing symptoms of chronic lead poisoning, even to a slight lead line on the gums. His teeth were bad and he tired quickly.

Two days of ascorbic acid treatment made him lazy and sleepy. After three weeks of ascorbic acid, he felt fine and energetic at the end of a hard day's work. His family noted the change from irritability to cheerfulness.

Urinary lead excretion fell from 0.4 mg. per liter to about 0.1 mg. Ascorbic acid in twenty-four hours' urine rose from 12 mg. to 86 mg.

Clinically Painter 3 seemed to one of us (E. J. A.) to be in good health, showing no signs of lead poisoning and having no complaints. During the period from May 9 to May 23 his blood picture was not reassuring and plans were made for ascorbic acid

PAINTER 2

ASCORBIC ACID TREATMENT CONTINUED THROUGH DELEADING

DATE	B. A. %	STIPPLING	RED BLOOD CELL MORPHOLOGY	MATURE NEUTROPHILES %	STABS %	JUVENILES %	MYELOCYTES %	LYMPHOCYTES %	EOSINOPHILES %
1938									
May 5	2.1	Negative	Anisocytosis slight	35	4	8	0	52 (?)	1
July 7	2.5	Negative	Anisocytosis moderate	28	6	10	0	54	2
July 7	. . . Began 200 mg. ascorbic acid daily								
July 14	2.1	3,500	Anisocytosis moderate, poikilocytosis slight, polychromasia slight	40	15	4	1	36	2
July 28	1.4	Negative	Normal	64	2	1	0	28	3
July 29	. . . Discontinued ascorbic acid. Deleading with potassium iodide began and continued three days until an attack of colic								
Aug. 9	2.3	3,000	Anisocytosis moderate, poikilocytosis slight, polychromasia slight	32	7	1	0	54	4

Basophiles, 2% Aug. 9. None on other dates.
Monocytes, 0 May 5, 0 July 7, 2% July 14, 2% July 28, 0 Aug. 9.

treatment. Urine examined for lead showed 0.5 mg. per liter (as compared to the average man's 0.085 mg.). Vitamin C excretion per twenty-four hours was very low, being only 10 mg.

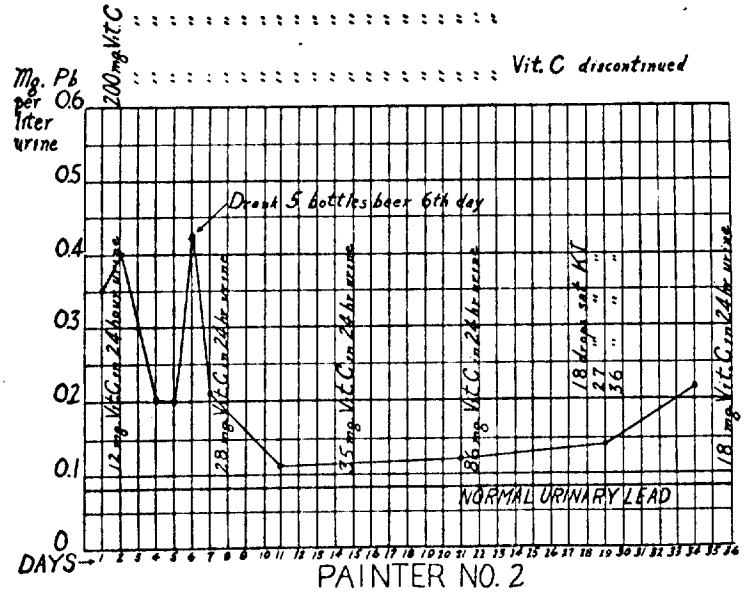
Two weeks of ascorbic acid treatment decidedly improved his blood picture and astonishingly lowered the urinary lead to 0.18 mg. per liter. Ascorbic acid excretion rose to the level of 31 mg. daily.

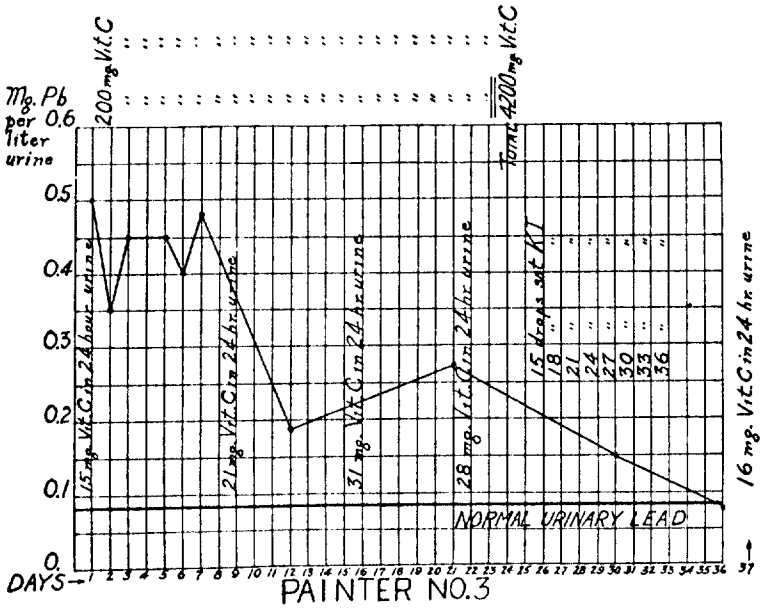
PAINTER 3

ASCORBIC ACID TREATMENT FOLLOWED BY DELEADING

DATE	B. A. %	STIPPLING	RED BLOOD CELL MORPHOLOGY	MATURE NEUTROPHILES %	STABS %	JUVENILES %	MYELOCYTES %	LYMPHOCYTES %	EOSINOPHILES %
1938									
May 9	1.8	30,000	Anisocytosis and polychromasia moderate	44	13	2	1	26	10
May 23	3.2			34	24	2	0	31	7
July 7	2.0	15,000	Anisocytosis moderate, poikilocytosis slight	30	12	3	0	47	6
July 7	. . . Began 200 mg. ascorbic acid daily								
July 21	1.6	Negative	Normal	58	2	2	0	34	3
July 29	. . . Discontinued ascorbic acid. Began deleading with potassium iodide (15 drops saturated solution, increasing by 3 drops daily)								
Aug. 8	1.0	Negative	Normal	57	4	1	0	35	3

Basophiles, 2% May 9, 2% May 23, 2% July 7, 1% July 21.
Monocytes, 2% May 9. None on other dates.





Eight days of deleading (ascorbic acid discontinued) brought urinary lead to normal (0.085 mg. per liter), but the ascorbic acid excretion unfortunately fell to 16 mg. per twenty-four hours. Clinically the man seemed in good health throughout the deleading treatment.

Painter 4.—Painter 4, a house painter for twenty-five years, showed the effects of a diet unusually rich in the best vitamin C foods as well as in milk. His health (except for a temporary tooth infection) was good, his urinary lead was only 0.1 mg. per liter, and his ascorbic excretion was 58 mg. per twenty-four hours, an exceptional figure.

Deleading with 80 grains ammonium chloride daily for six days was done (no milk in that period) and the urinary lead was cut to 0.04 mg. per liter.

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Painter 5.—The son of Painter 4, Painter 5 was brought up in the same dietary tradition as his father. He was in excellent health and painted for several years. He excreted 0.16 mg. lead per liter of urine. After six days of deleading with ammonium chloride (80 grains daily), his urinary lead excretion fell to 0.05 mg. He was comfortable during deleading. The usual vitamin C excretion had been 40 mg. per twenty-four hours urine.

Painter 6.—Painter 6 was a house painter who for fifteen years faithfully applied the dietary teachings of a religious organization and lived on a diet exceptionally rich in fruits and vegetables (many raw) and milk. He was in excellent health and possessed a good blood picture and a very good vitamin C excretion of 37 mg. per twenty-four hours. His lead excretion was 0.215 mg. per liter after four days of deleading with potassium iodide.

His wife on the same religious diet excreted 50 mg. of vitamin C per twenty-four hours, but in her case the effect of lead on vitamin C was not a factor. His pastor, a painter for thirty years, also living on a diet exceptionally rich in fruits, vegetables, and milk, enjoyed splendid health, and excreted only 0.13 mg. lead per liter in spite of his painting.

DISCUSSION

Administration of 100 mg. of vitamin C daily to each of 34 workmen exposed to factory lead hazards (and diagnosed as suffering from lead absorption) in general decidedly improved their blood picture and their health. Symptoms characteristic of chronic lead absorption usually disappeared. It seems reasonable to suppose that toxic lead ions were removed to a considerable extent from the blood stream. Calcium gluconate or lactate does this by storing the lead in the bones, presumably as lead phosphate. Experience shows some drawbacks to long-continued high calcium medication; hence bone storage should be followed by cautious deleading, with a respite from lead absorption.

A more detailed study of 3 house painters, diagnosed as suffering from chronic lead absorption, strongly reinforced the conclusions drawn from the factory experiments. The observation that 200 mg. daily of vitamin C as a dietary supplement decidedly lowered the urinary excretion of lead is astonishing. With it goes another observation that men actually suffering from lead absorption excrete less vitamin C in the urine than does the average man.

It is difficult to escape the conclusion that toxic lead compounds react with vitamin C to form a poorly ionized salt (lead ascorbate?) or a complex salt which yields a very low concentration of simple lead ions. If solutions of lead acetate and vitamin C are mixed in a test tube, no precipitate is observed; hence the product is soluble, not to be precipitated in the bones. Cautious addition of sodium hydroxide fails to precipitate lead hydroxide or oxide, normally termed insoluble.

This view of removal of toxic lead ions by reaction with ascorbic acid to form soluble, but poorly ionized, salts or complexes is strengthened by the observation of Greenwald[6] on the remarkable influence of ascorbic acid on the dissociation (and solubility products) of calcium phosphate and certain other calcium salts. He is of the opinion also that calcium gluconate, lactate, and citrate form complexes with calcium ions. Hastings and McLean[7] offer a parallel in the fact that citrates lower the concentration of free calcium ions through formation of a soluble complex.

In an excess of enthusiasm over the good diet, one of our painters drank a pint of milk for five days before starting the ascorbic acid treatment.

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His vitamin C excretion, previously only 10 to 13 mg., quickly rose to 25 mg. and, after he discontinued his daily pint of milk, quickly fell to 15 mg. A similar observation was made with another painter. Evidently sufficient dietary calcium spares vitamin C in the body of a man suffering from lead absorption, probably by forcing some of the lead into the bones as lead phosphate.

A natural fear arises that if ascorbic acid therapy reduces urinary excretion of lead this lead must be stored somewhere in the body, to be released later in toxic form in the blood stream as dietary or other conditions change. This was not true of four or five local house painters who enjoyed several, even many, years of excellent health while painting, evidently because of a diet that we know to be extremely rich in vitamin C. At any rate, we found that they excreted more of this vitamin than does the average healthy individual.

Judging from the statement by Sollman on deleading with potassium iodide, it seems a fair assumption that the compound formed by ascorbic acid and toxic lead ions is preferentially absorbed by the liver and, with the aid of the bile, excreted in the feces. We hope that others interested in this research will determine this by quantitative analysis. He[8] writes: "Renal excretion of lead depends directly upon the lead compounds circulating at the time in true solution in the blood. . . . The increased excretion (of lead) through use of potassium iodide has been noted by previous workers, although others have failed to confirm it, chiefly because they confined themselves to the urine while the excretion is mainly in the faeces. The iodide causes the reappearance of lead in the faeces from which it had been absent."

Note.—An extra 50 mg. more or less of vitamin C is furnished by approximately

4 oz. fresh orange juice	1 oz. raw green pepper (salad)
4 oz. grapefruit	8 oz. tomatoes, whole or juice
4 oz. raw cabbage	8 oz. cantaloupe
2 oz. raw spinach (salad)	4 oz. raw turnips
4 oz. spinach, cooked in least possible water	

In general, fresh fruits and vegetables (and canned tomatoes) are very good sources of vitamin C, but the average workman will find the above list definite and practical. On a limited income even raw cabbage and turnips with canned tomatoes are possible as sources of 50 mg. or 100 mg. vitamin C daily.

REFERENCES

1. McCord, C. P., and Johnson, J.: The Basophilic Aggregation Test, Am. J. Pub. Health **25**: 1089, 1935.
2. Ross, J. R., and Lucas, C. C.: A New Method for the Determination of Minute Amounts of Lead in Urine, J. Biol. Chem. **3**: 290, 1935.
3. Winter, O. B., Robinson, H. M., Lamb, F. W., and Miller, E. J.: The Determination of Lead, Indust. & Engin. Chem. Analyt. Ed. **7**: 265, 1935.
4. Harris, L. J., and Ray, S. N.: Diagnosis of Vitamin C in Subnutrition by Urine Analysis, Lancet **1**: 71, 1935.
5. Hawk, P. B., and Bergeim, O.: Practical Physiological Chemistry, ed. 11, Philadelphia, 1937, P. Blakiston's Son & Co., p. 733.
6. Greenwald, I.: The Dissociation of Some Calcium Salts, J. Biol. Chem. **124**: 437, 1938.
7. McLean, F. C., and Hastings, A. B.: A Biological Method for the Estimation of Calcium Ion, J. Biol. Chem. **107**: 351, 1934.
8. Sollman, T.: A Manual of Pharmacology, ed. 3, Philadelphia, 1928, W. B. Saunders Co., p. 1049.
9. Farley, D. L., St. Clair, H., and Reisinger, J. A.: Am. J. M. Sc. **180**: 336, 1930.

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J. DENTAL RES. 43: 433-436, 1964.

Serum Ascorbic Acid Levels of Guinea Pigs Following Soft- and Hard-Tissue Wounding

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Ascorbic acid deficiency is known to result in retardation of wound healing. But the extent to which wounding influences ascorbic acid metabolism is not completely understood.

A study was conducted to organize and evaluate the effect of wound infliction on ascorbic acid-serum levels in control and ascorbic acid-deficient guinea pigs.

Materials and Methods

Twenty female guinea pigs weighing initially approximately 450 gm. were used for this study. All animals were fed a basic diet[1] (Table 1) for a two-week period to establish

TABLE 1

BASIC DIET OF GUINEA PIGS*

	Per Cent by Weight
Rolled oats	39
Dry skim milk (heated)	30
Bran	20
Cod-liver oil	4
Cotton-seed oil	6
Sodium chloride	1
Raw cabbage-ascorbic acid source	..

* Modification of diet of Sherman and La Mer (*J. Amer. chem. Soc.*, **44**: 165, 1922).

pretest ascorbic acid-serum levels. Fresh raw cabbage was fed at the same time each morning to provide an adequate source of ascorbic acid. The feeding time was noted in relation to the time blood was drawn, since the work of Karel and Chapman[2] illustrated that this was a factor that influenced plasma ascorbic acid levels.

Animals were housed in individual cages under conditions of constant temperature and humidity. Fresh water was provided daily. All animals were weighed weekly.

Serum samples were obtained by drawing approximately 2 ml. of blood by intracardiac-puncture technique using a 20-gauge disposable needle while the animal was under ether anesthesia. Samples were immediately centrifuged at 2,000 rpm for 15 minutes. Freshly prepared 5 per cent trichloracetic acid was added to the supernatant (1:5 v/v). This mixture was again centrifuged. The supernatant fluid was drawn off using 60-mm.[3]

This investigation was supported in part by USPHS General Research Support Grant 1-GS-14, College of Dentistry, State University of Iowa.

Presented in part at the forty-first Annual Meeting of the IADR, Pittsburgh, Pennsylvania, March 21, 1963.

Received for publication September 4, 1963.

pipettes, transferred into micro test tubes, which were capped with parafilm,* and frozen until analyzed by the micro adaption of Lowery, Lopez, and Bessey[3] for ascorbic acid.

After establishing pretest serum-ascorbic acid levels (Table 2), the animals were divided into two equal groups. One group was maintained on the basic diet as controls. Cabbage was removed from the diet of the experimental animals, placing them on a scorbutic diet.

Each experimental animal was paired with a control animal. Blood samples were collected on the pairs a minimum of three times, namely: (1) when one mate was placed on the experimental diet, (2) when both animals were wounded, and (3) when both animals were sacrificed. Wounding was done on pairs when the experimental animals

* E. H. Sargent Co., Chicago, Illinois.

TABLE 2

SERUM-ASCORBIC ACID LEVELS OF CONTROL AND DEFICIENT ANIMALS AT THE THREE STAGES

(Mg. Per Cent)

Animals	Pretest*	Wounding	Sacrifice†
Control:			
Range	0.38–0.60	0.38–0.59	0.38–0.61
Mean	0.47	0.46	0.50
Deficient:			
Range	0.30–0.45	0.06–0.29	0.01–0.14
Mean	0.40	0.12	0.08

* Average of 7 control and 10 deficient animals.
† Average of 6 control and 8 deficient animals.

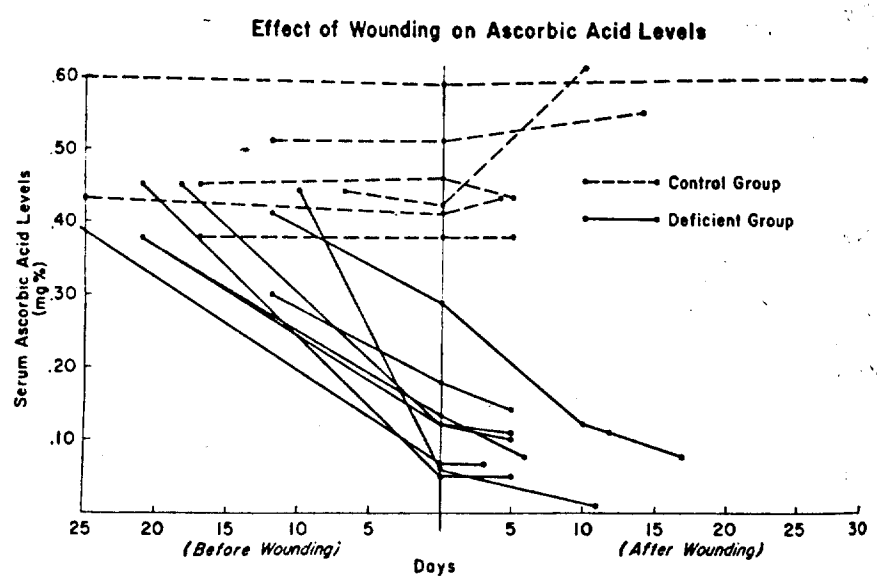

FIG. 1.—Serum-ascorbic acid levels of individual guinea pigs before wounding, at the time of wounding, and after wounding.

were from 7 to 30 days deficient. The pairs were sacrificed from 3 to 18 days postoperative depending in part upon the health and clinical appearance of the deficient mate (Fig. 1).

Wounding consisted of making a soft-tissue incision with a number 14 surgical scalpel. Using the best results of the unpublished research of Waite, Calderwood, and Hera* on the comparison of the healing rate of bone in dog mandible, after the production of defects by various rotary instruments, the number 8 round bur was used to create the bony wound.

Wounds were made on both hind limbs with the depth of the bony wound equal to the diameter of the head of the bur. Conventional speed was used on the left limb, and a high-speed Weber unit with an approximate speed of 200,000 rpm was used to make the wound on the opposite limb.

All surgery was conducted under as sterile conditions as possible for animal research.

Results

Female guinea pigs weighing approximately 450 gm. had pretest serum-ascorbic acid levels ranging from 0.30-0.60 mg. per cent (Table 2). After 25-35 days, serum-ascorbic acid levels of animals on a scorbutic diet fell to 0.06-0.29 mg. per cent, while serums of control animals fed a source of ascorbic acid maintained pretest levels. Following wounding, curves of deficient animals fell at a less accelerated rate than before wounding or leveled off gradually (Fig. 1). This was noted for animals deficient 10 days as well as for animals deficient as much as 35 days. Ascorbic acid levels of control animals remained the same or rose slightly after wounding in all but one animal.

The micro adaption of the colorimetric determination for serum ascorbic acid was found to give accurate, repeatable values when only 20 mm.^{3} of serum was used. Also, serum to which 5 per cent trichloracetic acid was added was frozen and stored up to three months without altering the measurable ascorbic acid levels.

One to two ml. of blood was taken as often as every third day from animals of this size without any adverse effects on weight or ascorbic acid curves. The loss of animals that died during the study was traced in part to hemorrhage due to poor cardiac-puncture technique or respiratory difficulty from ether anesthesia.

Discussion

The change in serum-ascorbic acid levels following soft- and hard-tissue wounding in this study is not compatible with findings reported by several authors. Chatak[4] reported that the ascorbic acid content of blood and urine was diminished in animals suffering from bone fracture. Giglio[5] observed a sharp decrease in blood ascorbic acid in dogs after the trauma of intestinal occlusion.

The consistent fall of serum-ascorbic acid levels of deficient animals at a less accelerated rate after wounding in this study suggests that stress from wounding triggered an outpouring of ascorbic acid into the blood stream from some "stored" source, possibly the adrenal gland. Rinfret[6] has reported a depletion of ascorbic acid in the adrenal glands of wounded animals. Clayton and Prunty[7] have shown in their research the importance of adrenocortical function in scurvy. Beaton[8] has suggested a possible connection be-

* D. E., Waite, R. G., Calderwood, and S. S. Hera. "A Comparison of the Healing Rate of Bone after the Production of Defects by Various Rotary Instruments." Unpublished data.

tween scurvy and thyroid gland function. Katsh, Katsh, and Osher[9] have shown that following a stress situation adrenal weight and ascorbic acid content were reduced.

Summary

Wounds were inflicted into the tibia of adult female guinea pigs in varying stages of ascorbic acid deficiency. Using an intracardiac-puncture technique, blood was drawn from control and deficient animals, then analyzed. After wounding, ascorbic acid–serum levels of deficient animals fell at a less accelerated rate than before wounding or leveled off. Ascorbic acid serum levels of control animals maintained pretest levels. The study needs further investigation before conclusions can be presented.

The authors wish to express appreciation to Evelyn Denzin and Karen Wyborny for care of animals and to Jai Kang Song for technical assistance with chemical determinations.

References

1. Sherman, H. C., La Mer, V. K., and Campbell, H. L. The Quantitative Determination of the Anti-Scorbutic Vitamin. *J. Amer. chem. Soc.*, **44**:165–72, 1922.
2. Karel, L., and Chapman, C. W. The Vitamin C Level of the Blood Plasma in Guinea Pigs, *J. Nutr.*, **28**:89–92, 1944.
3. Lowery, O. H., Lopez, J. A., and Bessey, J. The Determination of Ascorbic Acid in Small Amounts of Blood Serum. *J. biol. Chem.*, **160**:609–15, 1945.
4. Chatak, S. D., Dhor, S. C., Agarwala, S. C., and Shrivastava, D. L. Changes in Ascorbic Acid Metabolism in Guinea Pigs during Fracture, *J. sci. industr. Res.*, **13**:549–53, 1954.
5. Giglio, A. L. Content of Vitamin C in Blood Serum in Acute Experimental Intestinal Occlusion, *Pat. sper.*, **42**:275–84, 1954. *Chem. Abstr.*, **49**:7101, 1955.
6. Rinfret, A. P., and Hane, S. Depletion of Adrenal Ascorbic Acid Following Stress in the Infant Rat, *Endocrinology*, **57**:497–99, 1956.
7. Clayton, B. E., and Prunty, F. T. Relation of Adrenal Cortical Function to Scurvy in Guinea Pig. The Effect of Exogenous A.C.T.H. and Adrenalectomy, *J. Endocr.*, **9**:370–78, 1953.
8. Beaton, G. H., Hellebust, W. P., and Wright, A. M. The Effect of Scurvy on Thyroid Activity in the Guinea Pig, *J. Nutr.*, **70**:321–28, 1960.
9. Katsh, S., Katsh, G. F., and Osher, P. Adrenal, Pituitary, and Urinary Ascorbic Acid Levels in Rats Subject to Hypothermic Environment, *Amer. J. Physiol.*, **178**:457–61, 1954.

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Vitamin C Sources in Eskimo Food

It is the general experience among arctic explorers that when they 'live of the land', scurvy never occurs. Stefansson[1], who directed attention to this fact, cured three cases of scurvy with reindeer meat.

A Norwegian scientific expedition to Angmagssalik was made during 1936–37 for the purpose of examining nutrition, metabolism, physique and health of Eskimos. The accompanying table shows the vitamin C content of the available food. The numbers are means of at least two observations. We used the method of Emmerie and van Eekelen[2].

It must also be mentioned that 'mattaq' (epidermis of whale) which Bertelsen[3] especially has pointed out as a good antiscorbutic remedy, contained 20 mgm. ascorbic acid per 100 gm. (from narwhal). In skin of young seals from the coast of Norway we found such enormous amounts as 180 mgm. per 100 gm.

It is well known that marine algæ are used as food in Japan, China, Hawaii, and some algæ are eaten on Iceland and on the coast of certain parts of Great Britain. The Eskimos eat them raw or dipped in boiling water (or broth) or with blubber oil. From this source, the Angmagssalik Eskimo get 50 per cent of their vitamin C.

The other 50 per cent is mainly obtained from organs of mammals and birds and also from fish. On an exclusive lean meat diet one gets about 25 mgm. vitamin C a day, even when the meat is boiled. By boiling, 50 per cent is extracted or oxidized. If the broth also is used, one gets nearly 40 mgm. a day. This agrees with the experiences of Stefansson[1].

Vitamin C (*l*-ascorbic acid) in common Eskimo foods

(Mgm. per 100 gm.)

Fjord seal (*Ph. fœtida*)	
Adrenals	127
Thymus	26
Liver	18
Central nervous system	18
Epididymis	17
Kidney	13
Eye	10
Lymph gland	7
Pancreas	7
Ovary	7
Skin	3
Small intestine	3
Blood	3
Meat	2
Heart	2
Blubber	0
Other organs (average)	1
Plants	
Rosewort (*Sedum roseum*)	27
Mountain willow (*Salix* sp.)	18
Dandelion, leaves (*Taraxacum officinale*)	15
Angelica (*Archangelica officinalis*)	14
Crakeberries (*Empetrum hermafrodit.*)	9
Guillemot (*Uria grylle*)	
Liver	22
Brain	16
Eye	9
Intestine	5
Eiderduck (*Somateria mollissima*)	
Liver	17
Eye	14
Brain	9
Intestine	4
Invertebrates	
Sea mussel (*Mytilus edulis*)	6
Crayfish sp.	3
Cod (*Gadus callarias*)	
Liver	8
Meat	2
Roe	44
Cod liver oil	0
Marine plants	
Alaria sp.	45
Rhodymenia palm.	17
Fucus sp.	13
Ascophyllum nod.	11

In the opinion of one of the authors (A. H.), who has been on sledging journeys of long duration in the Arctic, less than 15 mgm. ascorbic acid a day effectively prevents scurvy.

The Angmagssalik Eskimo get a daily average dose of about 40 mgm. per adult. In agreement with this finding, scurvy is unknown among Angmagssalik Eskimos.

A detailed report on the results of the work of the expedition will appear in a book this autumn.

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May 6.

[1] Stefansson, "Observations on Three Cases of Scurvy", *J. Amer. Med. Assoc.*, **71**, 1715–1718 (1918).

[2] Emmerie, A., and van Eekelen, M., "Some Critical Remarks on the Determination of Ascorbic Acid", *Biochem. J.*, **30**, 25–27 (1936).

[3] Bertelsen, A., "Animalske antiscorbutica i Grønland", *Hospitalstidende*, **54**, 537 (1911).

Poult Sci 45: 100-4 Jan 66

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Effect of Dietary Ascorbic Acid and Hesperidin on Capillary Fragility of Broilers[1]

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(Received for publication July 5, 1965)

Discolorations of poultry carcasses caused by bruises or hemorrhages continue to be a serious problem to the poultry processing industry. According to Crothers and Helbacks (1960), bruises and hemorrhages accounted for the greatest and fourth greatest causes, respectively of undergrade broilers in Delmarva processing plants.

It was established by Hamdy *et al.* (1961a) that approximately 90 percent of the bruises on commercial broiler carcasses are inflicted within the 13 hours immediately preceding slaughter. Various handling techniques and equipment used to catch and transport chickens are factors partly determining the numbers of bruises inflicted. (Cox and May, 1964; Helbacka, 1962; Hough, 1954; Jewett, 1960; Jewett and Saunders, 1960; Kaiser and Smith, 1958). In addition to physical factors, certain physiological conditions of the bird such as age and previous bruising affect the incidence of bruising or rate of healing (Hamdy *et al.*, 1961b, c).

It would appear logical that the relative strength of capillaries would influence the ease of inflicting a bruise. Two compounds have been used rather extensively as therapeutic agents for humans with conditions involving capillary fragility and spontaneous hemorrhaging. These compounds are ascorbic acid and hesperidin (a citrus bioflavonoid). Many workers have shown beneficial results using either one or both of these compounds in mammals to strengthen capillaries (Scarborough and Edin, 1938; Sokoloff *et al.*, 1957; Bacharach *et al.*, 1942). Hamdy *et al.* (1961d) demonstrated that sodium ascorbate injected directly into the bruised tissue increased healing rate of broilers.

The present study was conducted to determine the influence of ascorbic acid and hesperidin administered to broilers in the feed on capillary fragility and rate of healing of bruises.

PROCEDURE

White Plymouth Rock male chicks were used in all experiments. The birds were started in battery brooders with wire floors and grown to four or five weeks of age then transferred to wire floored finisher batteries and held until 7 or 8 weeks of age. A commercial all-mash broiler starter was fed until birds were 5 weeks old and a commercial broiler finishing mash was fed for the remainder of the growing period. For treatment groups, ascorbic acid, crude hesperidin, or pure hesperidin[2] were added at varying levels, replacing the total ration.

Capillary fragility was measured, using a device very similar to that reported by Bacharach *et al.* (1942). The wing webs were clipped free of feathers then extended

[1] Journal Paper No. 441 College Experiment Station, College of Agriculture Experiment Stations, University of Georgia, Athens, Ga. Parts of this paper were taken from the M. S. theses of the second and third authors.

[2] Crude hesperidin was a commercial product containing 53% hesperidin and pure hesperidin was a semi-purified product containing 97% hesperidin as determined by the University of Florida Citrus Experiment Station, Winter Haven, Florida.

TABLE 1.—*Levels of hesperidin and ascorbic acid fed to chicks in Experiments 1, 2, and 3*

Experiment number	Ingredient fed	Levels of ingredient fed (hesperidin in kg./908 kg.; Ascorbic acid in mg./0.454 kg.)	Feeding period
1	Pure hesperidin	0.23, 0.45, 1.1, 2.3, 3.6, 5.4	Continuous
	Pure hesperidin	0.23, 0.45, 1.1	Last 2 weeks only
	Crude hesperidin	0.23, 0.45, 1.1, 2.3	Continuous
	Crude hesperidin	0.23, 0.45, 1.1	Last 2 weeks only
	Control	0.00	Continuous
2	Ascorbic acid	5, 10, 50, 100, 1,000	Continuous
	Ascorbic acid	5, 10, 50, 100, 1,000	Last week only
	Control	0.00	Continuous
3	Crude hesperidin	0.45, 1.1, 2.3	Continuous
	Crude hesperidin	1.1, 2.3	Last week only
	Ascorbic acid	5, 10, 50	Continuous
	Ascorbic acid	10, 50	Last week only
	Control	0.00	Continuous

over a glass plate which had a 100 watt light underneath. A glass suction cup was placed on the ventral side of the web and a negative pressure applied, by means of a vacuum pump, through a hose attached to the suction cup. Vacuum pressure was measured by means of a Hg manometer. Time until petechial hemorrhages appeared was measured by use of a stop watch. One operator made all observations of appearance of petechiae. Two observations were made on each bird and averaged for statistical analysis.

Bruises were inflicted using the device described by Hamdy *et al.* (1961b). The bruised birds were examined at 24 hour intervals after bruising with birds showing no visible signs of the bruise being recorded as healed. Observations continued until all bruises were healed.

Statistical analyses consisted of analysis of variance and tests of significant means by use of Duncan's multiple range test (Duncan, 1955) or the extended multiple range test of Kramer (1956).

Experiment 1. In this experiment 36 lots of 8 chicks each were used. Duplicate lots were fed rations with hesperidin levels as shown in Table 1, while four lots received the control ration. At 8 weeks of age all birds were tested individually for capillary fragility. In taking the capillary fragility readings, the suction cup was placed on the wing web when the manometer read 5.08 cm. of vacuum and the negative pressure was increased 5.08 cm. every 15 seconds until petechiae appeared. This time was then converted to total seconds for statistical analysis.

Experiment 2. Two separate trials were conducted using 33 lots of 9 chicks in each. In each trial, triplicate lots were fed rations with ascorbic acid levels as shown in Table 1. At 7 weeks of age the capillary fragility was measured using the same equipment as in Experiment 1 but with negative pressure preadjusted to 60.96 cm. before applying the suction cup to the wing web. Time required to form petechiae was recorded in seconds.

Relative, subjective vascularity scores were assigned to birds during the capillary fragility measurements in one trial. Scores ranged from 1 (no visible small capillaries) to 4 (highly vascular).

Two lots of birds on each diet were bruised by administering 3 blows on the breast with the bruising instrument.

Results of the two trials were combined for analysis.

TABLE 2.—*Significance of mean times required for appearance of petechiae, Experiment 1*[1]

Hesperidin Treatment (kg./908 kg. feed)	Mean time[2] (seconds)
0.45 kg., pure, continuous	55.3
0 kg., control	48.7
1.1 kg., crude, continuous	47.8
0.23 kg., pure, continuous	47.3
1.1 kg., crude, last 2 weeks	39.7
2.3 kg., pure, continuous	38.8
0.45 kg., crude, continuous	38.6
2.3 kg., crude, continuous	36.7
1.1 kg., pure, continuous	36.5
5.4 kg., pure, continuous	36.2
0.45 kg., crude, last 2 weeks	35.3
0.23 kg., crude, continuous	34.1
0.23 kg., crude, last 2 weeks	33.9
3.6 kg., pure, continuous	33.9
0.45 kg., pure, last 2 weeks	33.8
0.23 kg., pure, last 2 weeks	33.6
1.1 kg., pure, last 2 weeks	32.5

[1] As calculated by the Kramer test.
[2] Any 2 means not bordered (on the right) by the same line are significantly different at the 0.05 level of probability.

Experiment 3. Thirty-three lots of 9 birds each were fed rations containing either ascorbic acid or crude hesperidin as shown in Table 1. Each ration was fed to triplicate lots. At 8 weeks capillary fragility and vascularity were measured as in Experiment 2. One lot of birds from each ration were bruised on the breast using 3 blows of the bruising instrument.

RESULTS

Experiment 1. Capillary fragility measurements differed significantly among treatments and for the treatment × replicate interaction in the analysis of variance. When the means were tested (Table 2) it was found that nearly all hesperidin treatments had a significantly deleterious effect upon capillary fragility. Most differences among treated lots were not significant and no consistent trend appeared among levels or types of hesperidin fed or the length of the feeding period.

Experiment 2. Ascorbic acid in the feed significantly affected capillary strength but there were also significant variations among replicates. The multiple range analysis of mean times required for formation of petechiae showed that most treatment groups did not differ from the control group (Table 3). There was a tendency for treated groups to exhibit more fragile capillaries than the control, although only one lot (50 mg., last week) differed significantly.

Vascularity scores were found to differ significantly among treatments and replicates. Examination of mean scores indicated that all ascorbic acid treatments, except one (100 mg., last week), differed significantly from the control (Table 4). Treated birds had less vascular development of the wing web than did the controls.

The healing rate of bruised birds varied significantly among treated groups (Table 5). All lots of birds receiving ascorbic acid, except one (1000 mg., last week), healed significantly faster than the control lot. Birds receiving ascorbic acid for the entire feeding period healed faster than those treated only for the last week but differences were not significant in most cases.

Experiment 3. All lots of birds receiving either ascorbic acid or hesperidin formed petechiae in significantly less time than did controls (Table 6). Thus, both ascorbic acid and hesperidin weakened

TABLE 3.—*Significance of mean times required for appearance of petechiae, Experiment 2*[1]

Ascorbic acid treatment (mg./0.454 kg.)	Mean time[2] (seconds)
10 mg., last week	8.5
1,000 mg., last week	8.0
0 mg., control	7.6
10 mg., continuous	7.6
5 mg., continuous	7.4
100 mg., continuous	7.3
50 mg., continuous	7.2
1,000 mg., continuous	6.9
5 mg., last week	6.8
100 mg., last week	6.2
50 mg., last week	6.0

[1] As calculated by the Duncan test.
[2] Any 2 means not bordered (on the right) by the same line are significantly different at the 0.05 level of probability.

capillaries. In the groups fed crude hesperidin continuously, the strength of capillaries decreased significantly with increasing levels of hesperidin. In ascorbic acid fed lots the level did not have any evident effect.

No statistical evaluation of healing time was conducted on data in this experiment but treated groups, except for the hesperidin lots fed only the last week, had mean healing times less than those of the controls. Mean healing times in days were: control, 4.50; hesperidin fed last week, 4.53; ascorbic acid fed continuously 4.05; hesperidin fed continuously 3.48; and ascorbic acid fed last week, 3.47.

DISCUSSION AND CONCLUSION

It is evident from the data presented herein that both ascorbic acid and hesperidin administered to broilers via the feed tend to reduce the strength of capillaries as measured by the method used. This confirms the observation of May and Noles (1965) that hesperidin in the feed of broilers has no beneficial effect for prevention of bruises and hemorrhages of broilers handled in a conventional manner during catching and processing. However, since May and Noles (1965) did not report a high incidence of bruises or hemorrhages in hesperidin treated broilers, it may be that the decrease in capillary strength of treated birds in the present study is not of sufficient magnitude to cause problems in commercial handling. The decrease in capillary strength is in contrast to results with mammals in several studies including Sokoloff *et al.* (1957) and Bacharach *et al.* (1942).

Both ascorbic acid and hesperidin tended to increase healing rate of bruised broilers. This confirms the results with injected sodium ascorbate reported by Hamdy *et al.* (1961d). It is possible that some of the decrease in time required to heal could be attributed to a decrease in blood released in contused areas due to the decrease in vascu-

TABLE 4.—*Significance of mean degreee of vascularity in the wing web of broilers in Experiment 2*[1]

Ascorbic acid treatment (mg./0.454 kg.)	Mean vascularity scores[2]
0 mg., control	3.51
100 mg., last week	3.11
50 mg., last week	3.00
10 mg., last week	3.00
5 mg., last week	2.88
1,000 mg., continuous	2.85
5 mg., continuous	2.77
100 mg., continuous	2.76
10 mg., continuous	2.74
50 mg., continuous	2.73
1,000 mg., last week	2.59

[1] As calculated by the Duncan test.
[2] Any 2 means not bordered (on the right) by the same line are significantly different at the 0.05 level of probability. Possible range in score was 1 to 4 with higher scores indicating more vascularity.

TABLE 5.—*Significance of mean number of days required for bruised birds to heal, Experiment 2*[1]

Ascorbic acid treatment (mg./0.454 kg.)	Mean days required to heal[2]
0 mg., control	5.25
1,000 mg., last week	4.78
50 mg., last week	4.57
5 mg., last week	4.40
10 mg., last week	4.26
100 mg., last week	4.22
10 mg., continuous	4.20
5 mg., continuous	4.17
50 mg., continuous	4.00
1,000 mg., continuous	3.94
100 mg., continuous	3.94

[1] As calculated by the Duncan test.
[2] Any 2 means not bordered (on the right) by the same line are significantly different at the 0.05 level of probability.

TABLE 6.—*Kramer's extended multiple range test of mean time required to form petechiae in wing webs of birds, Experiment 3*

Treatment level	Mean time to form petechiae[1]
Control	8.05
2.3 kg. crude hesperidin/908 kg., last week	7.46
0.54 kg. crude hesperidin/908 kg., continuous	7.07
50 mg. ascorbic acid/0.454 kg., continuous	6.37
50 mg. ascorbic acid/0.454 kg., last week	6.19
1.1 kg. crude hesperidin/908 kg., continuous	5.65
10 mg. ascorbic acid/0.454 kg., continuous	5.39
10 mg. ascorbic acid/0.454 kg., last week	5.23
1.1 kg. crude hesperidin/908 kg., last week	4.70
2.3 kg. crude hesperidin/908 kg., continuous	4.56
5 mg. ascorbic acid/0.454 kg., continuous	4.30

[1] Any 2 means not bordered (on the right) by the same line are significantly different at the 0.05 level probability.

larity of the treated groups. This contention would require further investigation for confirmation. Also, added ascorbic acid in diets of bruised birds might be available to aid in healing in any one of several different ways as discussed by Hamdy *et al.* (1961d). The reason for the bird reacting differently than mammals when fed ascorbic acid or hesperidin is not known. Perhaps it is related to the fact that birds synthesize ascorbic acid readily.

It is concluded on the basis of the results herein that, although both ascorbic acid and hesperidin aid in healing of bruised tissue, neither compound is of benefit for prevention of bruising of chickens.

SUMMARY

White Plymouth Rock male chicks were fed varying amounts of ascorbic acid or hesperidin in their feed in a series of experiments. Both compounds tended to decrease the amount of time required for bruised birds to heal. However, both compounds tended (significantly in most instances) to decrease the strength of capillaries in the wing web as measured by a vacuum technique. It was concluded that neither ascorbic acid nor hesperidin are beneficial in prevention of bruising of chickens when added to feed.

ACKNOWLEDGMENTS

The authors are grateful to the National Institutes of Health Grant EF-00165; Kuder Citrus Feed Company, Lake Alfred, Florida, and Charles Pfizer & Company, Inc., New York, New York for partial financial support of this work.

REFERENCES

Bacharach, A. L., M. E. Coates and T. R. Middleton, 1942. A biological test for vitamin P activity. Biochem. J. 36: 407–411.

Cox, C. J., and K. N. May, 1964. Factors affecting bruising of commercial broilers. Georgia Agri. Expt. Sta., U. of Georgia College of Agri. Technical Bul. N. S. 35.

Crothers, J. L., and N. V. Helbacka, 1960. Evaluation of broiler grade defects by quality control methods. Misc. Ext. Publ. 226, U. of Maryland.

Duncan, D. B., 1955. Multiple range and multiple F tests. Biometrics, 11: 1–7.

Hamdy, M. K., K. N. May, J. P. Flannigan and J. J. Powers, 1961a. Determination of the age of bruises in chicken broilers. Poultry Sci. 40: 787–789.

Hamdy, M. K., K. N. May and J. J. Powers, 1961b. Some physical and physiological factors affecting poultry bruises. Poultry Sci. 40: 790–795.

Hamdy, M. K., K. N. May and J. J. Powers, 1961c. Some biochemical changes occurring in experimentally inflicted poultry bruises. Proc. Soc. Exp. Biol. Med. 108: 184–188.

Hamdy, M. K., K. N. May, J. J. Powers and D. E. Pratt, 1961d. Effect of ascorbate on healing of poultry bruises. Proc. Soc. Exp. Biol. Med. 108: 189–192.

Helbacka, N. V., 1962. Studies on bruising and breast blisters. Misc. Ext. Pub. 271, U. of Maryland.

Hough, J. W., 1954. Rough handling injures poultry profit. Farm Economics No. 192, New York State College of Agri.

Jewett, L. J., 1960. Handling and processing broilers in Maine, Part 1. Maine Agri. Exp. Sta. Bul. 592, University of Maine.

Jewett, L. J., and R. F. Saunders, 1960. Handling and processing broilers in Maine, Part 2. Maine Agri. Exp. Sta. Bul. 593, U. of Maine.

Kaiser, W. K., and R. C. Smith, 1958. Factors affecting the bruising of broilers. Delaware Agri. Exp. Sta. Tech. Bul. 323, U. of Delaware.

Kramer, C. Y., 1956. Extension of multiple range tests to group means with unequal numbers of replications. Biometrics, 12: 307–310.

May, K. N., and R. K. Noles, 1965. Effect of hesperidin and litter moisture on growth and carcass defects of broilers. Poultry Sci. 44: 844–848.

Scarborough, H., and M. B. Edin, 1938. Effect of hesperidin (Vitamin P) on capillary fragility. Lancet, 235: 610–612.

Sokoloff, B. T., W. C. Martin and C. C. Sachof, 1957. Capillary fragility in older people. The evaluation of bio-flavonoid therapy. J. Am. Geriat. Soc. 5: 306–318.

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Austral. J. Exp. Biol. Med. Sci., 25-17-23 1947

THE INFLUENCE OF FERROUS IRON IN THE DETERMINATION OF ASCORBIC ACID

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(Accepted for publication 5th September, 1946.)

Ascorbic acid in fresh and processed foods is now commonly determined by extraction with 3 p.c. metaphosphoric acid and titration with approximately 0·001 N 2·6 dichlorophenolindophenol. The oxidation of ascorbic acid is promoted both by enzymes and copper in the tissues and metaphosphoric acid preserves the vitamin by inactivating these catalysts. In processed foods the enzymes have already been inactivated by heat, and copper catalysis is generally of greater importance. This effect is probably determined by the concentration of free copper ions, which metaphosphoric acid reduces to a negligible level.

A disadvantage of metaphosphoric acid is that ferrous salts are oxidized quantitatively by the dye in this medium. Canned foods normally contain low concentrations of iron, which is probably retained mostly in the ferrous condition on account of the reducing conditions within the can. A concentration of iron of ten parts per million is quite common, and this could introduce a positive error of 1·6 mg./100 gm. into the determination of ascorbic acid. Errors of this order are significant in assays of many canned foods containing less than 15 mg./100 gm. of ascorbic acid and in studies of ascorbic acid retention in canned foods during storage when the iron content may increase as the ascorbic acid content decreases. A concentration of 50 parts of iron per million, which occurs less frequently, could increase the error to about 8 mg. of ascorbic acid per 100 gm. Higher concentrations, e.g. of the order of 100 parts of iron per million, are encountered only when there is serious internal corrosion of the can, usually associated with hydrogen swelling. Cases have occurred where a high level of iron contamination was derived from the equipment used in processing.

To correct for the presence of ferrous iron in metaphosphoric acid extracts, Lugg (1942) has proposed the use of formaldehyde, which at pH 3·5 condenses rapidly with ascorbic acid, but only slowly with ferrous salts. Hydrogen peroxide has been shown by Marston (1943) and Levy (1943) to eliminate interference by sulphur dioxide. In a pure solution of sulphur dioxide and ascorbic acid, the sulphur dioxide is oxidized rapidly by hydrogen peroxide with only slight attack on the ascorbic acid. The authors have found, however, that when ferrous iron is present in the ascorbic acid solution, the ferrous iron is oxidized by hydrogen peroxide and some of the ascorbic acid is also destroyed. Thus, in extracts containing ferrous iron, the use of hydrogen peroxide converts a positive error into a negative error of less magnitude. Hydrogen peroxide also promotes a slow reoxidation of the reduced dye and the titration should be completed as soon as possible after adding this reagent.

An alternative method of eliminating the interference of ferrous iron is to use extractants other than metaphosphoric acid. Lorenz and Arnold (1938) have shown that ferrous salts titrate with the dye in pure solutions of metaphosphoric or oxalic acid, but not in citric, acetic, phosphoric, sulphuric, hydrochloric, nitric or trichloracetic acids. The effect in metaphosphoric or oxalic acid solutions is probably due to displacement of the ferrous-ferric equilibrium by formation of complex ferric ions, as suggested by Michaelis and Friedheim (1931).

Feaster and Alexander (1944) claim that, if 8 p.c. acetic acid is used for extraction of canned foods, the titre is not affected by ferrous iron up to 100 parts

per million. We have investigated the effect of alternative extractants both on interference by ferrous iron and on the retention of ascorbic acid. Particular attention has been given to acetic and sulphuric acids. These acids reduce interference by ferrous iron, but Ponting (1943) has shown that they are definitely inferior to metaphosphoric and oxalic acids in retention of ascorbic acid.

EXPERIMENTAL.

Extraction with Metaphosphoric Acid.

Ferrous iron interference in metaphosphoric acid extracts and the effect of hydrogen peroxide were first studied. Six canned products—orange juice, tomato juice, broccoli, cabbage, silver beet and French beans—were selected for investigation. Twenty ml. of the liquid portion of each can were diluted to 100 ml. with 3 p.c. metaphosphoric acid. A suitable aliquot (5 ml.) was titrated with approximately 0·001 N dye solution. Similar aliquots were titrated after addition of:

(a) 1 ml. of 1·5 p.c. hydrogen peroxide.
(b) 2 ml. of 0·002 N ferrous ammonium sulphate and.
(c) 2 ml. of 0·002 N ferrous ammonium sulphate, followed by 1 ml. of 1·5 p.c. hydrogen peroxide.

The results were calculated as mg. of ascorbic acid per 100 ml. of the original liquor and are given in Table 1. The added ferrous ammonium sulphate was equivalent to 224 parts per million of ferrous iron or 35·2 mg. of ascorbic acid per 100 ml.

TABLE 1.

The effect of ferrous iron in metaphosphoric acid extracts.

	Apparent ascorbic acid (mg./100 ml.)			
Product.	Control.	H_2O_2.	Ferrous Iron.	Ferrous Iron. + H_2O_2.
Orange Juice	33·5	32·9	69·3	30·2
Tomato Juice	37·0	36·7	72·1	33·0
Broccoli	38·5	37·5	71·7	28·3
Cabbage	12·5	12·4	47·7	10·5
Silver Beet	13·5	13·3	49·5	9·0
French Beans	6·8	6·3	41·6	3·6

The addition of ferrous iron increased the apparent ascorbic acid content by 33–36 mg./100 ml. Hydrogen peroxide alone reduced the ascorbic acid figure by 0·1–1·0 mg./100 ml., but with ferrous iron the loss was increased to 2·0–19·0 mg./100 ml. The iron content of the original products was not more than ten parts per million. It is apparent that hydrogen peroxide cannot be used indiscriminately for elimination of iron interference in metaphosphoric acid extracts. The ascorbic acid figure obtained in the presence of hydrogen peroxide is sufficiently accurate when it is only slightly less than the figure obtained without hydrogen peroxide, *i.e.* when the difference is not more than 1 mg./100 ml. Where the difference is considerable, however, some other method of eliminating the interference of ferrous iron is required.

Extraction with Acetic and Sulphuric Acids.

Interference by ferrous iron. The effect of alternative extractants on ferrous iron interference was then investigated. Similar dilutions of the canned food liquors were made with 10 p.c. acetic acid and 0·5 N sulphuric acid and 5 ml. aliquots were titrated with and without the addition of 2 ml. of 0·002 N ferrous ammonium sulphate. The results are given in Table 2.

The use of 10 p.c. acetic acid in place of 3 p.c. metaphosphoric acid considerably reduced ferrous iron interference, except in the case of silver beet. Sulphuric acid (0·5 N) practically eliminated this interference in all products except silver beet. With silver beet, interference was still appreciable in 0·5 N sulphuric acid, but much less than in 10 p.c. acetic or 3 p.c. metaphosphoric acid.

The pH values of the extracts were approximately as follows:

3 p.c. metaphosphoric acid, 0·8; 10 p.c. acetic acid, 2·5; 0·5 N sulphuric acid, 0·8.

Ferrous iron does not titrate with the dye in pure solutions of acetic or sulphuric acid and the interference in extracts is probably due to low concentrations of oxalic or other organic acids from the tissues. The worst interference was obtained in extracts of silver beet, which

is known to be high in oxalate. The lesser interference in sulphuric as compared with acetic acid may be due to the effect of pH on the ionisation of organic acids from the tissues and on the formation of ferric complexes by these organic acids.

TABLE 2.

The effect of ferrous iron in acetic acid and sulphuric acid extracts.

Extracting solution.	Product.	Apparent ascorbic acid (mg./100 ml.) Control.	Ferrous Iron.
10 p.c. Acetic Acid	Orange Juice	32·4	41·7
	Tomato Juice	36·0	42·5
	Broccoli	37·0	38·1
	Cabbage	10·7	11·8
	Silver Beet	12·7	49·0
	French Beans	4·8	6·8
0·5 N Sulphuric Acid	Orange Juice	18·4	17·3
	Tomato Juice	35·3	35·2
	Broccoli	36·6	35·9
	Cabbage	10·5	10·0
	Silver Beet	11·8	21·4
	French Beans	5·2	5·7

To determine the effect of low concentrations of organic acids on ferrous iron interference, investigations were carried out in solutions containing 0·1 M sodium acetate adjusted to various pH levels from 0·4 to 3·0 with sulphuric acid. In these solutions ferrous iron did not react with the dye over the whole range of pH.

When the solutions contained in addition 0·1 M tartrate or citrate, ferrous iron gave appreciable titre at pH 3·0, but not at pH 2·0 or less.

With 0·1 M oxalate, ferrous iron reacted almost quantitatively with the dye over the whole range of pH. At pH 1·0 and above, an appreciable titre was obtained with as little as 0·005 M oxalate present, but at pH 0·4, there was no reaction with 0·02 M oxalate or less.

Loss of ascorbic acid. Alternative extractants, such as acetic or sulphuric acid, must ensure adequate retention of ascorbic acid as well as eliminate interference by ferrous iron. To investigate relative loss of ascorbic acid, extracts were titrated (a) immediately and (b) after standing for two hours at laboratory temperature. The results were expressed as percentage loss of ascorbic acid and are given in Table 3.

TABLE 3.

Loss of ascorbic acid in extracts after two hours.

Product.	P.c. loss of ascorbic acid in 3 p.c. metaphosphoric acid (pH 0·8).	10 p.c. acetic acid (pH 2·5).	0·5 N sulphuric acid (pH 0·8).
Orange Juice	2	1	43
Tomato Juice	1	0	30
Broccoli	4	10	15
Cabbage	7	5	60
Silver Beet	1	4	5
French Beans	22	26	53

Ascorbic acid was retained almost as well in 10 p.c. acetic as in 3 p.c. metaphosphoric acid. Retention in sulphuric acid, however, was very poor. This was quite unexpected in view of the low pH. It was decided to investigate further the effect of pH, copper and other factors on the oxidation of ascorbic acid.

Effect of copper on the oxidation of ascorbic acid. Apart from thermolabile enzymes, copper has been found to be the most important catalyst in the oxidation of ascorbic acid. Barron *et al.* (1936) showed that ascorbic acid is not autoxidisable in acid and neutral solutions up to pH 7·0. They investigated the effect of copper, iron, cobalt, nickel, manganese and calcium on the oxidation of ascorbic acid at pH 4–6, and found that copper alone had a marked catalytic effect. Copper catalysis was noticeable with as little as 0·05 parts per million.

We have investigated the oxidation of ascorbic acid in solutions containing 0·1 M sodium acetate adjusted to various pH levels from 0·4 to 3·0 with sulphuric acid. Studies were also made in solutions containing (a) 0·1 M tartrate, (b) 0·1 M citrate, (c) 0·01 M oxalate, or (d) 0·1 M sodium chloride in addition to sodium acetate and similarly adjusted with sulphuric acid. The effect of chloride was investigated because it is commonly used in canning vegetables and because Mapson (1941) has shown that, at laboratory temperatures, low concentrations increase oxidation. All solutions were prepared with glass distilled water.

The oxidation was studied at 40° C. by the following procedure. A 100 ml. florence flask containing 50 ml. of solution was brought to 40° C. in a constant temperature bath. Air was bubbled through at the rate of about 12 ml. per second. After two minutes, 2 ml. of 0·5 p.c. ascorbic acid solution were added. After mixing, 5 ml. of the solution were pipetted into 3 p.c. metaphosphoric acid (to stop oxidation) and titrated with 2·6 dichlorophenolindophenol. Further aliquots were removed at intervals and titrated. The results were expressed as p.c. oxidation of ascorbic acid after five minutes and are given in Table 4. Oxidation was studied without added copper and with the addition of ten parts of copper per million (as copper sulphate). Oxidation in the absence of copper was, however, negligible and the figures in Table 4 refer to the copper-catalysed oxidation.

The results of studies in 0·05 M phthalate buffers are included for comparison. These buffers were adjusted with sulphuric acid to avoid the pro-oxidant effect of chloride.

TABLE 4.

Oxidation of ascorbic acid (20 mg./100 ml.) in the presence of copper (10 p.p.m.)

Buffer.	Added substances.	P.c. oxidation in 5 mins. at pH. 0·4	1·0	2·0	3·0
0·05 M phthalate, adjusted with H_2SO_4	—	—	—	8·3	44·3
0·1 M sodium acetate, adjusted with H_2SO_4.	—	0	0·3	7·0	54·3
	0·1 M tartrate	0	0·3	2·7	8·3
	0·1 M citrate	0	0·3	3·4	10·5
	0·01 M oxalate	0	0	0	0·3
	0·1 M NaCl	0·3	6·1	81·8	84·6
	0·1 M NaCl + 0·01 M oxalate	0	1·2	0·9	1·8

The copper-catalysed oxidation was not significantly different in phthalate and acetate buffers. Oxidation was somewhat reduced by 0·1 M tartrate or citrate, but much more effectively by 0·01 M oxalate. In the presence of 0·01 M oxalate, oxidation was negligible at pH 0·4–2·0, and only slight at pH 3·0. Chloride (0·1 M) had a marked pro-oxidant effect, which was reduced but still appreciable in the presence of 0·01 M oxalate.

In all solutions, the copper-catalysed oxidation decreased with decreasing pH and hence another explanation is required for the rapid loss of ascorbic acid from sulphuric acid extracts of canned foods, as shown in Table 3.

Effect of ferrous iron on the oxidation of ascorbic acid. In the investigations on canned foods, loss of ascorbic acid from sulphuric acid extracts of pH 0·8 was much greater than from acetic acid extracts of pH 2·5. The same relationship was obtained when the drained liquors were diluted and adjusted to various pH levels with sulphuric acid. Canned orange juice was diluted five times and adjusted to pH 0·4, 1·0, 2·0 and 3·0 with sulphuric acid. The oxidation of ascorbic acid in these liquors was studied at 40° C. and expressed as p.c. oxidation in five minutes. The procedure was the same as for the buffer solutions. Measurements were made at each pH (a) without added copper and (b) with ten parts per million of added copper. The results are given in Table 5.

TABLE 5.

Oxidation of ascorbic acid (20 mg./100 ml.) in diluted canned orange juice.

Copper.	P.c. oxidation in 5 mins. at pH. 0·4	1·0	2·0	3·0
No added copper	13·7	3·6	0·6	0
Added copper (10 p.p.m.)	2·1	1·2	10·5	17·2

In the diluted juice without added copper, the p.c. oxidation was much greater at the lower pH, *i.e.* it was 13·7 at pH 0·4 and nil at pH 3·0. The addition of copper practically reversed the original order. It greatly increased the oxidation at pH 3·0 but reduced it at pH 0·4.

Some catalyst other than copper was obviously responsible for the high rate of oxidation at pH 0·4. The juice was ashed and an ash solution of pH 0·4 and of the same dilution as the original juice was prepared with sulphuric acid. Oxidation of ascorbic acid in this solution was very similar to the original juice. The percentage oxidation in five minutes without added copper was 17·7. With added copper (10 p.p.m.) it was 5·4. The diluted juice and ash solution contained one part per million of iron which appeared to be responsible for the catalytic effect. This was confirmed by adding this concentration of ferrous iron to a similar dilution of fresh orange juice. The per cent. oxidation in this liquor was 13·0 and ten parts per million of copper reduced it to 2·1.

Systematic studies were made of the catalytic effect of ferrous iron using solutions of 0·1 M sodium acetate adjusted to pH 0·4, 1·0, 2·0 and 3·0 with sulphuric acid. The solutions contained ten parts per million of ferrous iron, which were added as ferrous ammonium sulphate. At intervals aliquots were pipetted into 1 N sulphuric acid containing 0·1 p.c. oxalic acid and titrated immediately. This solution was used in place of metaphosphoric acid to avoid interference by ferrous iron. The results were expressed as percentage oxidation after five minutes and are given in Table 6.

The investigations have been confined to ferrous iron, which can be retained in equilibrium with ascorbic acid. Schulek and Floderer (1939) have shown that in acid solution ascorbic acid can reduce ferric iron to the ferrous condition.

TABLE 6.

Oxidation of ascorbic acid (20 mg./100 ml.) in the presence of ferrous iron (10 p.p.m.)

(Buffer 0·1 M sodium acetate adjusted with H_2SO_4.)

Added substances.	P.c. oxidation in 5 mins. at pH.			
	0·4	1·0	2·0	3·0
—	43·0	20·8	0·9	0·9
0·01 M Oxalate	2·7	2·0	0·4	0·4
0·1 M NaCl	13·5	3·0	0·3	0
0·01 M NaCl + 0·01 M Oxalate	5·1	0	0	0

The iron-catalysed oxidation was considerable at pH 0·4, but only slight at pH 2–3. This was in direct contrast to the copper-catalysed oxidation, which was negligible at pH 0·4 but rapid at pH 2–3. In the presence of 0·01 M oxalate, the iron-catalysed oxidation was reduced, but was still appreciable at pH 0·4. In this case, the rate was actually greater than appears from Table 6. The percentage oxidation in the first five minutes was 2·7, but after fifteen minutes it reached 29·8 as the rate increased considerably during the oxidation. Chloride reduced the catalytic effect of ferrous iron, again in contrast to its effect on copper.

These studies can now be related to the observed stability of ascorbic acid in extracts of canned foods. In solutions containing acetic and sulphuric acids, loss of ascorbic acid at pH 2–3 is largely due to copper catalysis. This effect, however, may be largely suppressed by low concentrations of oxalate or other substances in the tissues. Below pH 1 iron catalysis is of major importance, and this is affected much less by low concentrations of oxalate. The copper content of canned foods is usually only a fraction of the iron content. All these considerations explain why the loss of ascorbic acid was very much greater below pH 1, where iron catalysis predominated, than at pH 2–3, where most of the oxidation is due to copper catalysis.

In the investigations with diluted orange juice, added copper actually reduced the oxidation of ascorbic acid at pH 0·4. This is an effect of copper on iron catalysis, which was also demonstrated in pure solution. At pH 0·4 in the presence of 0·1 M sodium chloride, ten parts per million of ferrous iron gave 13·5 p.c. oxidation of ascorbic acid in five minutes at 40° C. Addition of ten parts per million of copper reduced the oxidation to 4·7 p.c. Thus at pH 0·4 traces of copper have practically no catalytic effect of their own and may actually reduce the catalytic effect of iron.

For comparison of reaction rates, the per cent. oxidation in five minutes rather than a velocity constant has been given, as the reaction kinetics were not similar in all cases. The rate of the copper-catalysed reaction decreased with time, being approximately proportional to the ascorbic acid concentration. That of the iron-catalysed reaction was usually approximately constant. In one case mentioned, the reaction rate increased considerably after the first five minutes, but otherwise the per cent. oxidation in this period formed a satisfactory basis for comparison.

SUGGESTED PROCEDURES FOR DETERMINING ASCORBIC ACID.

It is suggested that extraction with 3 p.c. metaphosphoric acid is the most suitable routine procedure for canned foods. Aliquots (5 ml.) can be titrated (a) alone and (b) immediately after the addition of 1 ml. of 1·5 p.c. hydrogen peroxide. The second titre is generally lower than the first and can be regarded as affording a sufficiently accurate estimation of ascorbic acid if the two results do not differ by more than 1 mg./100 gm. Otherwise an alternative procedure is required as a check.

An alternative determination by the method of Lugg (1942) is desirable when there is evidence of considerable interference by ferrous iron. It distinguishes "reductones" or apparent ascorbic acid as well as ferrous iron. This, however, may be an advantage as it is in long-stored products that both types of interference are likely to occur. Lugg's method depends on different rates of condensation with formaldehyde.

A procedure using alternative extractants is suggested by us as a result of the investigation described above. The extraction is made with 10 p.c. acetic acid containing 0·1 p.c. of oxalic acid. With silver beet, which is high in oxalate, the oxalic acid can be omitted. The acetic acid extracts have a pH of 2·2–2·5. This is reduced to approximately 0·4 by adding 1 ml. of 5 N sulphuric acid to a 5 ml. aliquot just before titration.

This procedure was tested with canned orange juice, tomato, broccoli, cabbage, silver beet and swede turnip. Addition of iron and copper equivalent to 100 and 10 parts per million respectively in the canned product resulted in a positive error of about 1 mg./100 gm. If the extracts containing iron and copper were allowed to stand for 30 minutes, the error was reduced to practically nil. On further standing the error became negative due to the slow loss of ascorbic acid.

In many canned products the ascorbic acid diffuses to equilibrium within a few days of preparation, and the liquid and solid portions do not differ greatly in ascorbic acid content. The liquid portion can be analysed by a very simple procedure. Pipette 5 ml. of 1 N sulphuric acid into a 20 ml. test tube and bubble carbon dioxide through the solution. Then pipette 1 ml. of liquor from the can immediately after opening and add to the sulphuric acid. Titrate with 2·6 dichlorophenolindophenol. Keep the carbon dioxide bubbling throughout the operation to prevent atmospheric oxidation. By this means the liquor can be brought immediately to a pH sufficiently low to prevent interference.

Where there is evidence of appreciable interference by ferrous iron, it is preferable to use more than one method of estimation. With our present somewhat limited knowledge of interfering substances, checks of this nature are still highly desirable.

FERROUS IRON AND ASCORBIC ACID

SUMMARY.

Ferrous iron introduces a positive error into the determination of ascorbic acid in metaphosphoric acid extracts. Addition of hydrogen peroxide converts this into a negative error of less magnitude.

In acetic and sulphuric acid extracts of canned foods, both iron interference and the stability of ascorbic acid are less at pH 0·4 than at pH 2–3. A similar effect of pH on iron interference is obtained with pure solutions of acetic and sulphuric acid containing 0·01 M oxalate.

The copper-catalysed oxidation of ascorbic acid is negligible at pH 0·4 but considerable at pH 2–3. The iron-catalysed oxidation is considerable at pH 0·4 and only slight at pH 2–3. At pH 0·4 copper actually reduces the iron-catalysed oxidation.

Oxalate (0·01 M) strongly inhibits the copper-catalysed oxidation and partially inhibits the iron-catalysed oxidation. Chloride (0·1 M) increases copper catalysis and reduces iron catalysis.

Various procedures are suggested for determining ascorbic acid in the presence of ferrous iron.

REFERENCES.

Barron, E. S. G. *et al.* (1936): J. biol. Chem., 112, p. 625.
Feaster, J. F. and Alexander, O. R. (1944): Ind. eng. Chem., 36, p. 172.
Levy, L. F. (1943): Biochem. J., 37, p. 713.
Lorenz, A. J. and Arnold, L. J. (1938): Ind. eng. Chem. Anal., 10, p. 687.
Lugg, J. W. H. (1942): Austral. J. exp. Biol., 20, p. 273.
Mapson, L. W. (1941): Biochem. J. 35, p. 1,332.
Marston, H. R. (1943): "The Estimation of Ascorbic Acid"—Austral. chem. Inst. Rpt.
Michaelis, L. and Friedheim, E. (1931): J. biol. Chem., 91, p. 343.
Ponting, J. D. (1943): Ind. eng. Chem. Anal., 15, p. 389.
Schulek, E. and Floderer, I. (1939): Angew. Chem., 52, p. 615.

J. Pharm Pharmacol 22(11): 823-827 Illus. 1970

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J. Pharm. Pharmac., 1970, **22**, 823–827 *Received May* 18, 1970

Some effects of experimentally-produced cigarette smoke on the growth, vitamin C metabolism and organ weights of guinea-pigs

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Guinea-pigs receiving a controlled dietary intake of L-xyloascorbic acid (ascorbic acid, vitamin C) inhaled experimentally produced cigarette smoke for periods of up to 20 min each day. Growth rate was significantly depressed by the smoke treatment, an effect at least in part attributable to a reduction in food intake. Growth of individual organs was not depressed to the same extent as that of the body as a whole. The lungs of the animals receiving smoke were significantly heavier than those of control animals ($P < 0{\cdot}05$). The concentration of ascorbic acid in the adrenal glands was significantly lower in the animals receiving smoke than in the controls ($P < 0{\cdot}01$). The smoke-induced depression of the adrenal gland ascorbic acid was apparent after 4 days; after 18 days marked adrenal hypertrophy accompanied the lowered ascorbic acid levels.

Lupu, Velican & Mihaescu (1954) found that exposure of guinea-pigs and rabbits to cigarette smoke resulted in a fall in the ascorbic acid concentration in the adrenal glands of both species. We have not found any reports of smoke-induced changes in the ascorbic acid content of any other organs. There is evidence that in man smokers have lowered blood levels of vitamin C (Venelut, 1954; Calder, Curtis & Fore, 1963).

The experiments now described were designed to cast further light on this relation. Guinea-pigs were used since their complete dependence upon dietary vitamin C enables tissue levels to be established at pre-determined values. The animals were exposed to controlled amounts of experimentally produced cigarette smoke and its effects on the growth rate and on the ascorbic acid content and weight of certain organs were recorded.

EXPERIMENTAL

Animals and diet

Male albino guinea-pigs, initial weight 250–300 g, were used. The diet had the following composition (g); ground oats 37, wheat bran 35, dried skim milk (1% fat) 10, dried full-cream milk (26·5% fat) 10, dried yeast 6·5, salt mixture 1·0, magnesium oxide 0·5. The dried milks were from Unigate (Milk Products) Ltd., the dried yeast from Distillers Co. (Yeast) Ltd. and the salt mixture from Glaxo Laboratories Ltd. Before incorporating the milk powders into the diet they were heated for 24 h at 110° to destroy any residual ascorbic acid. Each animal received a weekly supplement of 0·05 ml of menaphthone in arachis oil (5% w/v), 0·05 ml of DL-α-tocopherol acetate in arachis oil (5% w/v) and 0·05 ml of cod liver oil. Previous work had already indicated that this diet, when supplemented with ascorbic acid, supported normal

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growth of guinea-pigs; without the ascorbic acid the guinea-pigs developed scurvy (Hughes & Hurley, 1969). A daily dose of ascorbic acid was given orally on a body-weight basis to all animals.

The guinea-pigs were housed in individual galvanized zinc cages with removable 7/10-inch mesh bottoms. They were allowed to eat the diet freely. When measurements of food intake were required, 50 g of diet was given daily to each animal and the amount left uneaten 24 h later measured.

Organ weights and ascorbic acid analysis

At the end of each experiment the animals were killed by stunning and decapitation. The appropriate organs were rapidly dissected, dried once between filter paper and weighed. An extract for ascorbic acid determination was prepared by grinding the weighed organ with sand and 6% metaphosphoric acid; the reduction of a standard acid solution of phenol-indo-2,6-dichlorophenol by a portion of the extract was measured photometrically (Hughes, 1956).

Production of the cigarette smoke and its presentation to the guinea-pigs

The cigarettes were smoked in a standard smoking machine (R. W. Mason, Clevedon, Somerset). Each cigarette was drawn on once a minute by the machine for 2 s at a suction pressure of 350 mm of water. These values are based on measurements quoted for human smokers (Fabricant, 1946; Shepherd, 1951; Greenberg, Lester & Haggard, 1925; Hilding, 1956; van Proosdy, 1960). The cigarettes were burnt to a stub length of 20 mm (Doll, Hill & others, 1959; Korteweg, 1959).

The smoke entered an adaptor (manufactured and fitted by East & Co. Ltd., Cowley, Oxford) where it was mixed with air to give a composition similar to that known to be present in the lungs of smokers whilst smoking (about 500 ml of air containing 34 ml of smoke). The mixture was propelled along a Perspex tube containing a row of ports along each side fitted with rubber adaptors to accommodate heads of different sizes. The guinea-pigs were placed in wood–metal restrainers fitted with adjustable metal positioning rods and moved into position so that the head, up to, but excluding, the eyes, projected through the port into the tube. They appeared normal when replaced in their cages after being subjected to the smoke. The control animals were similarly treated but were not exposed to smoke. This procedure produces a situation where, instead of receiving a smoke–air mixture once a minute, the lungs are continuously filled with it. Methodological limitations make it difficult to simulate more closely the human situation.

Plan of experiments

Expt 1. Two groups of 12 animals were used. One was a control group and the other was exposed to the smoke–air mixture for a single period of 10 min daily, for 51 days, the animals being weighed daily. The animals were then killed, the adrenals, heart, lungs, kidney, brain, liver and testes removed and weighed and the ascorbic acid content of the adrenals, testes and brain determined.

Expt 2. Two groups of 15 animals were used. The experiment continued for 29 days and the animals were exposed to the smoke–air mixture for two periods of 10 min daily (at 09.30 and at 21.30 h). Food intake was measured and the ascorbic acid content of the liver, testes and adrenal glands determined.

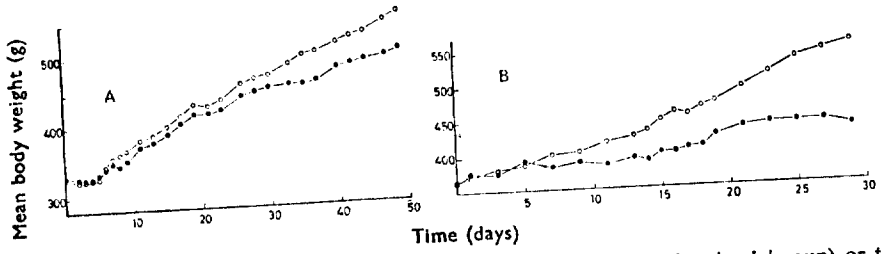

FIG. 1. Effect of smoke-air mixture [either one daily exposure (A, 12 animals/group) or two (B, 15 animals/group), each of 10 min] on growth of guinea-pigs. ○—○, control group; ●—●, smokers. Exposure to the smoke commenced on the fifth day.

Expt 3. Two groups of eight animals were used. The experimental conditions were as in Expt 2 but the experiment was terminated after 18 days. The weights and ascorbic acid contents of the adrenal glands and spleen were determined.

Expt 4. This was designed to determine how rapidly the smoking effects appeared. Two groups of animals were treated as in Expt 3 for 4 days.

RESULTS

These are given in Fig. 1 and in Table 1. Analysis of variance tests for significance were applied and a probability of $\leqslant 0{\cdot}05$ accepted as revealing a significant difference. Guinea-pigs exposed to the cigarette smoke for a single period of 10 min daily (Expt 1) had a lower growth rate than the corresponding control group (Fig. 1) the mean body-weights of the two groups being significantly different ($P \leqslant 0{\cdot}05$) from the 35th day of treatment. The ascorbic acid levels in the organs

Table 1. *Effect of smoke treatment (two 10-min exposures daily) on weight and ascorbic acid content of guinea-pig organs.* The daily intake of ascorbic acid was 0·5 mg/100 g. (Mean values with their standard errors)

Period of smoke treatment (days)	Guinea-pigs per group	Weight (g)	Weight as % of body-weight	Ascorbic acid (mg/100 g)
Adrenal glands				
C 4 (Expt 4)	8	0·127 ± 0·007	0·039 ± 0·002	30·5 ± 0·76†
S 4 (Expt 4)	8	0·129 ± 0·016	0·039 ± 0·005	21·9 ± 0·58
C 18 (Expt 3)	8	0·204 ± 0·004*	0·047 ± 0·009*	31·7 ± 0·76†
S 18 (Expt 3)	8	0·271 ± 0·007	0·067 ± 0·003	19·8 ± 0·88
C 29 (Expt 2)	15	0·236 ± 0·010	0·040 ± 0·002*	31·1 ± 1·5†
S 29 (Expt 2)	15	0·270 ± 0·011	0·062 ± 0·006	22·0 ± 2·2
Spleen				
C 4 (Expt 4)	8	0·409 ± 0·004	0·128 ± 0·001	8·6 ± 0·34
S 4 (Expt 4)	8	0·391 ± 0·005	0·169 ± 0·013	7·9 ± 0·24
C 18 (Expt 3)	8	0·300 ± 0·020	0·084 ± 0·004	8·1 ± 0·24
S 18 (Expt 3)	8	0·350 ± 0·015	0·085 ± 0·001	6·5 ± 0·24
Testes				
C 29 (Expt 2)	15	2·70 ± 0·08	0·46 ± 0·02	8·88 ± 0·16
S 29 (Expt 2)	15	1·96 ± 0·18	0·45 ± 0·034	8·09 ± 0·05

* Difference of means for controls (C) and smoke-treated animals (S) significant at 5% level.
† Difference of means for controls and smoke-treated animals significant at 1% level.

examined (adrenals, testes, brain) were not significantly different. Of the organs examined only with the lung was there a significant difference between the absolute weights. The mean absolute weight for the lungs of the controls was 3·16 g ± 0·11 (0·55 ±0·01 as % of body-weight) and for the smoke-treated animals 3·64 ± 0·19 (0·71 ± 0·02 as % of body-weight) ($P < 0·05$).

In Expts 2–4 the animals were subjected to two 10 min periods of smoke treatment daily. This intensification of the treatment produced a still greater divergence in the growth curves of the two groups, the difference in mean body-weight being significant ($P < 0·05$) from the 11th day of treatment (Fig. 1B). The adrenal ascorbic acid was in this experiment significantly lower in the animals receiving smoke than in the controls ($P < 0·01$; Table 1). During the period when food intake measurements were made (days 12–28) the controls ate significantly more food on a body-weight basis than the smoke-treated animals ($P < 0·01$).

Experiments 3 and 4 were short-term (18 and 4 days respectively). In the 18-day experiment exposure to the smoke produced both adrenal hypertrophy and a lowered adrenal ascorbic acid level (Table 1). In the 4-day experiment there was no hypertrophy of the adrenal glands although their ascorbic acid level was depressed ($P < 0·01$; Table 1). There was no significant change in the ascorbic acid content of the spleen (Expts 3 and 4) or of the testes (Expt 2) of the smoke-treated animals (Table 1).

DISCUSSION

Evidence suggestive of a possible relation in man between smoking and ascorbic acid derives primarily from measurements of blood levels of the vitamin (Venelut, 1954; Calder & others, 1963; Brook & Grimshaw, 1968). It is generally accepted that in man the level of ascorbic acid in the leucocytes (and to a less extent, in the plasma) provides a useful index to the ascorbic acid status of the body as a whole (Vilter, 1967). In guinea-pigs, however, this is not so, particularly at the sub-optimal dietary levels we used (Hughes & Jones, 1970). On the other hand, the ascorbic acid content of organs such as the adrenal glands and the spleen appears to reflect dietary intake over a very wide range and its determination in such organs presents none of the difficulties associated with its determination in leucocytes. In most experiments, the ascorbic acid content of the adrenals and spleen was, therefore, assumed to reflect the ascorbic acid status of the animal.

The guinea-pigs received a controlled daily intake of ascorbic acid of 0·3 mg/100 g body-weight (Expt 1) or 0·5 mg/100 g body-weight (Expts 2–4). This is sufficient to maintain growth and development but is well below the intake necessary to produce tissue saturation (Evans & Hughes, 1963). Restriction of the ascorbic acid intake to these pre-determined levels meant that any smoke-induced changes in the vitamin C economy of the body as a whole would be reflected as changes in the tissue levels.

The most striking result was a fall in the ascorbic acid concentration of the adrenal glands of the animals receiving smoke. Stressing agents such as low temperature, are known to produce a fall in adrenal ascorbic acid and Larson, Haag & Silvette (1961) suggested that smoking should be considered a stressing agent. This is now reinforced by two further findings; (*a*) the animals receiving smoke over the prolonged period had hypertrophied adrenal glands—this too being a condition characteristic of stressed animals, and (*b*) none of the other organs examined displayed

a fall in ascorbic acid. When the vitamin C status of the animal as a whole changes any change in the adrenal glands is paralleled by changes in other organs such as the spleen (Hughes & Jones, 1970).

It is unlikely that the lowered growth rate of the smoke-treated guinea-pigs is a consequence of changed tissue ascorbic acid levels. A large reduction of tissue ascorbic acid is necessary to produce the depressed growth rate characteristic of hypovitaminosis C (Evans & Hughes, 1963). Furthermore, the growth depression observed with the animals receiving smoke is attributable (at least in part) to a reduced food intake. Thus in Expt 2 from day 12 to day 28 of the experimental period, on a body-weight basis the food intakes of the controls were significantly higher ($P < 0{\cdot}01$) than those of the smoke-treated animals. This inanition presumably resulted from a direct effect of the smoke on the food intake control mechanism(s) although during the initial week of the experiment a depression of growth was recorded without any reduction of food intake (Fig. 1B).

Although the animals receiving smoke appeared to have larger organs on a body-weight basis, only the lungs (in absolute terms as well as on a body-weight basis) were significantly greater than in the controls. This would appear to indicate a true smoke-induced hypertrophy of the lungs in guinea-pigs, a phenomenon that does not appear to have been previously reported.

Acknowledgements

This work was made possible by a generous grant from Beecham Products (U.K.). The authors are indebted to Dr. Maurice Brook and his colleagues of Beecham Products for many useful discussions, and to the Tobacco Research Council for gifts of cigarettes.

REFERENCES

Brook, M. B. & Grimshaw, J. J. (1968). *Am. J. clin. Nutr.*, **21**, 1254.
Calder, J. H., Curtis, R. C. & Fore, H. (1963). *Lancet*, **1**, 556.
Doll, R., Hill, A. B., Gray, P. G. & Parr, E. A. (1959). *Br. med. J.*, **1**, 322–325.
Evans, J. R. & Hughes, R. E. (1963). *Br. J. Nutr.*, **17**, 251–255.
Fabricant, N. D. (1946). *Am. J. med. Sci.*, **211**, 505–509.
Greenberg, L. A., Lester, D. & Haggard, H. W. (1952). *J. Pharmac. exp. Ther.*, **104**, 162–168.
Hilding, A. C. (1956). *New Eng. J. Med.*, **254**, 775–781.
Hughes, R. E. (1956). *Biochem. J.*, **64**, 203–208.
Hughes, R. E. & Hurley, R. J. (1969). *Br. J. Nutr.*, **23**, 211–216.
Hughes, R. E. & Jones, P. R. (1970). *Br. J. Nutr.* In the press.
Korteweg, R. (1959). *Br. med. J.*, **1**, 645.
Larson, P. S., Haag, H. B. & Silvette, H. (1961). *Tobacco, Experimental and Clinical Studies*, p. 367. Baltimore: Williams and Wilkins.
Lupu, N. G., Velican, C. & Mihaescu, C. (1954). *Med. Sc. Sect. Rumanian Acad.*, Session Jan. 22–24, pp. 141–168 (quoted in Larson & others, p. 367).
Shepherd, J. M. (1951). *Br. med. J.*, **2**, 1007–1010.
Van Proosdy, C. (1960). *Smoking: Its Influence on the Individual and its Role in Social Medicine*, p. 14. Amsterdam: Elsevier.
Venelut, F. (1954). *Med. Klin. Berl.*, **49**, 959–962 (quoted in Larson & others, p. 336).
Vilter, R. W. (1967). In *The Vitamins*. Editors: Sebrell, W. H. Jnr. & Harris, Robert S., Vol. I, p. 472. Academic Press.

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Jap. J. Pharmac. 20, 52-60 (1970) March

INHIBITION OF Mg- AND Mg, Na, K-ACTIVATED ADENOSINE TRIPHOSPHATASE ACTIVITY BY L-ASCORBIC ACID AND L-CYSTEINE

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Received for publication July 30, 1969

It is now commonly postulated that the electrical excitability of neural cells depends on the concentration gradients of Na^+ and K^+ across the cell membrane and the gradients are maintained by active cation transport system. Further, a membrane-bound, Na^+ and K^+-stimulated and ouabain-sensitive adenosine triphosphatase (Na-K-ATPase) has been proved to be involved in the active cation transport system in the brain as well as in many other tissues (1). While cardiac glycosides have been well known to be specific inhibitors of Na-K-ATPase, some biogenic substances were also considered to participate in the transport mechanism. In this respect, two biogenic substances, ascorbic acid and cysteine, came to light. They were described as rather specific inhibitors of Na-K-ATPase in the electric organ of eel (2) and the liver of rat (3). The present report describes how ascorbic acid and cysteine affect Na-K-ATPase and Mg-ATPase of the rabbit brain, histochemically and biochemically. For reference the enzymes of rabbit liver were tested.

METHODS

1. *Histochemical demonstration of ATPase*

Male albino rabbits weighing 1.8 to 2.2 kg were used. Immediately after sacrifice of the animal by exsanguination, the cerebrum and the liver were removed. Further procedures for staining of the cerebral and liver ATPase were followed to the method of McClurkin (4). Small pieces of the cerebrum and the posterior part of the left lobe from the liver were cut out and fixed in an ice-cold 10% formalin with 0.25 M sucrose for 18 to 20 hours. Then the specimens were washed in ice-cold distilled water, placed into the test tubes and immediately frozen in a mixture of aceton and dry ice at about −70°C. The frozen tissues were sectioned at 10 μ thickness in a cryostat at −17°C. The sections were floated on cold 0.2 M Tris-HCl (pH 7.2) and stained for ATPase within 2 hours. The sections were incubated in a freshly prepared medium containing 3 mM ATP-Ba_2 (dissolved in 0.1 N HCl), 24 mM Tris-HCl (pH 7.8), 3 mM $Pb(NO_3)_2$ and 3 mM $MgSO_4$ (the final pH 7.2). Some sections were incubated in a medium added with 100 mM NaCl and 30 mM KCl. Incubation was carried out at 37°C for 10 to 30 minutes, depending on the enzyme activity of tissues. After incubation, the sections were rinsed

This work was partly supported by a research grant from the Ministry of Education, Japan.

in distilled water, floated on 1% ammonium sulfide solution for one minute, rinsed again in distilled water and mounted in buffered glycerin for microscopy.

In some experiments, instead of ATP-Ba_2 and $MgSO_4$ in McClurkin's incubation medium, Tris-ATP and $MgCl_2$ were used according to the reaction mixture in biochemical assay.

In the first series of experiments, agents to be tested were added to the incubation medium. The pH was adjusted to 7.2 with 0.1 M Tris after adding the agents. In the second series of experiments, tissue slices at 3 to 4 mm thickness were immersed in Ringer-Locke's solution containing the test agents and bubbling with oxygen. Control sections were incubated in a medium without substrate at 37 C for 10 to 30 minutes, or incubated in a complete medium after immersion in hot water at 90 C for 5 minutes to destroy the enzyme activity.

2. *Biochemical determinations of ATPase activity*

Preparation of a microsomal fraction from rabbit cerebrum: Male albino rabbits weighing 1.8 to 2.2 kg were killed, exsanguinated from the common carotid arteries. The brain was removed and the cerebral hemispheres were taken out in an isolation medium (0,25 M sucrose, 5 mM EDTA-Na_2, 30 mM histidine, pH 6.8) described by Skou (5). After removal of the pia-arachnoid membranes, 2 g of brain pieces were placed in an ice-cold Teflon homogenizer (A.H. Thomas Co.; No. 4228-B), added with 0.1% sodium desoxycholate and ground in 9 volumes of the isolation medium. Grinding involved 6 passages of the pestle, rotating at 2,800 rev/min for 1 minute in an ice bath. Immediately thereafter, the homogenates were centrifuged at 8,000×g for 10 minutes. The microsomal fraction was obtained as sedmients by centrifuging of 8,000×g supernatants for 30 minutes at 100,000×g in the isolation medium. The final sediments were suspended in 0.25 M sucrose containing 1 mM EDTA-Na_2 and 30 mM histidine at pH 7.0, as described by Skou (5), then kept frozen at −20°C as stock sample. Low speed centrifugation was carried out in an angle head of the usual centrifuge (Kubota), and for high speed centrifugation up to 100,000×g the no. 40 head of the Spinco model L centrifuge was used.

ATPase assay: Reaction mixtures of 2.0 ml were prepared in tubes in ice, containing 100 mM Tris-HCl (pH 7.8), 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$ and 0.1 mg protein of microsomal fraction. Preincubation was carried out at 37°C for 5 minutes, then 3 mM Tris-ATP was added. After shaking for 15 minutes, the tube was placed in ice and 0.5 ml of 40% trichloroacetic acid was added. After centrifugation, a 0.6 ml aliquot of the supernatant was provided for determination of inorganic phosphate as described by Fiske and Sabbarow (6). The activity of ATPase was expressed as μmoles Pi liberated/mg protein/hr. Protein was determined by the method of Lowry et al. (7), using crystalline bovine plasma albumine as standard.

Tris-ATP: A free form of ATP was made by a passage of disodium ATP through a Dowex-50 cation exchange resin column (H^+form). Concentration of ATP in the filtrate from the column was determined by reading the extinction at 260 mμ of a dilution, when a millimolar extinction of ATP at 260 mμ was 14.2 at pH 2.0 (8). The free ATP was made

to pH 7.2 with 0.5 M Tris, and water was added to make a final concentration of the solution 30 mM in ATP. The stock solution was stored at −20°C until use. The yeald of ATP with this method was 85 to 90% and the contents of inorganic phosphate and sodium ion in the 30 mM ATP solution were found to be less than 1.3 mM and 4 mM, respectievely.

Reagents: ATP-Ba_2 (Nutritional Biochemical Co.), ATP-Na_2 (Sigma Chemical Co.), L-glutathione (SH) (Daiichi Pharmaceutical Co., Tokyo) and ouabain (Merck) were used. Other agents were purchased from Nakarai Chemical Co., Kyoto. Water was distilled after treatment with ion excahnge resin.

RESULTS

1. *Histochemical demonstration of ATPase in rabbit cerebrum and liver*

Cerebrum: When ATP, Mg^{++}, Na^+ and K^+ were present in the incubation medium, the enzyme activity of ATPase was seen as dark stain in the plasma membranes of nerve cells and in the capillary endothelium. Nuclei of them were also stained darkly. In the absence of Na^+ and K^+ in the incubation medium, there was no stain in the plasma membranes of nerve cells and faint stain appeared in the cytoplasmic area of nerve cells and the capillary endothelium. Nuclei and nucleoli of nerve cells were also stained faintly.

Liver: In the presence of ATP, Mg^{++}, Na^+ and K^+ in the incubation medium, the activity of ATPase was seen in the plasma membranes of liver cells, the endothelial cells of central veins and other blood bessels and the sinusoids. The bile canaliculi, or the plasma membranes of hepatic cells fronting the bile canaliculi were stained heavily. Stellate cells in the sinusoids had also dark stain. In the nuclei and especially nucleoli of liver cells, a dark stain was seen. Sections incubated similarly but without Na^+ and K^+ showed a weak staining in the endothelial tissues. No stain occurred in the plasma membranes of hepatic cells and the bile canaliculi. The nuclei and nucleoli were not stained or, if any, extremely faint.

Controls: There was no stain in sections of cerebrum and liver incubated in a medium without substrate. Immersion of sections in hot water at 90°C inactivated the enzyme activity and made unstained.

2. *Effects of L-ascorbic acid and other agents on ATPase staining in rabbit cerebrum and liver*

L-Ascorbic acid, L-cysteine and, as reference agents, L-glutathione-SH, L-methionine and ouabain were examined. In the presence of ATP, Mg^{++}, Na^+ and K^+, the addition of L-ascorbic acid (10^{-4}M) reduced the ATPase activity in all regions of the cerebrum (Table 1) and the liver (Table 2). There was no stain in the plasma membranes of the liver cells and the neural membranes in the cerebrum. The bile canaliculi were also unstained. L-Cysteine (10^{-4}M) and L-glutathione-SH (10^{-4}M) did not affect or, if any, slightly reduced the Na-K-ATPase staining in the cerebrum and the liver. In the absence of Na^+ and K^+, L-ascorbic acid, L-cysteine and L-glutathione-SH did not significantly affect ATPase staining. The addition of L-methionine (10^{-4}M) did not affect the staining, irrespective of Na^+ and K^+. Ouabain (10^{-4} M) inhibited the Na-K-ATPase activity in the plasma membranes of the liver cells, the bile canaliculi and the neural membranes in

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TABLE 1. Effects of drugs on adenosine triphosphatase staining of rabbit brain cortex.

Drugs	ATPase staining	Neuronal cells: Plasma membranes	Neuronal cells: Cytoplasmic area	Endothelial cells of capillaries
—	Mg^{++}	−	−∼+	+
	$Mg^{++}+Na^{+}+K^{+}$	++	+	++
Ouabain 10^{-4}M	Mg^{++}	−	−	+
	$Mg^{++}+Na^{+}+K^{+}$	−	+	+∼++
L-Ascorbic acid 10^{-4}M	Mg^{++}	−	−	+
	$Mg^{++}+Na^{+}+K^{+}$	−	−∼+	+
L-Cysteine 10^{-4}M	Mg^{++}	−	−	+
	$Mg^{++}+Na^{+}+K^{+}$	−∼+	+	+∼++
L-Glutathione (SH) 10^{-4}M	Mg^{++}	−	−∼+	+
	$Mg^{++}+Na^{+}+K^{+}$	+∼++	+	+∼++
L-Methionine 10^{-4}M	Mg^{++}	−	−∼+	+
	$Mg^{++}+Na^{+}+K^{+}$	++	+	++

++ : strong staining, + : weak staining, − : no staining.

TABLE 2. Effects of drugs on adenosine triphosphatase staining of rabbit liver.

Drugs	ATPase staining	Liver cells: Plasma membranes	Liver cells: Cytoplasmic area	Bile canaliculi	Endothelial cells of central veins	Stellated cells
—	Mg^{++}	−	+	−	+	+
	$Mg^{++}+Na^{+}+K^{+}$	++	++	++	++	++
Ouabain 10^{-4}M	Mg^{++}	−	+	−	+	+
	$Mg^{++}+Na^{+}+K^{+}$	−	++	−	+∼++	++
L-Ascorbic acid 10^{-4}M	Mg^{++}	−	+	−	+	+
	$Mg^{++}+Na^{+}+K^{+}$	−	++	−	+	+
L-Cysteine 10^{-4}M	Mg^{++}	−	+	−	+	+
	$Mg^{++}+Na^{+}+K^{+}$	−∼+	+	−∼+	++	++
L-Glutathione (SH) 10^{-4}M	Mg^{++}	−	+	−	+	+
	$Mg^{++}+Na^{+}+K^{+}$	+∼++	++	+∼++	++	++
L-Methionine 10^{-4}M	Mg^{++}	−	+	−	+	+
	$Mg^{++}+Na^{+}+K^{+}$	++	++	++	++	++

++ : strong staining, + : weak staining, − : no staining.

the cerebrum.

The essentially similar results were obtained on tissue slices incubated in Ringer-Locke solution containing above described agents. The reducing action of ATPase staining was most obvious in the regions around blood vessels. The use of the incubation medium containing Tris-ATP for ATP-Ba_2 (see Methods) did not alter the results described above.

3. *Activity of ATPase in microsomal fraction of rabbit cerebrum*

According to the observations of Schwartz et al. (9), 75% of Na-K-ATPase activity in the guinea pig cerebral cortex is present in a microsomal fraction. Therefore, the microsomal fraction was used in the present study. The conditions for ATPase assay were based on those of Skou (5), as described under Methods. There was a linear relationship between

liberation of inorganic phosphate and incubation time for at least 17 minutes. In further experiments, the reaction was carried out for 15 minutes. In order to know the amount of inorganic phosphate liberated from non-enzymatic hydrolysis of ATP or from sources other than substrate ATP, a reaction mixture without microsomal enzyme or ATP was incubated. In a reaction mixture without ATP, even a trace of inorganic phosphate was undetectable at 0 and 15 minutes of incubation, indicating that inorganic phosphate derived from sources other than ATP preparation was negligible. In a reaction mixture without microsomal protein as enzyme, the amount of inorganic phosphate at the beginning of incubation, which was probably due to a contamination of ATP preparation, did not change after 15-minute incubation. This fact indicates that non-enzymatic hydrolysis of ATP within 15 minutes at 37°C was negligible. Inorganic phosphate liberated by enzymatic hydrolysis was thus estimated by subtracting the amount of the phosphate in a reaction mixture without microsomal protein from that in a complete reaction system. There was no linear relationship between the initial velocity of enzymatic reaction and the amount of enzyme between 0.1 and 1.0 mg protein/tube. In further experiments, microsomal protein 0.1 mg/tube was used. Changes in pH of reaction mixture affected only slightly the activity of Mg-ATPase but very markedly Na-K-ATPase. The optimal pH of Na-K-ATPase was 7.8. The effects of substrate concentration of ATPase activity were examined and a nearly maximal activity was obtained with 3 mM ATP for both Mg- and Na-K-ATPase. The concentration of Mg^{++} required for maximal activity was 1.5 mM for Mg-ATPase and 3 mM for Na-K-ATPase. For maximal activity of Na-K-ATPase, 100 mM of Na^{+} and 20 mM of K^{+} were sufficient, and at higher concentrations of these ions Mg-ATPase activity was rather somewhat inhibited. The activity of both enzyme increased proportionally to a rise of incubation temperature from 20°C to 45°C.

Thus, suitable conditions for maximal ATPase activities at a physiological temperature were indicated from data obtained above. In next experiments, 100 mM Tris-HCl (pH 7.8), 3 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, Tris-ATP 3 mM and 0.1 mg of microsomal protein were used in the reaction mixtures. Incubation was carried out for 15 minutes at 37°C (Table 3). Under these conditions, it was found that ouabain 2×10^{-5} M inhibited the activity of Na-K-ATPase almost completely, while it had no effect on the activity of Mg-ATPase.

TABLE 3. Conditions for assay of adenosine triphosphatase.

Reaction mixture	Tris-HCl (pH 7.8)	100 mM
	$MgCl_2$	3 mM
	NaCl	100 mM
	KCl	20 mM
	ATP (Tris salt)	3 mM
	Microsomal protein	0.1 mg
Incubation time	15 min	
Incubation temperature	37°C	

4. *Effects of L-ascorbic acid and other agents on the microsomal ATPase*

The assay conditions were described above, and the effects of L-ascorbic acid, L-cysteine, L-glutathione (SH) and L-methionine were examined (Table 4). L-Ascorbic acid 10^{-4} M inhibited both Mg-ATPase and Na-K-ATPase to 64.7% and 23.2%, respectively. L-Cysteine 10^{-4} M also inhibited Mg-ATPase and Na-K-ATPase to 60.1% and 43.8% of control, respectively. The activities of Mg-ATPase and Na-K-ATPase were plotted against the concentration of L-ascorbic acid and L-cysteine in a representative experiment with microsomal preparation (Figs. 1 and 2). L-Ascorbic acid and L-cysteine inhibited almost completely Na-K-ATPase at a concentration or 10^{-4} M. Unlike L-cysteine, L-glutathione 10^{-4} M showed statistically non-significant effects on Mg-ATPase and Na-K-ATPase. Similar results were obtained with three different preparations of microsomal

TABLE 4. Effects of drugs on adenosine triphosphatase activities of microsomal fractions from rabbit brain cortex.

Drugs		ATPase activities (μ moles Pi/mg protein/hr) Mg-ATPase activity (%)	Mg-Na-K-ATPase activity-Mg-ATPase activity (%)
—		32.6 ± 2.8 (100.0)	55.5 ± 22.0 (100.0)
L-Ascorbic acid	10^{-4}M	21.1 ± 1.6* (64.7)	12.9 ± 10.7* (23.2)
L-Cysteine	10^{-4}M	19.6 ± 2.9* (60.1)	24.3 ± 3.1* (43.8)
L-Glutathione (SH)	10^{-4}M	29.2 ± 5.6 (89.6)	50.1 ± 16.2 (90.3)
L-Methionine	10^{-4}M	30.2 ± 5.2 (92.6)	65.0 ± 18.9 (117.1)

Mean in three observations ± standard error.
* statistically significant ($P<0.05$)

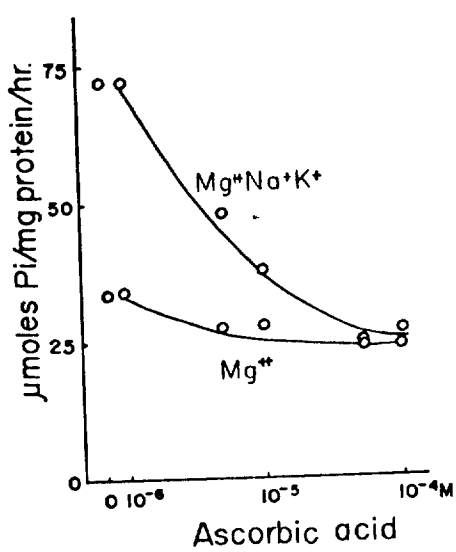

FIG. 1. Relationship between the concentration of L-ascorbic acid and the activities of Mg-ATPase and Na-K-ATPase in a rabbit brain microsomal fraction.

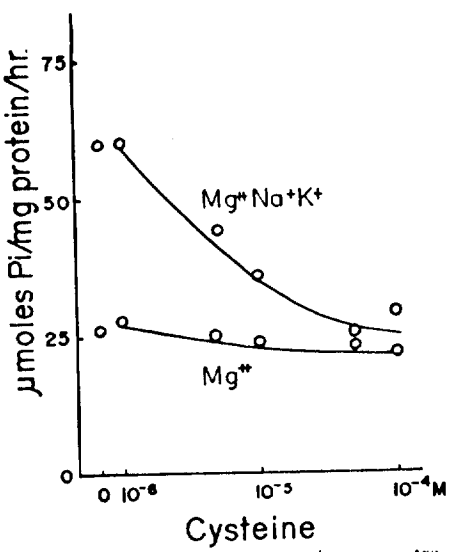

FIG. 2. Relationship between the concentration of L-cysteine and the activities of Mg-ATPase and Na-K-ATPase in a rabbit brain microsomal fraction.

fraction. L-Methionine 10^{-4} M inhibited neither Mg-ATPase nor Na-K-ATPase in all microsomal preparations tested.

DISCUSSION

In the histochemical demonstration of ATPase, omission of Na^+ and K^+ from the medium resulted in a loss of stain in the plasma membranes of neural cells in the cerebrum, the plasma membranes of liver cells and the bile canaliculi of the liver. In a medium containing Na^+ and K^+, ouabain inhibited the staining in these structures. The observations were in agreement with those of McClurkin (4), indicating the presence of ouabain-sensitive Na-K-ATPase in these structures. In the endothelial tissues of the cerebrum and liver, the ATPase staining was made stronger by the addition of Na^+ and K^+. But the staining was unaffected by the addition of ouabain. These observations may suggest the possible presence of ouabain-insensitive Na-K-ATPase in the endothelial tissues.

On the phosphatase staining of nuclei, Gomori (10) considered the stain in the nuclei to be a result of diffusion of reaction product and selective affinity of nuclear substance to lead complexes. In experiments with the nuclei isolated in non-aqueous media for preventing the the diffusion of reaction product, Stern and Mirsky (11) showed the absence of acid phosphatase, alkaline phosphatase and ATPase, while Fisher et al. (12) and Siebert (13) demonstrated the presence of ATPase. According to Gomoli, the staining of nucleus with a lead salt method for the demonstration of phosphatase does not always show the enzymatic activity, and the effects of drugs on stain in the nuclei were not necessarily due to the effects on the nuclear enzyme. The significance of the staining of nucleus and the effects of drugs on it must be studied by chemical and biochemical approach.

The distribution of ATPase in the subcellular fractions of brain has been studied by Skou (5), Schwartz (9), Jänefelt (14), Deul and McIlwain (15) and Aldridge (16). The Na-K-ATPase was mostly present in the microsomal fraction. In the rabbit brain microsomal fraction of this study, there were 25 to 35 μmoles Pi/mg protein/hr of specific activity of Mg-ATPase and 35 to 80 μmoles Pi/mg protein/hr of Na-K-ATPase activity. The addition of Na^+ and K^+ to the reaction system approximately tripled the activity of Mg-ATPase, giving ratio of 2.4 to 4.2. The activation of enzyme by alkali metals averaging threefold was described by Deul and McIlwain (15) and the activation up to eightfold by Skou (5). The latter author used a reaction mixture containing 6 mM Mg^{++} for Na-K-ATPase assay, but in this study the activation ratio did not increase with 6 mM Mg^{++}.

Histochemical and biochemical studies were performed for testing the effects of L-ascorbic acid and L-cysteine on ATPase activity. In the histochemical studies, both agents 10^{-4} M seemed to inhibit only a Na-K-ATPase staining and had likely no effect on the Mg-ATPase staining in the brain and liver. Biochemically, these substances inhibited both Mg-ATPase and Na-K-ATPase in the brain microsomal fraction with a predominat effect on the latter enzyme. Since the ouabain-insensitive Na-K-ATPase, described in a histochemical study, was not detectable in microsomal fraction, the effects of L-ascorbic acid and L-cysteine on this enzyme were not examined biochemically. Regarding the inhibitory

effect of L-ascorbic acid and L-cysteine on Mg-ATPase, the histochemical results were inconsistent with those obtained biochemically. It was possible that the histochemically demonstrable Mg-ATPase was inhibited by formaldehyde fixation and stained weakly, thus further inhibition of the enzyme activity by L-ascorbic acid or L-cysteine was made obscure. L-Glutathione-SH 10^{-4} M showed a slight inhibition on Na-K-ATPase, and L-methionine 10^{-4} M had no effect on ATPase either histochemically or biochemically.

Glynn (2) showed that L-ascorbic acid 10 mM with p-phenylenediamine 1 mM inhibited Na-K-ATPase by 18% without showing any significant effect on Mg-ATPase in electric organ of eel. Emmelot and Bos (3) described the inhibitory effect of L-cysteine on Na-K-ATPase by 30 to 60% and a slight inhibition on Mg-ATPase by 0 to 10%, using rat liver cytoplasmic membranes. Thus, L-ascorbic acid and L-cysteine were recognized as specific inhibitors of Na-K-ATPase. A slight or no effect of ascorbic acid and cysteine on Mg-ATPase may perhaps be due to species or organ difference of nature of Mg-ATPase itself.

Rajalakshmi and Patel (17) reported that a tranquilizer increased the synthesis of ascorbic acid by liver homogenate and raised the concentration of ascorbic acid in the liver, adrenals and brain. It is interesting to investigate whether L-ascorbic acid in higher concentration in the brain would produce a tranquilizing effect and, if so, it might be related to the inhibition of the brain Na-K-ATPase by the acid. Thus, further question remains whether the physiological level of L-ascorbic acid in the brain would play an important role in normal function of the brain.

SUMMARY

1. In the histochemical demonstration of Mg-ATPase and Na-K-ATPase, stain location was described in cerebral and liver sections. With cerebral sections, in the presence of Mg^{++} and ATP in the incubating medium, faint stain of Mg-ATPase was observed in cytoplasmic area of neuronal cells and capillary endothelium. When Na^+ and K^+ were added to the incubating medium, dark stain occurred in plasma membranes of neuronal cells and capillary endothelium. With liver sections, stain and faint stain of Mg-ATPase were also observed in cytoplasmic area and endothelial tissues. When Na^+ and K^+ were added, dark stain was seen in plasma membranes of liver cells, bile canaliculi and endothelial tissues.

2. L-Ascorbic acid (10^{-4} M) reduced the staining of Na-K-ATPase in all regions in cerebral and liver sections, especially in plasma membranes of neuronal cells, and bile canaliculi, but the acid did not affect staining of Mg-ATPase. The effects of L-cysteine and L-glutathione (SH) were similar in nature, but less in intensity on the staining of ATPase. L-Methionine had no effect on the staining of Mg-ATPase and Na-K-ATPase. When tissue slices were incubated in a Ringer-Locke's solution containing test agents, the similar results were obtained especially in regions around blood vessels.

3. In the biochemical assay of ATPase in microsomal fractions of rabbit brain, assay conditions were selected as suitable for maximal activities. The optimal pH was 7.8,

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maximal activity of Mg-ATPase was obtained at Mg^{++} concentration of 3 mM and maximal stimulation of Mg-ATPase by Na^+ and K^+ occurred at Na^+ and K^+ concentrations of 100 mM and 20 mM, respectively. Ouabain (2×10^{-5} M) inhibited completely the Na-K-ATPase, but did not the Mg-ATPase.

4. L-Ascorbic acid (10^{-4} M) and L-cysteine (10^{-4} M) inhibited both Mg-ATPase and Na-K-ATPase, but the latter was more greatly inhibited by these agents. L-Glutathione and L-methionine had not statistically significant effects on Mg-ATPase and Na-K-ATPase.

Acknowledgment: The author wishes to express her appreciation to Drs. H. Yamada, K. Shimamoto, M. Fujiwara and G. Tanaka, Kyoto University, for their valuable advices and encouragement throughout this study, and to Prof. T. Murano, Wakayama Medical College, for his usefull suggestion about the conditions of ATPase assay.

REFERENCES

1) Dengler, H.J., Spiegel, H.E. and Titus, E.O.: *Science, N.Y.* **133**, 1072 (1961)
2) Glynn, I.M.: *J. Physiol.* **169**, 452 (1963)
3) Emmelot, P. and Bos, C.J.: *Biochem. biophys. acta* **120**, 369 (1966)
4) McClurkin, L.T.: *J. Histochem. Cytochem.* **12**, 654 (1964)
5) Skou, J.C.: *Biochem. biophys. acta* **58**, 314 (1962)
6) Fiske, C.H. and Sabbarow, Y.: *J. biol. Chem.* **81**, 629 (1929)
7) Lowry, D.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: *J. biol. Chem.* **193**, 265 (1951)
8) Volkin, E. and Cohn, W.G.: *Methods of Biochemical Analysis*, Interscience Publishers, Inc., Vol. **1**, 287 (1954)
9) Schwartz, A.: *Biochem. biophys. acta* **67**, 331 (1963)
10) Gomori, G.: *J. Lab. Clin. Med.* **37**, 526 (1951)
11) Stern, H. and Mirsky, A.E.: *J. gen. Physiol.* **36**, 181 (1952)
12) Fisher, F., Siebert, G. and Adelhoff, E.: *Biochem. Z.* **332**, 131 (1959)
13) Siebert, G.: *Fifth Int. Congr. Biochem. Symp. II* (1961)
14) Jansteelt, J.: *Biochem. biophys. acta* **48**, 104 (1961)
15) Deul, D.H. and McIlwain, H.: *J. Neurochem.* **8**, 246 (1961)
16) Aldridge, W.N.: *Biochem. J.* **83**, 527 (1962)
17) Rajalakshmi, R. and Patel, A.: *J. Neurochem.* **15**, 195 (1968)

J. Pha[illegible]rmac., 1968, 20, 210–214 Received July 10, 1967

The effect of ascorbic acid on the cerebral and adrenal catecholamine content in the male rat

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The intraperitoneal administration of ascorbic acid (500 mg/kg) to rats produces a significant decrease in the concentration of dopamine, and an increase in noradrenaline, in the cerebral hemispheres, cerebellum, diencephalon, mesencephalon and medulla oblongata. The only change in the concentration of adrenaline was a decrease observed in the cerebral hemispheres. Ascorbic acid produces an increase in the concentration of ascorbic acid in the brain; this rise was not observed in the adrenal medulla where the concentrations of catecholamines were also unchanged.

ASCORBIC acid increases significantly the concentration of catecholamines, expressed as noradrenaline, in cerebral hemispheres, cerebellum and mesencephalon of rats (Izquierdo, Jofré & Dezza, 1964). We have since found that the acid decreases significantly the adrenaline concentration but does not modify the noradrenaline contents in the guinea-pig heart (Izquierdo & Jofré, 1965).

According to McLean & Cohen (1963) ascorbic acid releases adrenaline from adrenal granules *in vitro*. Levin, Levenberg & Kaufman (1960) and Levin & Kaufman (1961) considered that the acid participated in the β-hydroxylation of dopamine in the adrenal gland.

These results, together with the discrepancy between our findings on guinea-pig heart (Izquierdo & Jofré, 1965) and on rat brain (Izquierdo & others, 1964), led us to reinvestigate the effect of ascorbic acid on the cerebral and adrenal catecholamine contents in the rat.

Experimental

One hundred and thirty adult male albino rats, weighing 84–126 g, were used. They were killed by decapitation and the dopamine, noradrenaline, adrenaline and ascorbic acid contents or the amines or acid alone in brain structures and in the whole adrenal glands were estimated. There were 41 normal rats; there were 4 groups, each of 16 animals, killed 5, 10, 20 and 60 min after intraperitoneal injection of ascorbic acid (500 mg/kg). A further 4 groups, this time of 2 rats each, were injected intraperitoneally with nialamide (100 mg/kg) 4 hr before death, and pyrogallol (10 mg/kg) and ascorbic acid in separate injections 20 min before death, in which the amines only were assessed. Finally there were 4 groups of 2 rats each, and 3 groups of 3 rats each, killed 20 min after injection of ascorbic acid in which the acid alone was assessed.

Drugs used were: ascorbic acid (Roche) dissolved in twice distilled water and adjusted to pH 5·5–6·0 with 5N sodium hydroxide; nialamide (Pfizer); pyrogallol (Mallinckrodt). Solutions were prepared extemporaneously for each animal.

Brains were removed, washed immediately with cold Tyrode solution

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and dissected into component parts on ice. Adrenals and the adrenal-medulla were similarly treated. The structures were weighed and then adrenaline and noradrenaline were determined spectrofluorimetrically according to Bertler, Carlsson & Rosengren (1958), dopamine according to Laverty & Sharman (1965), and ascorbic acid by the photocolorimetric method of Roe (1954).

Results

From Table 1 it can be seen that ascorbic acid decreased dopamine and at the same time increased noradrenaline in some cerebral structures, as early as 5 min after injection. The effect increased at 10 min and reached a maximum at 20 min in all the brain structures. At 60 min there was still a decreased content of dopamine in hemispheres, mesencephalon, pons and medulla oblongata, but with no corresponding increase

TABLE 1. EFFECT OF THE INTRAPERITONEAL ADMINISTRATION OF 500 MG/KG OF ASCORBIC ACID ON THE CONTENT OF THE CATECHOLAMINES IN DIFFERENT PARTS OF THE BRAIN AND ADRENAL GLANDS OF THE RAT

	Wt (g)	DA	NA	A	DA	NA	A	DA	NA	A
		Hemispheres			Cerebellum			Diencephalon		
Normal	103 (24)* (11)†	0·21 ±0·03	0·16 ±0·03	0·04 ±0·002	0·18 ±0·04	0·12 ±0·01	0·05 ±0·01	1·66 ±0·12	0·34 ±0·02	0·11 ±0·03
20 min	113 (16) (4)	0·06 ±0·01 P<0·001 73·2 (D)	0·20 ±0·01 P<0·01 22·9 (I)	0·01 ±0·001 P<0·001 83·2 (D)	0·06 ±0·0054 P<0·001 69·3 (D)	0·17 ±0·01 P<0·01 41·9 (I)	0·07 ±0·01 P<0·05 54·9 (I)	0·89 ±0·07 P<0·001 46·4 (D)	0·51 ±0·005 P<0·001 48·7 (I)	0·14 ±0·01 P<0·01 25·5 (I)
60 min	104 (16) (4)	0·15 ±0·01 P<0·02 26·4 (D)	0·16 ±0·01 4·4	0·04 ±0·004 7·3	0·14 ±0·01 22·7	0·13 ±0·01 6·5	0·05 ±0·001 P<0·01 13·7 (I)	1·44 ±0·13 13·1	0·35 ±0·019 2·8	0·12 ±0·004 6·0
		Mesencephalon			Pons-medulla oblongata			Adrenal glands		
Normal	103 (24)(11)	0·80 ±0·07	0·31 ±0·03	0·05 ±0·01	0·72 ±0·06	0·42 ±0·04	0·09 ±0·003	0·03 ±0·004	0·13 ±0·02	0·52 ±0·05
20 min	113 (16) (4)	0·57 ±0·03 P<0·001 29·7 (D)	0·49 ±0·04 P<0·02 57·4 (I)	0·07 ±0·006 25·2 (I)	0·45 ±0·03 P<0·001 37·7(D)	0·69 ±0·02 P<0·001 58·5 (I)	0·11 ±0·01 16·4 (I)	0·03 ±0·003 13·4 (I)	0·12 ±0·008 7·5 (D)	0·55 ±0·02 4·5 (I)
60 min	104 (16) (4)	0·69 ±0·02 P<0·01 13·4 (D)	0·35 ±0·03 12·6 (I)	0·05 ±0·003 8·8 (D)	0·63 ±0·01 P<0·01 12·4 (D)	0·53 ±0·03 21·7 (I)	0·10 ±0·003 5·5 (I)	0·03 ±0·002 14·0 (I)	0·13 ±0·007 2·5 (D)	0·56 ±0·02 7·8 (I)

Values are means and standard errors in μg/g of fresh tissue, in adrenals μg/mg.
* Number of animals
† Number of determinations
(I) % increases
(D) % decreases
DA Dopamine
NA Noradrenaline
A Adrenaline

in noradrenaline, although adrenaline was still significantly high in the cerebellum.

Adrenaline content was decreased in the hemispheres after 5 min by injection of the acid but it was not modified in the mesencephalon, pons and medulla oblongata, and was increased significantly in the diencephalon and cerebellum.

On a percent basis, only in the diencephalon, and at 10 min after injection of acid, was dopamine decreased by 44%; this corresponds to the increase of noradrenaline (29%) and of adrenaline (16%) (Table 2).

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TABLE 2. EFFECT OF THE ADMINISTRATION OF ASCORBIC ACID (500 MG/KG I.P.) ON THE CONTENT OF CATECHOLAMINES IN THE DIENCEPHALON

	Dopamine	Noradrenaline	Adrenaline
Normal	1·66 ± 0·12*	0·34 ± 0·02	0·11 ± 0·03
At 10 min	0·92 ± 0·01 44·3 (D) P < 0·001	0·44 ± 0·02 28·9 (I) P < 0·02	0·13 ± 0·01 15·7 (I)

* Values are means and standard errors of 4 determinations in µg/g of fresh tissue.
(I) % increases; (D) % decreases.

After 20 min the noradrenaline increases were proportionally larger than the decreases in dopamine in the mesencephalon, pons and medulla oblongata.

The adrenal content of the three amines did not change significantly for 60 min after injection of the acid (Table 1).

Injection of ascorbic acid produced an increase in its concentration in brain structures; and even more so in the whole adrenal glands (Table 3).

TABLE 3. ASCORBIC ACID CONTENT IN BRAIN AND ADRENAL GLANDS. NORMAL AND INJECTED WITH ASCORBIC ACID 500 MG/KG I.P.

	Normal	20 min	% increase
Brain hemispheres	82·40 ± 3·65*	125·35 ± 2·40	52†
Cerebellum	80·59 ± 4·00	145·85 ± 3·46	81†
Diencephalon	66·38 ± 2·95	105·25 ± 6·39	58†
Pons and medulla oblongata ..	41·32 ± 2·70	85·92 ± 3·26	108†
Mesencephalon	60·52 ± 2·57	102·95 ± 7·10	70†
Adrenal glands	471·78 ± 13·00	973·60 ± 6·58	106†

* Values are means and standard errors of 4 determinations in µg/g of fresh tissue.
† P<0·001.

TABLE 4. ASCORBIC ACID AND DOPAMINE CONCENTRATION OF ADRENAL GLANDS AND ADRENAL MEDULLA IN NORMAL RATS AND RATS INJECTED WITH ASCORBIC ACID 500 MG/KG I.P.

	Normal	20 min
	Ascorbic acid	
Adrenal gland	471·10 ± 12·57*	988·14 ± 14·10 109 (I) P<0·001
Adrenal medulla ..	368·66 ± 12·05	402·15 ± 5·32 9·1 (I)
Cortex (deduced value) ..	102·44	585·99 472 (I)
	Dopamine	
Adrenal gland	0·03 ± 0·004	0·03 ± 0·003 13·4 (I)
Adrenal medulla ..	0·03 ± 0·001	0·04 ± 0·003 6·8

* Values are means and standard errors of 4 determinations in µg/g of fresh tissue.
(I) % increases.

This increase reached its peak at 20 min and declined at 60 min. No modification of adrenal medullary ascorbic acid was detected (Table 4), thus the increase in adrenal ascorbic acid appeared to occur only in the cortex of the gland.

Twenty min after rats pretreated with nialamide and pyrogallol were given ascorbic acid, a significant increase in the concentration of noradrenaline and adrenaline occurred in the diencephalon alone compared with the concentration of the amines in animals not treated with nialamide or pyrogallol. This pretreatment did not modify the effect of the acid on the concentration of dopamine in the diencephalon or in the adrenals (Table 5).

TABLE 5. EFFECT OF THE ASCORBIC ACID (500 MG/KG I.P.) ADMINISTRATION ON THE DIENCEPHALON AND ADRENALS CONTENT OF CATECHOLAMINES IN RATS PREVIOUSLY TREATED WITH NIALAMIDE AND PYROGALLOL

	Diencephalon			Adrenal—gland		
	DA	NA	A	DA	NA	A
Normal	1·66 ± 0·12	0·34 ± 0·02	0·11 ± 0·03	0·03 ± 0·004	0·13 ± 0·02	0·52 ± 0·05
Ascorbic acid (20 min)	0·89 ± 0·07 46·4 (D) P≤0·001	0·51 ± 0·01 43·7 (I) P≤0·001	0·14 ± 0·01 25·5 (I) P<0·02	0·03 ± 0·003 11·3 (I)	0·12 ± 0·01 7·5 (D)	0·55 ± 0·02 4·6 (I)
Nialamide Pyrogallol Ascorbic acid (20 min)	0·92 ± 0·05 44·8 (D) P<0·001	0·65 ± 0·03 89·8 (I) P<0·001	0·17 ± 0·01 49·8 (I) P<0·001	0·03 ± 0·003 11·5 (I)	0·11 ± 0·01 15·3 (D)	0·57 ± 0·02 9·1 (I)

Values are means and standard errors of 4 determinations in µg/g of fresh tissue, in adrenal µg/mg.
(I) % increases.
(D) % decreases.

Discussion

The simplest explanation for the decrease in dopamine and the increase in noradrenaline produced by ascorbic acid may be the possible participation of the acid in the β-hydroxylating mechanism of dopamine (Levin & others, 1960; Levin & Kaufman, 1961). According to these authors, ascorbic acid acts as coenzyme to dopamine β-hydroxylase in adrenal gland *in vitro* (Levin & others, 1960; Kaufman, 1966). But, except in the diencephalon at 10 min after injection of the acid, close agreement between dopamine decrease and noradrenaline increase is not apparent. Furthermore, adrenaline also appears to be decreased in brain hemispheres (Table 1) and we found the acid not to affect the three amines in the adrenal gland. This suggests that, *in vivo*, factors may be involved other than those which participate in the *in vitro* effect of the acid on catecholamine concentration.

It has been suggested that the hypophyso-corticoadrenal system regulates the adrenal medullary synthesis of adrenaline (Wurtman & Axelrod, 1965; Axelrod, 1966). There is a relation between adrenal cortical function and response to catecholamines (Brodie, Davies & others, 1966). However, neither ACTH nor corticoids modify the adrenaline content of adrenal glands (Linét & Hertting, 1966). Our results

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suggest the acid to have an effect independent of cortical and medullary function, inasmuch as the acid is markedly increased in cortical tissue after intraperitoneal injection, but there is no change in medullary ascorbic acid or in adrenal dopamine, noradrenaline or adrenaline. This is suggested by the fact that in brain structures, which are normally relatively low in ascorbic acid, its injection not only increases its concentration but also markedly affects catecholamine concentration.

Acknowledgement. Supported by research grant of the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

References

Axelrod, J. (1966). *Pharmac. Rev.*, **18** (1), 95–113.
Bertler, A., Carlsson, A. & Rosengren, E. (1958). *Acta physiol. scand.*, **44**, 273–283.
Brodie, B. B., Davies, J. I., Hyme, S., Krishna, G. & Weiss, B. (1966). *Pharmac. Rev.*, **18** (1), 273–289.
Friedman, S. & Kaufman, S. (1965). *J. biol. Chem.*, **240**, 552–554.
Izquierdo, J. A., Jofré, I. J. & Dezza, M. A. (1964). *Medna Exp.*, **10**, 45–55.
Izquierdo, J. A. & Jofré, I. J. (1965). *J. Pharm. Pharmac.*, **17**, 391–392.
Kaufman, S. (1966). *Pharmac. Rev.*, **18** (1), 61–69.
Laverty, R. & Sharman, D. F. (1965). *Br. J. Pharmac. Chemother.*, **24**, 759–772.
Levin, E. Y. & Kaufman, S. (1961). *J. biol. Chem.*, **236**, 2043–2049.
Levin, E. Y., Levenberg, B. & Kaufman, S. (1960). *Ibid.*, **235**, 2080–2086.
Linèt, O. & Hertting, G. (1966). *Archs int. Pharmacodyn. Thér.*, **159**, 407–415.
McLean, J. R. & Cohen, R. (1963). *Life Sci.*, **4**, 261–265.
Roe, J. H. (1954). In *Methods of Biochemical Analysis*, Vol. 1, p. 115. Editor Glick, D. New York: Interscience.
Weber, L. J. (1966). *Proc. Soc. exp. Biol. Med.*, **123**, 349–352.
Wurtman, R. J. & Axelrod, J. (1965). *Science, N.Y.*, **150**, 1464–1465.

J. Pharm. Pharmac., 1968, **20**, 215–225 Received July 28

Role of pH in the coacervation of the systems: gelatin–water–ethanol and gelatin–water–sodium sulphate*

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Phase boundary determination, coacervate volume measurements and analysis of the phases have been made to assess the influence of pH on the coacervation of gelatin solutions by ethanol and sodium sulphate. Coacervation was found to be pH dependent. In the ethanol system coacervation was noticeable only within a pH range in the vicinity of the isoionic point; at other pH values either a viscous gel phase or floccules occurred. In the sodium sulphate system, coacervation occurred at all pH values examined. The effect of pH in changing the overall charge on the gelatin molecule is explained in relation to the formation of gelatin coacervates. Finally, the role of the coacervate phase in the microencapsulation of oil and solid particulates is discussed.

THE term coacervation was introduced by Bungenberg de Jong & Kruyt (1929) to describe the various cases of partial miscibility occurring in macromolecular systems where two isotropic liquid phases co-exist. Under optimum conditions the colloid-rich phase, the coacervate, contains the bulk of the colloidal components and the equilibrium liquid contains only a negligible amount of the colloid. Two main types of coacervation have been classified by Bungenberg de Jong (1949); simple coacervation occurs as the result of a decrease in the solubility of the colloidal components caused by the addition of non-solvents, as in the systems: isoelectric gelatin–water–ethanol and isoelectric gelatin–water–sodium sulphate, first investigated by Holleman, Bungenberg de Jong & Modderman (1934). Complex coacervation, on the other hand, results from the interaction of oppositely charged colloids and has been of academic interest in relation to some biological phenomena (Bungenberg de Jong & Booij, 1956). Recently a quantitative formulation to the theory of complex coacervation was attempted by Veis (1963).

Simple coacervation of gelatin systems has received little attention as it has been assumed that the phenomenon occurs as a result of dehydration and that the charge effects play no active role.

In the past few years, attention has focussed on the application of both types of gelatin coacervation to the microencapsulation of pharmaceuticals (Luzzi & Gerraughty, 1964; Phares & Sperandio, 1964) and as a superior method for the fractionation of heterodisperse polymers (Stainsby, 1954). The present paper examines the effects of pH on the simple coacervation of gelatin by ethanol and sodium sulphate.

Experimental

MATERIALS

Gelatin. Two samples were used having the characteristics given in Table 1. The gelatins were dried in thin layers at 110° for 12 hr and

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* This work forms part of a thesis presented by S.A.H.K. for the degree of Ph.D. in the University of London.

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Source: Klinische Wochenschrift 13: 845-846, 1934.

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The Depigmenting Action of l-Ascorbic Acid (Vitamin C). Hyperpigmentation caused by Follicular Hormone and Vitamin C

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The observation that administration of vitamin C can cause a recession of the hyperpigmentation observed in Addison's disease (v. Szent - Gyorgyi, Szule, Morawitz) is of very great theoretical interest. From a pratical standpoint, this observation is less important, since the course of Addison's disease is not affected by vitamin C.

It is worthwhile to investigate whether other hyperpigmentations can be made to regress by administration of vitamin C, or whether at least their appearance can be delayed. This is not only important from a theoretical standpoint to clarify the action mechanism of vitamin C in reducing the hyperpigmentation of a patient suffering from Addison's disease, but this question is also of practical importance for the dermatologist.

We have especially considered the question as to whether there is any chance of success in attempting to treat chloasma uterinum with vitamin C, or to prevent the formation of such hyperpigmentations during pregnancy by administration of vitamin C. From a practical standpoint, this could be done, since, according to Szule, extensive depigmentation of a patient with Addison's disease can be achieved by the daily administration of juice from only 2 lemons.

As a result of the studies published by Morawitz and Schroder while our tests were already in progress, such a therapy of chloasma appears promising. Morawitz writes: "The same phenomenon, namely the disappearance of a chloasma-like melanosis, was observed by me in a patient with chronic enterocolitis who had contracted scurvy, during administration of orange and lemon juice". The same author further mentions another scurvy patient who exhibited chloasma-like pigment spots, located primarily on the face.

According to Schroder, v. Szent-Gyorgyi had already shown that, in a test tube, melanin formation is inhibited by ascorbic acid, and he was

able to demonstrate himself that Bloch's dopa reaction is negative when vitamin C is added to the substrate. The crucial question now is whether vitamin C prevents the conversion of melanogen into melanin in the skin, which is accomplished (effected) by dopaoxidase. If this is the case, then it is highly probable that vitamin C can remove (eliminate) not only the Addison pigmentation, but also the chloasma uterinum.

For the formation of chloasma uterinum tests carried out by Lipschutz, Bloch and Schrafl and by Bloch and Guldberg are important, since they showed that, along with a great increase in size of nipples, the areolae of castrated and noncastrated male guinea pigs exhibit a great hyperpigmentation as a result of ovary implantation (Lipschutz) and also of follicular hormone injections (Bloch et.al.). We have new investigated whether such a hyperpigmentation does not take place when large doses of vitamin C are injected along with follicular hormone.

Tests were carried out on 2 groups of animals. Castrated male guinea pigs received a daily subcutaneous injection for one month of 0.8 - 1.00 cc Ostroglandol Roche. This dose is sufficient to impart a dark brown color to the areolae during this time. In the first group, the test animals received, along with Ostroglandol, a daily subcutaneous injection of 5 mg l-ascorbic acid. In the second group, the animals received daily 50 mg l-ascorbic acid.

For injection, ascorbic acid solutions prepared as prescribed and neutralized with the calculated amount of soda were used.

The results of these tests were clearly negative, i.e. the guinea pigs treated in the manner described above could not be distinguished from the controls treated only with Ostroglandol. Their areolae had the same dark pigmentation as those of the Ostroglandol animals. In our tests, the amount of vitamin C injected was equal to 5-100 times the amount given orally to protect guinea pigs against scurvy. Only a doubling of the protective dose is required to reduce the pigmentation (bleach) of an Addison disease patient. Thus, we have tested much higher doses than those used in Addison disease patients.

From these tests we can draw the following conclusions: large doses of vitamin C do not prevent the hyperpigmentation of guinea pig areolae caused by follicular hormone. Therefore, in the very close relationship between this hyperpigmentation and chloasma uterinum pointed out by Bloch,

it is very unlikely that the therapeutic use of vitamin C in chloasma will be successful.

It is difficult to assume that the depigmenting action of vitamin C in the Addison disease patient takes place as a result of the influence exerted on the system melanogen-dopaoxidase-melanin in the skin, since in our tests this system was not affected by relatively much larger amounts of vitamin C. In spite of the fact that vitamin C *in vitro* (redox compound) suppresses the dopa reaction, the depigmenting action of ascorbic acid cannot act on the pigment-forming skin cell, since otherwise a hyperpigmentation of areolae would not have taken place in our tests. In the Addison patient, vitamin C must act at some other location, perhaps in the adrenal gland itself, if we do not want to assume that only in the Addison patient is vitamin C able to penetrate into the pigment apparatus in the skin, but is unable to do so in an animal with healthy adrenal glands.

Bibliography

Br. Bloch, Handbuch der Haut-und Geschlechtskrankheiten I, Tl, I, 434 (1927).

Br. Bloch u. G. Guldberg, Klin. Wschr. 1933, 734.

Br. Bloch u. A. Schrafl, Arch. i. Dermat. 165, 268 (1932).

A. Lipschutz, Virchows Arch. 276, 676 (1930).

P. Morawitz, Klin. Wschr. 1934, 324.

F. Schaaf, Zbl. Hautkrkh. 46, 657 (1933).

H. Schroeder, Klin. Wschr. 1934, 553.

A. V. Szent-Gyorgyi, Science (N.Y.) 72, 125 (1930)

Dtsch. med. Wschr. 1932, 852.

KLIN. WOCHSCHR. 13: 845-46, 1934.

ZUR FRAGE DER DEPIGMENTIERENDEN WIRKUNG DER L-ASCORBINSÄURE (VITAMIN C).

(Durch Follikelhormon erzeugte Hyperpigmentation und Vitamin C.)

Von

W. JADASSOHN und F. SCHAAF.

Die Feststellung, daß es durch Verabfolgung von Vitamin C gelingt, die Hyperpigmentierung beim Morbus Addison zum Zurückgehen zu bringen (v. SZENT-GYÖRGYI, SZÜLE, MORAWITZ), ist von sehr großem theoretischen Interesse. Praktisch ist diese Feststellung von geringerer Bedeutung, denn der Verlauf der Addisonschen Krankheit wird durch das Vitamin C nicht beeinflußt.

Es ist der Untersuchung wert, ob andere Hyperpigmentierungen durch Vitamin C-Gabe zum Rückgang gebracht werden können oder wenigstens ihre Entstehung hintangehalten werden kann. Es ist dies nicht nur theoretisch wichtig für den Wirkungsmechanismus des Vitamins C bei der Verminderung der Hyperpigmentierung des Addisonkranken, sondern es ist diese Frage für den Dermatologen von praktischer Bedeutung.

Wir haben uns speziell die Frage vorgelegt, ob es Aussicht auf Erfolg hätte zu versuchen, das Chloasma uterinum mit Vitamin C zu behandeln oder durch Vitamin C-Gabe während der Schwangerschaft die Ausbildung solcher Hyperpigmentierungen zu verhindern. Praktisch wäre das durchführbar, denn zur weitgehenden Depigmentierung der Addisonpatienten bedarf es nach SZÜLE nur der täglichen Gabe des Saftes von 2 Citronen.

Durch die Arbeiten von MORAWITZ und von SCHRÖDER, die erschienen sind, während unsere Versuche schon im Gange waren, erscheint eine solche Chloasmatherapie aussichtsreich. MORAWITZ schreibt: „Dieselbe Erscheinung, nämlich Verschwinden einer chloasmaartigen Melanose sah ich bei einer Kranken mit chronischer Enterocolitis, die Skorbut bekommen hatte, während der Darreichung von Apfelsinen- und Citronensaft". Er erwähnt ferner einen weiteren Skorbutkranken, der chloasmaartige Pigmentflecke, vorwiegend im Gesicht, zeigte.

Nach SCHROEDER hat schon v. SZENT-GYÖRGYI nachgewiesen, daß im Reagensglas die Melaninbildung durch Ascorbinsäure gehemmt wird. Ihm selbst gelang der Nachweis, daß die Blochsche Dopareaktion negativ ausfällt, wenn dem Substrat Vitamin C zugesetzt wird. Die Kardinalfrage ist nun die, ob das Vitamin C in der Haut den Übergang von Melanogen in Melanin, der durch die Dopaoxydase bewirkt wird, verhindert. Wäre dies der Fall, so müßte sich mit großer Wahrscheinlichkeit durch das Vitamin C nicht nur die Addisonpigmentierung, sondern auch das Chloasma uterinum beseitigen lassen.

Für die Entstehung des Chloasma uterinum sind Versuche von LIPSCHÜTZ, BLOCH und SCHRAFL und BLOCH und GULDBERG wichtig, die gezeigt haben, daß neben einer starken Vergrößerung der Zitzen die Warzenhöfe kastrierter und nichtkastrierter Meerschweinchenmännchen, sowohl durch Eierstockimplantation (LIPSCHÜTZ) als auch durch Follikelhormoninjektionen (BLOCH und Mitarbeiter) stark hyperpigmentiert werden. Wir haben nun untersucht, ob diese Hyperpigmentierung ausbleibt, wenn den Tieren neben Follikelhormon große Dosen Vitamin C injiziert werden.

Die Versuche wurden in 2 Gruppen durchgeführt. Kastrierte männliche Meerschweinchen erhielten während eines Monates täglich 0,8—1,0 ccm Östroglandol Roche, subcutan injiziert. Diese Östroglandolgabe genügt, um in dieser Zeit die Warzenhöfe dunkelbraun werden zu lassen. In der ersten Gruppe erhielten die Versuchstiere neben Östroglandol täglich 5 mg l-Ascorbinsäure subcutan injiziert. In der zweiten Gruppe erhielten die Tiere täglich 50 mg l-Ascorbinsäure*.

Zur Injektion wurden lege artis hergestellte und mit der berechneten Sodamenge neutralisierte Ascorbinsäurelösungen verwendet.

Das Resultat dieser Versuche war ein eindeutig negatives, d. h. die in der oben angegebenen Weise behandelten Meerschweinchen ließen sich nicht von den nur mit Östroglandol behandelten Kontrollen unterscheiden. Ihre Warzenhöfe waren genau so dunkel pigmentiert, wie die der Östroglandoltiere. Wir haben in unseren Versuchen das 5—100fache der das Meerschweinchen bei oraler Gabe vor Skorbut schützenden Vitamin C-Menge injiziert. Zur Bleichung des Addisonpatienten ist nur eine Verdoppelung der schützenden Dosis erforderlich. Wir haben also mit sehr viel größeren Dosen experimentiert als beim Addisonpatienten verwendet wurden.

Aus diesen Versuchen ergibt sich:

Große Gaben von Vitamin C verhindern die durch Follikelhormon erzeugte Hyperpigmentierung der Warzenhöfe des Meerschweinchens nicht. Es ist daher bei den von BLOCH betonten sehr nahen Beziehungen dieser Hyperpigmentierung zum Chloasma uterinum sehr unwahrscheinlich, daß die therapeutische Verwertung von Vitamin C beim Chloasma Erfolg haben würde.

Es ist schwer anzunehmen, daß die depigmentierende Wirkung des Vitamin C beim Addisonpatienten durch Beeinflussung des Systems Melanogen-Dopaoxydase-Melanin in der Haut zustande kommt, da dieses System in unseren Versuchen mit relativ sehr viel größeren Vitamin C-Mengen unbeeinflußt blieb. Trotzdem das Vitamin C in vitro (Redoxsubstanz) die Dopareaktion aufhebt, kann die depigmentierende Wirkung der Ascorbinsäure nicht an der pigmentbildenden Hautzelle angreifen, denn sonst wäre in unseren Versuchen die Hyperpigmentierung der Warzenhöfe nicht zustande gekommen. Das Vitamin C muß bei Addisonpatienten anderswo, vielleicht in der Nebenniere selber, angreifen, wenn man nicht annehmen will, daß nur beim Addisonpatienten das Vitamin C bis zum Pigmentapparat in die Haut gelangte, nicht aber beim nebennierengesunden Tier.

(Aus der Dermatologischen Klinik Zürich [Direktor: Prof. Dr. Micscher].)

Literatur: BR. BLOCH, Handbuch der Haut- und Geschlechtskrankheiten 1, Tl. 1, 434 (1927). — BR. BLOCH u. G. GULDBERG, Klin. Wschr. 1933, 734. — BR. BLOCH u. A. SCHRAFL, Arch. f. Dermat. 165, 268 (1932). — A. LIPSCHÜTZ, Virchows Arch. 276, 676 (1930). — P. MORAWITZ, Klin. Wschr. 1934, 324. — F. SCHAAF, Zbl. Hautkrkh. 46, 657 (1933). — H. SCHROEDER, Klin. Wschr. 1934, 553. — A. v. SZENT-GYÖRGYI, Science (N. Y.) 72, 125 (1930) — Dtsch. med. Wschr. 1932, 852.

Biochim. Biophys. Acta 121(2):269-280, 1966, 3963

BBA 25575

THE ROLE OF ASCORBIC ACID IN THE BIOSYNTHESIS OF COLLAGEN

I. ASCORBIC ACID REQUIREMENT BY EMBRYONIC CHICK TIBIA IN TISSUE CULTURE

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(Received November 22nd, 1965)

SUMMARY

Embryonic chick tibias, when incubated in a chemically defined medium, grow and synthesize collagen for several days. The tissues display a marked dependence upon the presence of L-ascorbic acid in the culture medium for normal growth. Deletion of ascorbic acid reduces collagen synthesis by the bones after the first 2 days in culture. A less drastic reduction in the synthesis of other proteins, DNA and RNA, was also observed. The rate of synthesis of these substances was rapidly restored by the addition of ascorbic acid.

INTRODUCTION

Several workers have attempted to demonstrate a requirement for ascorbic acid by tissues cultured *in vitro*. JENEY AND TORO[1] and QUERIDO AND GAILLARD[2] have reported an enhancement of collagen synthesis upon addition of vitamin C to cultures of various embryonic chick cells. HASS AND MACDONALD[3] and SACERDOTE DI LUSTIG[4], however, observed no diminution of collagen formation in fibroblasts cultured in scorbutic plasma. The latter author found that fewer cells were produced in cultures containing little or no ascorbic acid, but the amount of collagen synthesized per cell was unchanged. He concluded that ascorbic acid affected cell health and proliferation, but had no specific effect on collagen formation.

WOESSNER AND GOULD[5] conducted studies on the ascorbic acid requirements of embryonic chick tissue in culture and could find no effect of ascorbic acid depletion on either cell maintenance or collagen formation. They pointed out, however, that since these tissues are able to store ascorbic acid, it was not completely certain that the system was completely free of ascorbic acid. Moreover, WOESSNER AND GOULD suggested the possibility that the media used in their studies was not completely deficient in ascorbic acid. More recently, GREEN AND GOLDBERG[6] have reported that

* Taken in part from a thesis submitted in partial fulfilment of the requirements for the Ph.D. degree in Chemistry at Georgetown University, Washington, D.C., 1965.

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ascorbic acid stimulates collagen synthesis by established lines of mammalian fibroblasts.

In an attempt to clarify the role of ascorbic acid in collagen biosynthesis, we have studied the influence of ascorbic acid on collagen producing tissues *in vitro*. We have found that embryonic chick tibias, when cultured in a chemically defined medium, undergo rapid growth while maintaining their structure and their ability to synthesize collagen. Ascorbic acid is required for normal growth as well as for collagen synthesis in these tissues. Similar observations have been made by McCarty and his co-workers[7,8].

EXPERIMENTAL

Fertile eggs were obtained from the Duckworth Hatchery and Feed Company, Hanover, Maryland, and stored in an incubator at 37° until needed. Tibias were excised from 8-day-old chick embryos. Aortic tissue was obtained from 16-day-old embryos. The tissues were fixed to a small rectangular piece of 0.45-μ Millipore filter with a clot formed by mixing two drops of chick plasma (Difco, Inc.) and one drop of bovine thrombin solution (kindly supplied by Dr. A. J. Osbahr). Each tissue was placed in a 16 × 150-mm test tube with 2 ml (tibias) or 4 ml (aortas) of a chemically defined medium. The test tubes were stoppered and placed in a roller drum in a 37° incubator. The medium was replaced every 2 days. The basic medium used was that of Morgan, Morton and Parker[9] (TC 199) modified by omitting hydroxyproline and ascorbic acid. 2 ml of penicillin–streptomycin solution (5000 units of each per ml) (Microbiological Associates) were routinely added per 100 ml of medium.

Uniformly labeled L-[^{14}C]proline and L-[^{14}C]cysteine were obtained from the Nuclear Chicago Corporation and uniformly labeled DL-hydroxy[^{14}C]proline was obtained from New England Nuclear Corporation. [2-^{14}C]Uridine and [*Me*-^{3}H]thymidine were obtained from Schwartz BioResearch, Inc.

After incubation, bones were washed free of medium with distilled water and homogenized in cold 5 % trichloroacetic acid in a glass homogenizer. The suspension was dialyzed free of trichloroacetic acid and free [^{14}C]proline, and the precipitate washed twice with water. Suspensions of the precipitate were made 6 N in HCl by the addition of an equal volume of the concentrated acid, and hydrolyzed in sealed tubes at 121° for 18 h. The hydrolyzates were evaporated to dryness under a stream of air and the residue dissolved in distilled water. In cases where total protein radioactivity was measured, an aliquot of the dialyzed precipitate was plated on stainless-steel planchets and counted in a Nuclear-Chicago low background thin-window gas-flow counter (Nuclear-Chicago Corporation, Des Plaines, Ill.).

Proline and hydroxyproline radioactivity were estimated by the method of Prockop *et al.*[10]. An aliquot of the first toluene extract of the mixture of oxidized imino acids was counted in a Tri-Carb Liquid Scintillation Counter (Packard Instrument Company, La Grange, Ill.) to assay proline radioactivity. Hydroxyproline radioactivity was assayed in the same sample as described by Prockop *et al.*[10].

Prior to the assay of amino acid activating enzymes, 8-day-old chick embryos and tibias from 8-day embryos were homogenized in a glass homogenizer in 0.05 M Tris (pH 7.6), and the homogenates centrifuged at 100000 × *g* for 60 min. Aliquots of the supernatant from bone homogenates were assayed directly for proline- and

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hydroxyproline-dependent [^{32}P]pyrophosphate–ATP exchange by a modification of the [^{32}P]ATP exchange method of BERGMANN[11]. In these experiments the radioactive [^{32}P]ATP was not eluted from the Norit. Instead, the charcoal suspension was filtered through Millipore filters (0.45 μ pore diameter), and washed with 100 ml of 5% trichloroacetic acid. The filter discs were affixed to stainless-steel planchets with rubber cement and ^{32}P activity measured in a Nuclear Chicago low-background thin-window gas-flow counter. The supernatant from whole chicks was found to give very high blank values presumably because of the presence of high concentrations of free amino acids. In order to overcome this problem, 1- or 2-ml aliquots of the supernatant were applied to a 50–75 ml column of Sephadex G-25 equilibrated with 0.05 M Tris (pH 7.6). After gel filtration aliquots of the high molecular weight fraction were assayed as above.

RESULTS

Tibias of 8-day-old chick embryos, when incubated in tissue-culture medium 199 in roller tubes, grew to almost double their length in a 6-day incubation period (Fig. 1). There was a concomitant increase in shaft diameter during this time. When ascorbic acid was omitted from the medium, normal growth was impaired.

Fig. 2 shows the amount of peptide-bound hydroxyproline in normal bones after various times in culture. In this experiment approx. 1.4 μg of hydroxyproline

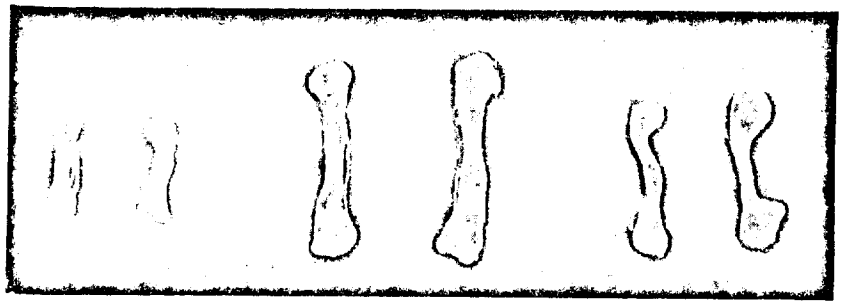

Fig. 1. Chick tibias grown with and without ascorbic acid in the culture medium. On the left are tibias frozen and thawed after removal from the 8-day-old embryo. In the center are tibias incubated for 6 days in medium containing ascorbic acid. Tibias on the right were grown for 6 days without ascorbic acid.

was synthesized per bone per day. After 6 days of incubation this amounted to an 8-fold increase in the hydroxyproline content of the tissue. Since peptide-bound hydroxyproline is generally accepted as a marker for collagen, this finding indicates that the bones synthesized this protein.

Bones grown in medium containing ascorbic acid incorporated [^{14}C]proline into protein and, in addition, converted some of the [^{14}C]proline to peptide-bound hydroxy[^{14}C]proline (Table I). When corrections were made for counting efficiency and recovery of the imino acids in the analytical procedure, it was found that approx. 10% of the amount of [^{14}C]proline incorporated into bone protein was converted to hydroxy[^{14}C]proline.

In the absence of ascorbic acid, incorporation of [^{14}C]proline into protein was

approximately constant for the first 4 days of incubation (Table I). From the fourth to the sixth day, however, proline incorporation was reduced by 63 %. Hydroxy-[^{14}C]proline formation, in contrast to proline incorporation, was diminished by 50 % from the second to the fourth day of incubation. After this time depleted bones

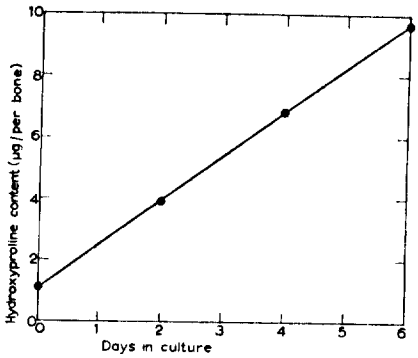

Fig. 2. Hydroxyproline content of chick tibias grown in tissue culture. The hydroxyproline content was determined by the method of PROCKOP AND UDENFRIEND[18]. Each value represents the mean of three determinations.

TABLE I

SYNTHESIS OF COLLAGEN AND OTHER PROTEINS BY CHICK TIBIAS GROWN FOR VARIOUS TIMES IN TISSUE CULTURE. EFFECTS OF ASCORBIC ACID DEPLETION

0.2 μC uniformly labeled L-[^{14}C]proline added per ml of medium. Each value represents the average of duplicate analyses on groups of three bones. In the "Recovery" group [^{14}C]proline and ascorbic acid are added simultaneously.

Group	*Time of [^{14}C]proline addition (days)*	*Trichloroacetic acid-precipitable (counts/min per bone)*	
		[^{14}C]Proline	*Hydroxy-[^{14}C]proline*
Control	0–2	31 700	3860
	2–4	67 240	8504
	4–6	77 160	9512
	6–8	84 610	13 432
Depleted	0–2	44 850	5152
	2–4	54 790	4464
	4–6	22 970	334
	6–8	31 280	1176
Recovery	0–2	—	—
	2–4	63 970	8364
	4–6	90 075	11 806
	6–8	106 010	14 356

synthesized less than 10 % as much hydroxyproline as did control bones. The addition of ascorbic acid to previously depleted bones restored the ability of the tissue to synthesize both protein and hydroxyproline. In fact, in most experiments in which ascorbic acid was added to depleted bones, the rate of isotope incorporation exceeded that observed in control bones by 10–30 %.

The extent to which free hydroxyproline was incorporated into tibia protein was measured by incubating bones with DL-hydroxy[^{14}C]proline. One group of bones was incubated in Medium 199 containing the normal proline concentration (40 μg/ml), a second group in Medium 199 containing no proline, and a third in Medium 199 containing no proline, but supplemented with 40 μg/ml of unlabeled hydroxyproline. The amount of protein-bound hydroxy[^{14}C]proline formed under these conditions was compared to the amount of hydroxy[^{14}C]proline formed in three additional groups of bones incubated in the media just described, but with L-[^{14}C]proline instead of DL-hydroxy[^{14}C]proline. Twice as much DL-hydroxy[^{14}C]proline as L-[^{14}C]proline was added to the media in order to obtain equal concentrations of L isomer radioactivity. All groups were incubated for 2 days, after which time the bones were removed and analyzed for protein-bound [^{14}C]proline and hydroxy[^{14}C]proline. The results (Table II) indicate that very little free hydroxyproline is incorporated into bone protein. The amount of peptide-bound hydroxy[^{14}C]proline produced by bones incubated with hydroxy[^{14}C]proline in proline-free medium is approx. 2 % of the amount of peptide-bound hydroxy[^{14}C]proline formed when bones are incubated with [^{14}C]proline. Addition of unlabeled proline to the tissue-culture medium lowered this value to about 0.5 %.

TABLE II

INCORPORATION OF [^{14}C]PROLINE AND HYDROXY[^{14}C]PROLINE INTO CHICK-TIBIA PROTEIN

Each value represents the average of duplicate analyses on groups of three bones.

[^{14}C]Amino acid in medium		*Incubation medium*	*Amino acid incorporated trichloroacetic acid-insoluble (counts/min per bone)*	
			[^{14}C]Proline	*Hydroxy-[^{14}C]proline*
DL-Hydroxyproline	0.5 μC/ml	TC199, 40 μg/ml L-proline	185	35
DL-Hydroxyproline	0.5 μC/ml	TC199, no proline	303	290
DL-Hydroxyproline	0.5 μC/ml	TC199, no proline + 40 μg/ml L-hydroxyproline	597	315
L-Proline	0.25 μC/ml	TC199, 40 μg/ml proline	55 317	6 555
L-Proline	0.25 μC/ml	TC199, no proline	163 370	16 140
L-Proline	0.25 μC/ml	TC199, no proline + 40 μg/ml L-hydroxyproline	179 707	16 330

The data from this experiment further show that addition of unlabeled hydroxyproline to the medium of bones incubated with [^{14}C]proline has no effect on the incorporation of [^{14}C]proline into protein or on hydroxy[^{14}C]proline formation. As expected, addition of unlabeled proline to the medium reduced [^{14}C]proline incorporation into protein and conversion to hydroxy[^{14}C]proline.

To determine if the lack of free hydroxyproline incorporation into bone protein is the result of the absence of an activating enzyme for this imino acid, the amount of hydroxyproline-dependent [^{32}P]pyrophosphate–ATP exchange was measured in the soluble fraction of this tissue. Hydroxyproline appeared to be activated by enzymes in the soluble fraction of both whole chick and tibia (Table III). The amount of hydroxyproline-dependent [^{32}P]ATP production ranged from 20 to 50 % that of

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proline-dependent [^{32}P]ATP formation. Extracts prepared from tibias grown in culture for 4 days with and without ascorbic acid were also assayed for proline-activating enzymes. Proline was found to be activated to the same extent in normal and depleted bones.

It was of interest to determine whether compounds resembling ascorbic acid could substitute for it in this system. Bones grown for 4 days in a medium containing no ascorbic acid were incubated for an additional 2 days in medium containing [^{14}C]proline and either ascorbic acid, isoascorbic acid (L-araboascorbic acid), glucoascorbic acid, or no addition (Table IV). Ascorbic acid produced the expected stimulation of both proline incorporation and hydroxyproline synthesis. Isoascorbic acid

TABLE III

PROLINE AND HYDROXYPROLINE ACTIVATION IN EXTRACTS OF TIBIAS AND WHOLE CHICK EMBRYO

Each value represents the average of triplicate analyses. Normal tibias, tibias incubated 4 days in ascorbic acid-supplemented medium. Depleted tibias, tibias incubated 4 days in ascorbic acid-free medium.

Enzyme source	*Amino acid added*	*Total [^{32}P]pyrophosphate (counts/min) exchanged*	*Net amino acid-dependent [^{32}P]pyrophosphate (counts/min) exchanged*
Tibia	Proline	570	195
Tibia	Hydroxyproline	487	102
Tibia	None	385	—
Whole embryo	Proline	502	431
Whole embryo	Hydroxyproline	166	95
Whole embryo	None	71	—
Normal tibia	Proline	559	209
Normal tibia	None	350	—
Depleted tibia	Proline	569	243
Depleted tibia	None	326	—

TABLE IV

EFFECT OF ASCORBIC ACID ANALOGUES ON THE SYNTHESIS OF COLLAGEN AND OTHER PROTEINS IN CHICK TIBIAS

Each value represents the average of duplicate analyses on groups of 3 bones. [^{14}C]Proline added as 0.2 μC uniformly labeled L-[^{14}C]proline per ml of medium. All other compounds added in a concentration of 50 μg per ml of medium.

Group	*Incubation conditions*		*Trichloroacetic acid-precipitable (counts/min per bone)*	
	0–4 days	*4–6 days*	*[^{14}C]Proline*	*Hydroxy-[^{14}C]proline*
1	—Ascorbic acid	+ [^{14}C]Proline	417	0
2	—Ascorbic acid	+ [^{14}C]Proline + ascorbic acid	4301	340
3	—Ascorbic acid	+ [^{14}C]Proline + isoascorbic acid	1440	280
4	—Ascorbic acid	+ [^{14}C]Proline + glucoascorbic acid	595	0

was approx. 50 % as effective as ascorbic acid, whereas glucoascorbic acid was inactive in stimulating either protein synthesis or hydroxyproline formation. In another experiment, 2,6-dichlorophenolindophenol, a compound which replaces ascorbic acid in some systems, was added to depleted bones as described above. In the tibia system, 2,6-dichlorophenolindophenol is toxic to normal bones and inhibits both [^{14}C]proline incorporation and hydroxy[^{14}C]proline formation.

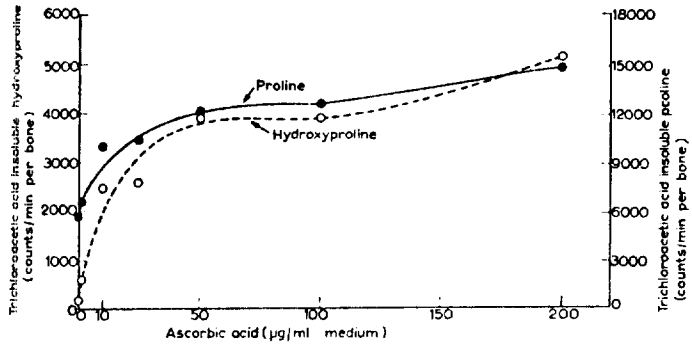

Fig. 3. Effect of ascorbic acid concentration on the synthesis of collagen and other proteins in chick tibias. Tibias grown 4 days without ascorbic acid and final 2 days with uniformly labeled [^{14}C]proline and various levels of ascorbic acid.

The effectiveness of varying concentrations of ascorbic acid in stimulating protein synthesis and hydroxyproline formation was studied. Bones depleted of the vitamin for 4 days were incubated in medium containing 1–200 µg/ml of ascorbic acid, and [^{14}C]proline (0.2 µC/ml). The extent of recovery, as assayed by measuring the amount of peptide-bound [^{14}C]proline and hydroxy[^{14}C]proline formed by the bones, was progressive with increasing ascorbic acid concentrations (Fig. 3). Most of the increased incorporation took place at ascorbic acid concentrations from 1 to 50 µg/ml, although some further stimulation was observed at a concentration of 200 µg/ml. At the latter concentration, however, it was difficult to control the pH of the tissue-culture medium. Subsequent studies, therefore, routinely employed an ascorbic acid concentration of 50 µg/ml of medium.

TABLE V

EFFECT OF ASCORBIC ACID DEPLETION IN EMBRYONIC CHICK AORTAS

Each value represents the average of duplicate analyses on groups of four aortas. L-[^{14}C]Proline (0.2 µC per ml medium) was added to all groups for the last 2 days of incubation.

Group	*Trichloroacetic acid insoluble (counts/min per aorta)*	
	[^{14}C]*Proline*	*Hydroxy-*[^{14}C]*proline*
4-day control	14 487	2253
4-day depleted	14 109	1296
6-day control	17 660	1930
6-day depleted	14 514	256

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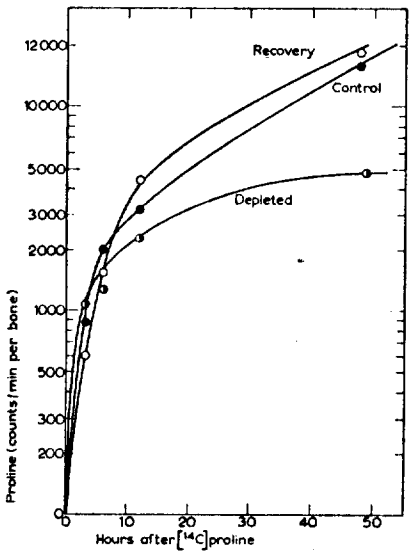
In order to determine if the effects of ascorbic acid depletion are confined to bone, or can be observed in other tissues, aortas were dissected from 16-day-old chick embryos and placed in tissue culture in exactly the same fashion as tibias, except that twice as much medium (4 ml) was added to the tubes. The aortas were incubated for 4 or 6 days, with and without ascorbic acid. [^{14}C]Proline was added to the medium 2 days before removing the tissues from culture. The results of a typical experiment are shown in Table V. Between the second and fourth days of incubation without ascorbic acid, [^{14}C]proline incorporation was not affected, whereas hydroxy[^{14}C]-proline synthesis was depressed 42 %. From the fourth to the sixth day [^{14}C]proline incorporation is inhibited 18 %, while during this time hydroxy[^{14}C]proline formation drops by 87 %. We have also observed a similar decrease in collagen synthesis in calvaria obtained from 5-day-old mice, when cultured under similar conditions in the absence of ascorbic acid.

The rate of formation of peptide-bound [^{14}C]proline and hydroxy[^{14}C]proline was studied in ascorbic acid-depleted and supplemented bones. In these studies three groups of bones were incubated in culture for 4 days. One group was incubated in normal medium containing 50 μg/ml of ascorbic acid, the other two in ascorbic acid-free medium. On the fourth day [^{14}C]proline was added to the medium of all bones. The first group continued to receive ascorbic acid, the second group received no



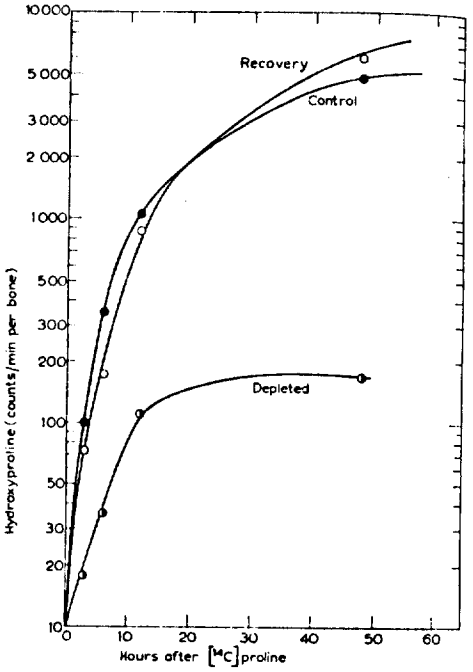


Fig. 4. Effect of ascorbic acid on protein synthesis in chick tibias. Bones were grown for 4 days in culture. Control bones were grown with 50 μg ascorbic acid per ml medium. Recovery and depleted bones did not receive ascorbic acid during this period. Uniformly labeled [^{14}C]proline was added after the fourth day and control and recovery bones received ascorbic acid (50 μg/ml).

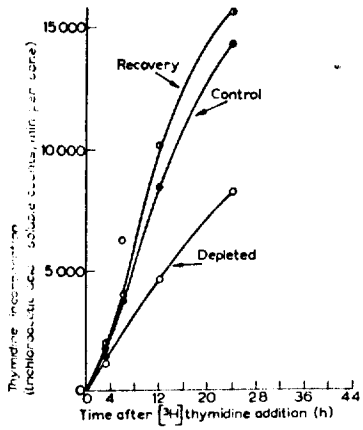
Fig. 5. Effect of ascorbic acid on collagen synthesis in chick tibias. Conditions are as described in Fig. 4.

ascorbic acid, and the third, depleted of the vitamin for the first 4 days, received 50 μg/ml of ascorbic acid. Bones were removed from each group at various times and the amounts of peptide-bound [^{14}C]proline and hydroxy[^{14}C]proline determined. The results of such an experiment are summarized in Figs. 4 and 5.

Control bones incorporated [^{14}C]proline into protein and synthesized hydroxy-[^{14}C]proline at a linear rate throughout the incubation period. In depleted bones the rate of hydroxy[^{14}C]proline formation was much slower than that of control bones throughout the incubation period. After 12 h of incubation deficient bones synthesized only about 10% as much hydroxy[^{14}C]proline as control bones. Little additional synthesis occurred between 12 and 48 h of incubation. [^{14}C]Proline incorporation into protein in depleted bones, however, continued at a normal rate for at least the first 3 h of incubation and, after 12 h, [^{14}C]proline incorporation was approx. 70% that observed in control bones. Between 12 and 48 h of incubation [^{14}C]proline incorporation in depleted bones doubled whereas in control bones there was a 4-fold increase during this time.

Addition of ascorbic acid to previously depleted bones caused a stimulation of both [^{14}C]proline incorporation and hydroxy[^{14}C]proline synthesis. At early incubation times (3 and 6 h) the amounts of both imino acids incorporated into protein appeared to be somewhat less than those in control bones, but by 12 h the recovery values were equal to or even greater than those of control bones. At 48 h the amounts of both radioactive imino acids in protein are greater in recovery than in control bones.

In order to investigate the effect of ascorbic acid depletion on the incorporation into protein of an amino acid which is not found in vertebrate collagens, control, depleted, and recovery bones were incubated with [^{14}C]cysteine from day 4 to day 6. The bones were removed on the sixth day and the amount of trichloroacetic acid-insoluble radioactivity was assayed. [^{14}C]Cysteine incorporation into depleted bone protein was depressed about 50% and the depression was reversed upon the addition of ascorbic acid.



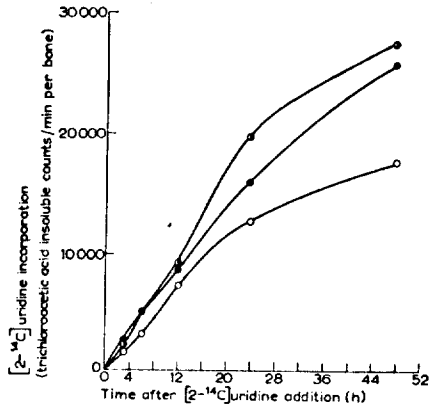


Fig. 6. Effect of ascorbic acid depletion on DNA synthesis in chick tibias. Groups were treated as described in Fig. 4 except that [^{14}C]thymidine was added to the medium.

Fig. 7. Effect of ascorbic acid depletion on RNA synthesis in chick tibias. Groups were treated as listed in Fig. 4 except that [^{14}C]uridine was added to the medium.

The effect of ascorbic acid-depletion on DNA synthesis in tibias was assessed by incubating control, depleted, and recovery bones for various lengths of time with [*Me*-^{3}H]thymidine. After incubation the bones were homogenized in cold 5% trichloroacetic acid, the precipitate washed, extracted with hot 10% trichloroacetic acid for 30 min and the extracts assayed for tritium radioactivity. In this experiment, summarized in Fig. 6, the incorporation of [^{3}H]thymidine was less in depleted bones than in control or recovery bones. The rate of thymidine incorporation in recovery bones exceeded that of control bones at all times.

In order to assess the effect of ascorbic acid depletion on RNA synthesis, control, depleted and recovery bones were incubated with [2-^{14}C]uridine for various lengths of time, and the amount of trichloroacetic acid-insoluble radioactivity in the bones determined. Fig. 7 summarizes the results of this experiment. RNA synthesis appears to be diminished by ascorbic acid deficiency. The rate of RNA synthesis in recovery bones was somewhat higher than in control bones.

DISCUSSION

The ability of embryonic chick tibias to undergo rapid growth and to synthesize collagen for several days, *in vitro*, suggested the possibility that this system could be a useful one for the study of the pathways of collagen biosynthesis. That the tibias are actively synthesizing collagen is evident from the rate at which the tissue content of protein-bound hydroxyproline increases throughout the 6 days of incubation. The 8-fold increase which occurs represents the synthesis of approx. 11 μg of collagen per bone per day. This amount compares favorably with the amount of collagen synthesized by isolated mouse fibroblasts in a system recently reported by GREEN AND GOLDBERG[12]. In this system each inoculum of fibroblasts, consisting of about 10^7 cells, synthesizes approx. 15 μg of collagen per day.

A marked dependence of this tissue upon ascorbic acid for growth and proper metabolism is readily demonstrated. Histological examination of bones grown for 6 days in the absence of ascorbic acid reveals that the amount of extracellular ground substance present in this tissue is drastically reduced. In fact, the appearance of this tissue is quite similar to the histological appearance of connective tissues from animals depleted of ascorbic acid *in vivo*.

It seems clear that ascorbic acid is required as a vitamin for the tibia *in vitro* even though it is not a vitamin in the chicken. The reason for this may lie in the fact that ascorbic acid synthesis in the chicken occurs in the kidney, very little or no synthesis occurring in other tissues[13]. Apparently, in tissue culture, the ascorbic acid stores of the tibias can be depleted, although the possibility cannot be excluded by our data that a small amount of ascorbic acid is synthesized by the tibias at all times. If this is the case, the amount must be very small, since ascorbic acid concentrations as low as 1 μg per ml of medium caused a stimulation of collagen synthesis in the bones.

The isotope incorporation data indicate that there is drastic depression of collagen synthesis in the tibias greater than the diminution of other protein synthesis. This depression is apparent by the fourth day in culture, and after this time collagen synthesis is inhibited by up to 100%, while the synthesis of other proteins continues to take place, although at reduced rates.

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Incubation of chick aortas and mouse calvaria without ascorbic acid, in tissue culture, reveals a similar dependence of these tissues upon the presence of the vitamin. Hence it appears that the effect of ascorbic acid depletion on tibias is not limited to the bone, but is apparent in other connective tissues.

The data from isotope-incorporation studies reveal that the effect of ascorbic acid depletion is not limited to collagen production. After 6 days of vitamin deprivation, the incorporation of [^{14}C]proline into protein is reduced by at least 60%. This reduction is more than can be accounted for by the failure of the tissue to synthesize collagen. The depression observed in [^{14}C]cysteine incorporation into depleted tibias is essentially the same as that observed in [^{14}C]proline incorporation. In addition to the effects of amino acid depletion on protein synthesis, DNA and RNA synthesis are also depressed in vitamin-depleted bones, indicating that ascorbic acid is in some way involved in the maintenance of a broad range of reactions in the cell.

Addition of ascorbic acid to previously depleted tibias results in an immediate resumption of both [^{14}C]proline incorporation and hydroxy[^{14}C]proline synthesis. In almost every experiment the rates of incorporation of both imino acids into protein in recovery tibias exceeded the rates in control bones. This finding is analogous to that of ROBERTSON AND SCHWARTZ[14] as well as GOULD AND WOESSNER[15] in scorbutic guinea-pigs. Administration of ascorbic acid to these animals stimulated an immediate synthesis of collagen at a rate which exceeded that observed in normal animals. The cause of this elevated rate is unknown.

Although several studies have indicated that free hydroxyproline is not incorporated as such into collagen, MITOMA AND SMITH[16] have reported that this imino acid is incorporated into chick-embryo collagen hydroxyproline about 10% as efficiently as is proline. For this reason we wished to determine whether free hydroxyproline is incorporated into the protein of chick tibias in our system. In tibias incubated in proline-free medium, the incorporation of free hydroxy[^{14}C]proline into protein is negligible.

In spite of the fact that little or no free hydroxyproline appears to be incorporated into tibia protein, significant activation of this imino acid appears to occur in extracts of bone and whole embryo. In tibia extracts, the amount of hydroxyproline-dependent ATP–[^{32}P]pyrophosphate exchange ranged from 30 to 50% of proline-dependent exchange. The fact that hydroxyproline appears to form hydroxyproline adenylate, yet is not incorporated into protein must mean that hydroxyproline is unable to participate in one of the subsequent reactions of protein synthesis. It is tempting to suggest, in view of the recent work of NORRIS AND BERG[17], that in the transfer of hydroxyproline from the adenylate to sRNA, the hydroxyproline–adenylate–enzyme complex becomes unstable and dissociates. In any case, the data suggest that proline is not hydroxylated as prolyl adenylate, since the resulting hydroxyprolyl adenylate apparently fails to participate further in protein synthetic reactions.

ACKNOWLEDGEMENT

The authors are grateful to Dr. H. M. FULLMER for the preparation and interpretation of the histological specimens.

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REFERENCES

1 A. V. JENEY AND E. TORO, *Arch. Pathol. Anat. Physiol.*, 298 (1937) 87.
2 A. QUERIDO AND P. J. GAILLARD, *Acta Brevia Neerl. Pharmacol. Microbiol.*, 9 (1939) 70.
3 G. HASS AND F. MCDONALD, *Am. J. Pathol.*, 16 (1940) 525.
4 E. SACERDOTE DI LUSTIG, *Res. Soc. Arg. Biol.*, 20 (1944) 170.
5 J. F. WOESSNER AND B. S. GOULD, *J. Biophys. Biochem. Cytol.*, 3 (1957) 685.
6 H. GREEN AND B. GOLDBERG, *Proc. Soc. Exptl. Biol. Med.*, 117 (1964) 258.
7 J. ROBERTS AND K. S. MCCARTY, *Thirteenth Annual Meeting of the Tissue Culture Association, Washington, D.C., 1962.*
8 R. S. BHATNAGAR, *Thesis*, Duke University, Durham, N.C., 1963.
9 J. F. MORGAN, H. J. MORTON AND R. C. PARKER, *Proc. Soc. Exptl. Biol. Med.*, 73 (1950) 1.
10 D. J. PROCKOP, B. PETERKOFSKY AND S. UDENFRIEND, *J. Biol. Chem.*, 237 (1962) 1581.
11 F. H. BERGMANN, in S. P. COLOWICK AND N. O. KAPLAN, *Methods of Enzymology*, Vol. 5, Academic Press, New York, 1962, p. 708.
12 H. GREEN AND B. GOLDBERG, *Nature*, 200 (1963) 1097.
13 I. B. CHATTERJEE, N. C. KAR AND B. C. GUHA, *Ann. N.Y. Acad. Sci.*, 92 (1961) 36.
14 W. ROBERTSON AND B. SCHWARTZ, *J. Biol. Chem.*, 235 (1960) 426.
15 B. S. GOULD AND J. F. WOESSNER, *J. Biol. Chem.*, 226 (1957) 289.
16 C. MITOMA AND T. E. SMITH, *J. Biol. Chem.*, 235 (1960) 426.
17 A. T. NORRIS AND P. BERG, *Proc. Natl. Acad. Sci. U.S.*, 52 (1964) 330.
18 D. J. PROCKOP AND S. UDENFRIEND, *Anal. Biochem.*, 1 (1960) 228.

Biochim. Biophys. Acta. 121(2): 281-291 1966 3964

BBA 25576

THE ROLE OF ASCORBIC ACID IN THE BIOSYNTHESIS OF COLLAGEN

II. SITE AND NATURE OF ASCORBIC ACID PARTICIPATION

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(Received November 22nd, 1965)

SUMMARY

Studies have been conducted on collagen biosynthesis by embryonic chick tibias in tissue culture and on the nature of the action of ascorbic acid in this system. Inhibitors of electron transport and uncouplers of oxidative phosphorylation were found to inhibit the synthesis of peptide-bound hydroxyproline and other proteins to a similar extent.

Although depressed DNA and RNA synthesis by the bones is an early effect of ascorbic acid depletion, the effect of ascorbic acid on collagen synthesis did not appear to be on either the stability or synthesis of collagen mRNA.

Puromycin was found to inhibit the synthesis of peptide-bound hydroxyproline more than it inhibited the incorporation of proline into protein, indicating that the incorporation of proline into peptide linkage preceded hydroxylation. Simultaneous addition of ascorbic acid and puromycin to bones prelabeled with [^{14}C]proline increased the amount of peptide-bound hydroxyproline in the absence of peptide synthesis. These results indicate that ascorbic acid exerts a direct action on collagen synthesis by stimulating the hydroxylation of peptide-bound proline.

INTRODUCTION

In the preceding paper[1], we described the influence of ascorbic acid depletion on the growth and metabolism of embryonic chick tibias *in vitro*. Of the parameters studied, the rate of collagen synthesis was particularly sensitive to ascorbic acid deficiency. After 4 days in culture without ascorbic acid, collagen synthesis virtually ceased. Addition of ascorbic acid to previously depleted tibias rapidly restored the rate of collagen synthesis to normal.

In this paper, we report the results of our studies intended to delineate the site and the nature of the participation of ascorbic acid in collagen synthesis.

* Taken in part from a thesis submitted in partial fulfilment of the requirements for the Ph.D degree in Chemistry at Georgetown University, Washington, D.C., U.S.A.

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EXPERIMENTAL

The tissue-culture procedures used in these studies were those previously described[1]. Preparation of the tissues and the analyses for proline and hydroxyproline were also carried out as previously described.

Metabolic inhibitors. Amytal, dicoumarol, antimycin A, oligomycin and 2,4-dinitrophenol were kindly supplied by Dr. ELLIOTT SCHIFFMANN. SKF-525A* was kindly supplied by Dr. J. KAMM.

Puromycin. Puromycin dihydrochloride was a gift of the National Cancer Chemotherapy Service.

Actinomycin D. Actinomycin D was kindly supplied by Drs. E. KATZ AND J. A. R. MEAD.

RESULTS

Effect of metabolic inhibitors on proline and hydroxyproline incorporation into protein

In order to investigate the possibility that collagen synthesis is more sensitive than the synthesis of other proteins to a metabolic defect caused by the lack of ascorbic acid, bones were incubated with ascorbic acid and one of a number of inhibitors of electron transport. In general, inhibitors of electron transport, such as KCN, Amytal, antimycin A and dicoumarol and uncouplers of electron transport, such as dinitrophenol and oligomycin, inhibit both [^{14}C]proline incorporation and hydroxy[^{14}C]proline synthesis to approximately the same extent (Table I). In addition, the compound SKF-525A, which has been shown to inhibit a number of microsomal hydroxylation reactions[2], produces similar inhibitions. Hydrocortisone, which has been said to exert a specific inhibitory effect on the production of granulation tissue[3], does not selectively inhibit hydroxy[^{14}C]proline synthesis in the tibia.

TABLE I

EFFECT OF METABOLIC INHIBITORS ON PROLINE AND HYDROXYPROLINE INCORPORATION INTO TIBIA PROTEIN

Inhibitor and [^{14}C]proline (0.2 μC per ml medium) added simultaneously. All bones then incubated for 2 days. Each value represents the average of at least duplicate analyses on groups of three bones.

Inhibitor	*Concentration (M)*	*Radioactive imino acid incorporated into tibia protein (% of control)*	
		[^{14}C]Proline	*Hydroxy[^{14}C]proline*
KCN	$1 \cdot 10^{-4}$	79	81
KCN	$5 \cdot 10^{-4}$	37	21
Amytal	$2.5 \cdot 10^{-3}$	36	26
Antimycin A	$4 \cdot 10^{-6}$	71	50
Oligomycin	$1.6 \cdot 10^{-5}$	56	43
2,4-Dinitrophenol	$1 \cdot 10^{-4}$	54	41
Dicoumarol	$3 \cdot 10^{-5}$	25	18
SKF-525A	$1 \cdot 10^{-4}$	20	14
SKF-525A	$1 \cdot 10^{-5}$	76	89
Hydrocortisone	$7 \cdot 10^{-4}$	46	42

* β-Diethylaminoethyl diphenylpropylacetate hydrochloride.

Inhibition of protein synthesis and hydroxyproline formation by actinomycin D

Since RNA synthesis was found to be depressed in depleted bones[1], we investigated the possibility that the observed defect in collagen biosynthesis in ascorbic acid-deficient tissue is caused by a defect in RNA production. We considered the possibility that collagen synthesis is more sensitive than the synthesis of other proteins to a general impairment of RNA production. Consequently, the effect of actinomycin D on protein synthesis and hydroxyproline production in tibias was studied. Actinomycin D is an antibiotic which has been shown to inhibit DNA-dependent RNA synthesis. Normal bones were incubated for 2 days with [^{14}C]proline and with several concentrations of actinomycin D. After incubation, the bones were assayed for protein-bound [^{14}C]proline and hydroxy[^{14}C]proline. The results (Fig. 1) show that, at each concentration of antibiotic, hydroxy[^{14}C]proline formation and [^{14}C]proline incorporation into protein are inhibited to the same extent. The inhibition, however, never reached 100%. At the two higher concentrations of the antibiotic, the bones still retain approx. 20% of their total collagen synthetic activity in spite of the fact that these concentrations of actinomycin D are sufficient to lower [^{14}C]uridine incorporation into trichloroacetic acid-insoluble material (presumably RNA) to less than 1% of control.

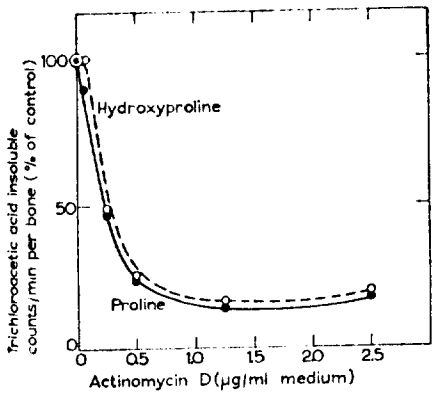

Fig. 1. Inhibition of the synthesis of collagen and other proteins by actinomycin D.

It seemed possible that the residual incorporation of isotope into bones in the presence of high concentrations of actinomycin D was due to relatively stable messenger RNA (mRNA) present in the bones at the time of addition of the antibiotic. Presumably, preincubation of the bones with actinomycin D before addition of [^{14}C]proline would abolish all incorporation when the preincubation time is longer than the lifetime of mRNA in the tissue. At shorter preincubation times, incorporation should occur to an extent proportional to the amount of mRNA remaining in the cells. Accordingly, an experiment was performed in which bones were preincubated for various times with 5 μg/ml of actinomycin D. At zero time all bones received [^{14}C]proline and the incubation was continued for an additional 24 h in the presence of the antibiotic. At the end of the incubation the bones were analyzed for trichloroacetic acid-insoluble [^{14}C]proline and hydroxy[^{14}C]proline radioactivity as usual. As expected, the residual incorporation of the radioactive imino acids declines as actino-

mycin preincubation times lengthen (Fig. 2). The rate of decline of hydroxy[^{14}C]proline formation parallels that of [^{14}C]proline incorporation, and in samples preincubated for more than 12 h both processes were essentially abolished.

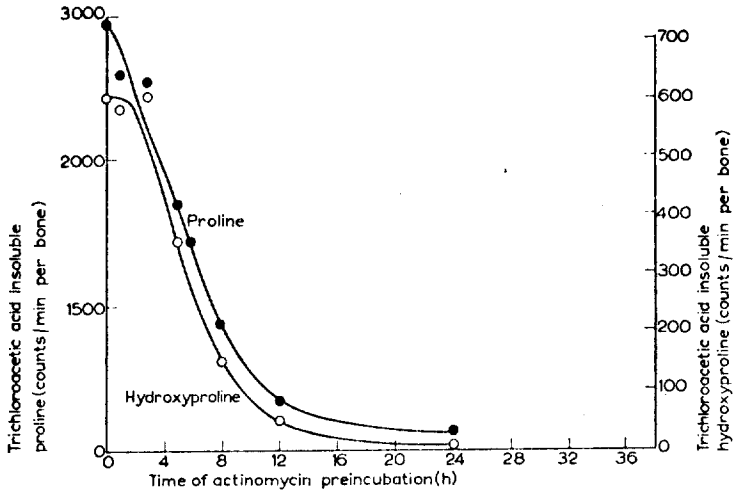

Fig. 2. Effect of actinomycin D preincubation on the synthesis of collagen and other proteins by chick tibias.

The results of these experiments indicate that collagen biosynthesis is no more sensitive to a general diminution of RNA production than is the synthesis of other proteins. Further, the results suggest that in chick tibia the messenger RNA for collagen is at least as long-lived as the mRNA for other proteins. Similar data has been obtained by BLOOM, GOLDBERG AND GREEN[4] for collagen and cell protein synthesis in an established mammalian cell line.

Ascorbic acid stimulation of hydroxyproline synthesis in the presence of actinomycin D

We have investigated the possibility that the depression of RNA synthesis observed in ascorbic acid depletion represents the failure of the cell to synthesize the specific messenger RNA for collagen. If this hypothesis were correct, new RNA synthesis would be required in order for ascorbic acid to enhance collagen synthesis in bones previously depleted of the vitamin. In order to test this hypothesis we have studied the effectiveness of ascorbic acid in stimulating collagen synthesis in bones treated with actinomycin D. In this experiment bones were incubated for 4 days, either with or without ascorbic acid. On the fourth day the bones were divided into three groups: control, depleted and recovery. All bones received [^{14}C]proline at this time, and half the bones in each of the three groups received 5 μg/ml of actinomycin D. (Table II shows the extent to which the antibiotic inhibits RNA synthesis in the tibia. In this experiment, 5 μg/ml of actinomycin D inhibited [^{14}C]uridine incorporation into trichloroacetic acid-insoluble material by 99 %.) After a further 16-h incubation all groups were analyzed for protein-bound [^{14}C]proline and hydroxy[^{14}C]-proline. The results are summarized in Table II. Actinomycin D inhibits the formation of hydroxy[^{14}C]proline in all three groups. In the recovery group, however, ascorbic

TABLE II

STIMULATION OF HYDROXYPROLINE FORMATION BY ASCORBIC ACID IN ACTINOMYCIN D-TREATED CHICK TIBIA

All bones were incubated for 6 days in TC medium 199. Uniformly labeled [^{14}C]proline (0.2 μC per ml medium), [2-^{14}C]uridine (0.2 μC per ml medium) and actinomycin D (5 μg per ml medium) were added on the fourth day. Control bones received 50 μg/ml ascorbic acid throughout the experiment. Depleted bones received no ascorbic acid during this time, and recovery bones received no ascorbic acid for the first 4 days, then 50 μg/ml ascorbate. Each value represents the average of duplicate analyses on groups of at least three bones.

Group	*Incubation conditions*		*Hydroxy-[^{14}C]proline (trichloroacetic acid-insoluble counts/min per bone)*	*[^{14}C]Uridine incorporation (trichloroacetic acid-insoluble counts/min per bone)*
	Isotope	*Actinomycin*		
Control	[^{14}C]Proline	0	1355	—
Control		+	588	—
Depleted		0	224	—
Depleted		+	60	—
Recovery		0	1160	—
Recovery		+	460	—
Control	[^{14}C]Uridine	0	—	12 700
Control		+	—	123

acid stimulates the formation of hydroxy[^{14}C]proline in spite of the presence of actinomycin D. The amount of hydroxy[^{14}C]proline produced by actinomycin D-treated recovery bones is equal, expressed as per cent of the value in bones receiving no actinomycin, to the amount produced in control bones treated with the antibiotic. Compared with actinomycin D-treated depleted bones, the treated recovery bones show greater than a 7-fold stimulation of hydroxy[^{14}C]proline formation. The results of this experiment indicate that normal amounts of the mRNA for collagen are present in depleted bones after 4 days in culture. Ascorbic acid depletion apparently does not specifically depress collagen mRNA formation in this tissue.

Inhibition of protein synthesis and hydroxyproline formation by puromycin

Several workers[5-8] have suggested that prolyl sRNA is hydroxylated in the cell to yield hydroxyprolyl sRNA, and that the latter compound is an intermediate in collagen biosynthesis. If hydroxyprolyl sRNA is indeed the major product of the proline hydroxylation reaction, hydroxyproline formation should occur independently of protein synthesis. In order to investigate this possibility, hydroxyproline formation was measured in bones treated with the antibiotic puromycin. Puromycin has been shown to inhibit protein synthesis in a number of systems by attaching to the ends of growing peptide chains on the mRNA template, preventing further growth of the chains. In these experiments normal bones were incubated for 2 days with [^{14}C]proline and various concentrations of puromycin. After incubation the bones were analyzed for trichloroacetic acid-insoluble [^{14}C]proline and hydroxy[^{14}C]proline radioactivity.

The characteristics of puromycin inhibition of [^{14}C]proline incorporation and hydroxy[^{14}C]proline formation in chick tibias are shown in Fig. 3. Puromycin, unlike actinomycin, inhibits these processes completely at the high concentrations of antibiotic. Further, puromycin appears to inhibit hydroxy[^{14}C]proline formation to a

greater extent than it does [^{14}C]proline incorporation. This differential inhibition is apparent at every concentration of puromycin studied. We attempted to determine if the differences observed are the result of an actual decrease in the amount of hydroxy[^{14}C]proline synthesized by the bones by studying the distribution of hydroxyproline in various fractions obtained from normal and puromycin-treated

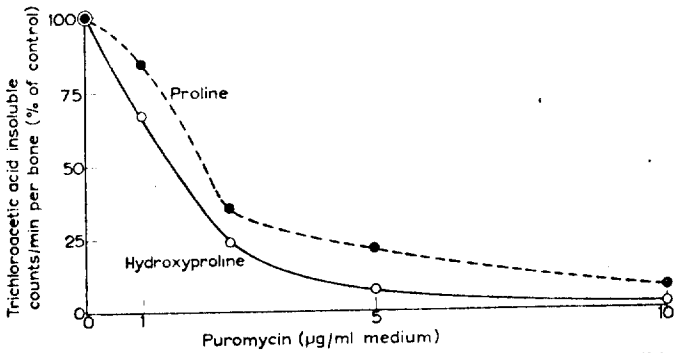

Fig. 3. Inhibition of collagen and other protein synthesis in chick tibias by puromycin.

bones. Tibias were incubated for 2 days with [^{14}C]proline; half of the bones also received 5 µg/ml of puromycin in their incubation medium. After incubation the bones were homogenized in distilled water and the insoluble fractions collected by centrifugation. The supernatants were further fractionated by the addition of a 40% trichloroacetic acid solution to a final concentration of 10%. The trichloroacetic acid-insoluble residue was collected by centrifugation. The two insoluble residues and the trichloroacetic acid-soluble fraction were analyzed for [^{14}C]proline and hydroxy-[^{14}C]proline radioactivity. These analyses (Table III) show that, in both normal and puromycin-treated bones, almost all the hydroxyproline is present in the two insoluble residues. There appears to be very little change in the hydroxyproline content of these fractions after puromycin treatment, although the amount of hydroxyproline in the trichloroacetic acid-soluble fraction of treated bones is decreased. There is,

TABLE III

DISTRIBUTION OF HYDROXYPROLINE IN VARIOUS FRACTIONS OF NORMAL AND PUROMYCIN-TREATED CHICK TIBIAS

All values represent the average of duplicate analyses of groups of 10 bones.

Fraction	*Normal bones*			*Puromycin-treated bones*		
	[^{14}C]Proline (counts/min per bone)	*Hydroxy-[^{14}C]proline (counts/min per bone)*	*Hydroxy-proline (µmole per bone)*	*[^{14}C]Proline (counts/min per bone)*	*Hydroxy-[^{14}C]proline (counts/min per bone)*	*Hydr proli (µmol per b*
H_2O-insoluble	47 034	18 629	0.708	4310	426	0.635
Trichloroacetic acid-insoluble	12 222	5 640	0.098	1526	204	0.100
Trichloroacetic acid-soluble	11 808	508	0.015	566	62	0.002

however, a large decrease in the amount of both proline and hydroxyproline radioactivity in all three fractions of treated bones. Similar data has been obtained by JUVA AND PROCKOP[9]. It appears, therefore, that hydroxyproline formation in chick tibias does not continue when peptide bond formation is prevented.

Stimulation of hydroxyproline synthesis by ascorbic acid in puromycin-treated tibias

The previous studies with puromycin support the hypothesis that hydroxyproline formation takes place after the incorporation of proline into peptide linkage. An attempt to demonstrate the existence of such a proline–peptide precursor was made by labeling ascorbic acid-depleted tibias with [^{14}C]proline, followed by simultaneous addition of puromycin and ascorbic acid, in order to determine if ascorbic acid would stimulate hydroxy[^{14}C]proline synthesis in the absence of protein synthesis. Presumably, under these conditions, increased formation of hydroxy[^{14}C]-proline would be due to the hydroxylation of the hypothetical peptide precursor.

In these experiments tibias were incubated in roller tubes for approx. 4 days without ascorbic acid. At the end of this time the bones were transferred under sterile conditions to small erlenmeyer flasks. Each flask contained twenty tibias. The bones were then incubated for 6 h with [^{14}C]proline in a small amount of medium (0.25 ml per bone instead of 2 ml per bone in roller tubes). At the end of this time the medium

TABLE IV

STIMULATION OF PROLINE HYDROXYLATION BY ASCORBIC ACID IN THE ABSENCE OF PROTEIN SYNTHESIS

The bones in Groups 1–4 were grown for 4 days in culture without ascorbic acid. Groups of 20 bones were then placed in stoppered flasks with 5 ml of media containing 2.0 μC/ml L-[^{14}C]proline except in Expt. 3 in which 0.2 μC/ml [^{14}C]proline was added. After 6 h of incubation at 37°, the bones were rinsed twice with non-radioactive media and the incubation continued for 6 h with media containing the additions indicated. Bones in Groups 5 and 6 were grown for 4 days in culture with ascorbic acid and then placed in flasks in groups of 20 with 5 ml of media containing ascorbic acid. During the last 6 h of incubation the bones received media containing the additions indicated.

Group	*Additions*		[^{14}C]*Imino acid (counts/min per bone)*					
	0–6 h	*6–12 h*	*Expt. 1*		*Expt. 2*		*Expt. 3*	
			Proline	*Hydroxyproline*	*Proline*	*Hydroxyproline*	*Proline*	*Hydroxyproline*
1	[^{14}C]Proline	None	4012	370	3530	465	212	24
2	[^{14}C]Proline	Ascorbic acid 50 μg/ml	6631	551	7820	780	396	38
3	[^{14}C]Proline	Puromycin 40 μg/ml	3975	213	2180	112	218	13
4	[^{14}C]Proline	Ascorbic acid 50 μg/ml Puromycin 40 μg/ml	3412	298	2266	179	164	31
5	Ascorbic acid	Ascorbic acid 50 μg/ml [^{14}C]Proline	6977	1187	—	—	—	—
6	Ascorbic acid	Ascorbic acid 50 μg/ml Puromycin 40 μg/ml [^{14}C]Proline	102	9	—	—	—	—

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was removed, the bones rinsed twice with 5 ml of Medium 199 to remove excess radioactivity, and divided into the following groups for an additional 6 h of incubation. Group 1 (Table IV) was incubated in ascorbic acid-free medium with no further addition, Group 2 received 50 μg/ml ascorbic acid, Group 3 received 40 μg/ml puromycin, Group 4 received 50 μg/ml ascorbic acid plus 40 μg/ml puromycin. As in the first 6-h period, bones were incubated in 0.25 ml of Medium 199 per bone. Two additional groups (5 and 6) of bones received medium containing ascorbic acid throughout the entire time of culture. They were incubated with [^{14}C]proline only during the last 6 h, and one of the groups received puromycin in addition to the radioactive imino acid. After incubation all groups of bones were removed and analyzed for trichloroacetic acid-insoluble [^{14}C]proline and hydroxy[^{14}C]proline.

The results of three such experiments are shown in Table IV. The addition of ascorbic acid to bones preincubated with [^{14}C]proline stimulated the incorporation of [^{14}C]proline into protein as well as its conversion to peptide-bound hydroxy-[^{14}C]proline (Group 2 *vs.* Group 1). This result indicated that ascorbic acid was stimulating the utilization of residual free [^{14}C]proline. In the presence of puromycin, however, ascorbic acid increased hydroxyproline formation 40–100% (Group 4 *vs.* Group 3), but not the incorporation of [^{14}C]proline as such into protein. This result indicated that ascorbic acid was enhancing the conversion of peptide-bound proline to peptide-bound hydroxyproline in the absence of protein synthesis. LUKENS[10] has recently reported that hydroxyproline synthesis can occur in the absence of protein synthesis and previous studies by PETERKOFSKY AND UDENFRIEND[11] also indicate this.

DISCUSSION

An attempt was made to identify pathways essential for collagen biosynthesis by the use of metabolic inhibitors, since it was possible that a simulation of ascorbic acid depletion might be obtained by blocking an ascorbic acid-dependent step. In this regard several workers have described the existence of an ascorbic acid-dependent oxidation of DPNH in microsomes. Oxygen has been shown to be the terminal electron acceptor in this pathway. STAUDINGER *et al.*[12] have suggested that ascorbic acid may exert an effect on biological hydroxylation reactions by participating in the transfer of electrons to oxygen. In the process the "active" oxygen, responsible for the hydroxylation of such substrates as steroids, norepinephrine, and certain drugs is formed. If hydroxyproline is produced by this pathway, blocking the flow of electrons to oxygen could result in the cessation of proline hydroxylation.

Accordingly, tibias were incubated with several electron transport inhibitors (KCN, Amytal, oligomycin, antimycin A) and uncouplers of oxidative phosphorylation (2,4-dinitrophenol, dicoumarol) and the inhibition of protein synthesis and proline hydroxylation in these bones was measured. None of the compounds used in this study appeared to have a particularly selective effect on hydroxyproline formation. Moreover, the compound SKF-525A, which has been found to specifically inhibit TPNH-O_2-requiring hydroxylating systems in microsomes, appeared to exert no selective effect on proline hydroxylation in tibias. It seems, therefore, that the specific function of ascorbic acid in collagen biosynthesis does not reside in a participation of ascorbic acid in electron-transport processes. It is conceivable, however, that the effect of ascorbic acid depletion upon general protein synthesis and other

cellular processes could be ascribed in part to a defect in an ascorbic acid-linked electron-transport system.

Since a depression of DNA and RNA synthesis was one of the earliest changes noted in ascorbic acid-deficient bones, we investigated the possibility that ascorbic acid has its primary action in the maintenance of normal nucleic acid synthesis. In this regard, recent electron-micrographic studies by ROSS AND BENDITT[13] reveal that the number of ribosomal particles aggregated on the endoplasmic reticulum is decreased in scorbutic cells. This defect disappears very rapidly after administration of ascorbic acid. Since recent studies on protein biosynthesis indicate that ribosomes aggregate in the presence of mRNA to form the primary site of protein synthesis in the cell, these authors suggest the possibility that mRNA synthesis is impaired in scorbutic animals. Since collagen synthesis is more sensitive to a lack of ascorbic acid than is the synthesis of other proteins, we considered the possibility that the mRNA for collagen is more sensitive than others to a general impairment of RNA synthesis or that it is less stable than other mRNA species. A second possibility considered is that ascorbic acid is specifically required for the production of collagen mRNA. Studies with actinomycin D indicate that the synthesis of collagen and other proteins are inhibited equally at all concentrations of inhibitor. It therefore appears that collagen mRNA is not more sensitive than the mRNA of other proteins synthesized by the tibia. Furthermore, preincubation of tibias with a concentration of actinomycin D sufficient to inhibit all RNA synthesis results in the decline of proline incorporation and hydroxyproline synthesis at equal rates as the preincubation time lengthens. Thus, no difference was detected between the stability of collagen mRNA and the mRNA of other proteins. Lastly, conclusive evidence that ascorbic acid does not exert its effect on collagen synthesis by way of an effect on mRNA was provided by the finding that ascorbic acid is able to stimulate hydroxyproline formation in the absence of RNA synthesis. Thus, essentially normal amounts of collagen mRNA must be present in bones depleted of ascorbic acid for 4 days.

The experiments with actinomycin D indicate a close relationship between proline hydroxylation and protein synthesis. This correlation would not be expected if, for example, sRNA hydroxyproline were a major intermediate in collagen biosynthesis. Further investigations of this relationship were conducted using puromycin, which inhibits protein synthesis by preventing the transfer of amino acids from sRNA to the growing peptide chain. When the inhibition of protein synthesis and hydroxyproline formation by various concentrations of puromycin is measured, it is seen that hydroxyproline formation is inhibited to a greater extent than is protein synthesis. Such a result is difficult to explain if a hydroxyprolyl sRNA intermediate exists in collagen biosynthesis. Even if the steady-state concentration of hydroxyprolyl sRNA were quite low, and its formation could not continue in the absence of protein synthesis, the inhibition of hydroxyproline formation should not be greater than that of protein synthesis. That is, the inhibition of hydroxyproline formation by puromycin should be less than or equal to that of general protein synthesis.

A possible explanation for the results is that proline is hydroxylated after it is incorporated into peptide linkage. In this case the proline destined to become hydroxyproline would presumably be specified by its adjacent amino acids. Hydroxylation could occur on the mRNA template or at some separate site after completion of the peptide chain. If this hypothesis were true, it could explain the results

of the puromycin-inhibition experiments. At any given dose of puromycin some protein molecules are completed and released from the template, others are stopped after various amounts of growth. As the puromycin concentration increases, fewer and fewer completions occur and more and more incomplete chains will result. Assuming that either translocation of completed chains or completion of a specific amino acid sequence in a growing chain is required for hydroxylation, it can be seen that, as the puromycin concentration is increased, the fraction of peptide chains which are suitable substrates for hydroxylation becomes smaller and smaller.

Ascorbic acid appears to stimulate hydroxyproline formation in depleted bones prelabeled with [^{14}C]proline and then treated with puromycin to block further protein synthesis. In these experiments, ascorbic acid did not stimulate the incorporation of [^{14}C]proline into puromycin-treated bones, indicating that hydroxylation was taking place after peptide-bond formation.

Recent studies by PETERKOFSKY AND UDENFRIEND[14] and PROCKOP AND JUVE[15] on hydroxyproline formation in cell-free systems strongly support a pathway for the hydroxylation of proline in which the substrate for hydroxylation is peptide-bound proline. Such a pathway is schematically represented in Fig. 4. Our results support the conclusions of these workers and, further, suggest a role for ascorbic acid in collagen biosynthesis. PETERKOFSKY AND UDENFRIEND[14] have fractionated their hydroxylation complex into an enzyme fraction and two heat-stable fractions, one of which can be replaced by ascorbic acid, a folic acid derivative, and to a lesser extent by TPNH. In the tissue-culture system, folic acid is present in the medium, hence it would seem that folic acid itself cannot substitute for ascorbate in the hydroxylation reaction. It is, of course, conceivable that a folic acid derivative is required for hydroxylation and that the enzymic pathways for its production from folic acid are not present in the tibia.

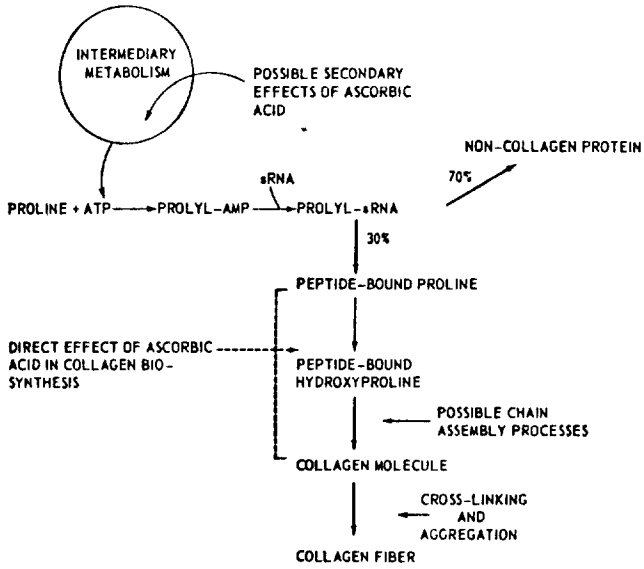

Fig. 4. Pathway of proline hydroxylation in collagen biosynthesis. Site of ascorbic acid participation.

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REFERENCES

1 J. J. JEFFREY AND G. R. MARTIN, *Biochim. Biophys. Acta*, 121 (1966) 269.
2 J. R. GILLETTE, *Progr. Drug Res.*, 6 (1963) 11.
3 G. ASBOE-HANSEN, in L. C. MILLS AND J. H. MOYER, *Inflammation and Disease of Connective Tissue*, Saunders, Philadelphia, 1961, p. 460.
4 S. BLOOM, B. GOLDBERG AND H. GREEN, *Biochem. Biophys. Res. Commun.*, 19 (1965) 317.
5 G. MANNER AND B. S. GOULD, *Biochim. Biophys. Acta*, 72 (1963) 243.
6 A. CORONADO, E. MARDONES AND J. E. ALLENDE, *Biochem. Biophys. Res. Commun.*, 12 (1963) 257.
7 A. CORONADO, E. MARDONES, J. CELIS AND J. E. ALLENDE, *Abstr. 6th Intern. Congr. Biochem.*, New York, 1964, Part 1, p. 35.
8 D. S. JACKSON, D. WATKINS AND A. WINKLER, *Biochim. Biophys. Acta*, 87 (1964) 152.
9 K. JUVA AND D. J. PROCKOP, *Biochim. Biophys. Acta*, 91 (1964) 174.
10 L. N. LUKENS, *J. Biol. Chem.*, 240 (1965) 1661.
11 B. PETERKOFSKY AND S. UDENFRIEND, *J. Biol. Chem.*, 238 (1963) 3966.
12 H. J. STAUDINGER, K. KRISCH AND S. LEONHAUSSEN, *Ann. N.Y. Acad. Sci.*, 92 (1961) 195.
13 R. ROSS AND E. P. BENDITT, *J. Cell Biol.*, 22 (1964) 365.
14 B. PETERKOFSKY AND S. UDENFRIEND, *Proc. Natl. Acad. Sci. U.S.*, 53 (1965) 335.
15 D. J. PROCKOP AND K. JUVA, *Proc. Natl. Acad. Sci. U.S.*, 53 (1965) 661.

Toxicol Appl Pharmacol. 18 (1): 53-59 1971 3970

TOXICOLOGY AND APPLIED PHARMACOLOGY 18, 53-59 (1971)

Effect on Experimental Animals of Long-Term Inhalation Exposure to Mineral Spirits

II. Dietary, Sex and Strain Influences in Guinea Pigs[1]

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Received January 12, 1970

Effect on Experimental Animals of Long-Term Inhalation Exposure to Mineral Spirits. II. Dietary, Sex, and Strain Influences in Guinea Pigs. JENKINS, L. J., JR., COON, R. A., LYON, J. P., and SIEGEL, J. (1971). *Toxicol. Appl. Pharmacol.* 18, 53-59. Male and female NMRI:(ASH) and FTD: Hartley guinea pigs were exposed to mineral spirits at a concentration of approximately 900 mg/m^3 continuously for 90 days, during which time the animals were fed diets fortified with ascorbic acid in the range from 0.00015 to 2.00%. It was found that the animals on the higher ascorbic acid diets were less susceptible to the toxicity of mineral spirits. Males were shown to be more susceptible than females and the NMRI:(ASH) strain was more susceptible than the FTD:Hartley strain.

In reporting their studies on the inhalation toxicity of mineral spirits in rats, guinea pigs, rabbits, dogs, and monkeys, Rector *et al.* (1966) noted significant mortality in guinea pigs exposed to concentrations of mineral spirits that were relatively noninjurious to the other species studied. They were not, however, able to explain this apparent increased susceptibility. An examination of the diets used by Rector and his co-workers indicated that the guinea pigs were receiving an apparently adequate diet and although the dry chow they used was somewhat low in ascorbic acid content, the daily supplementation with 1/4 head of lettuce provided each animal with enough vitamin C to meet the requirements cited by Reid (1962). Studies were undertaken to assess the effects of diets containing large excesses of vitamin C on the toxicity of mineral spirits in 2 strains of guinea pigs.

METHODS

NMRI:(ASH) Princeton-derived guinea pigs, as used by Rector *et al.* and FTD: Hartley-derived guinea pigs of both sexes were used as test animals. They were housed in individually compartmented cages within the chamber and observed throughout the course of exposure for visible manifestations of toxicity such as alterations in the behavior, locomotor activity, and general physical condition. Blood specimens were

[1] The opinions expressed herein are those of the authors and do not necessarily reflect the view of the Navy Department or the naval service at large. The experiments reported herein were conducted according to the principles enunciated in "Guide for Laboratory Animal Facilities and Care" prepared by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences—National Research Council, Washington, D. C.

TABLE 1

EXPERIMENTAL PARAMETERS EMPLOYED IN EXPERIMENTS I AND II

Concentration of mineral spirits: Group	Experiment I 892 ± 64 mg/m³			Experiment II 892 ± 56 mg/m³		Experiment II Control	
	A	B	C	A	B	C	D
NMRI:(AHS)							
Female	5[a]	5	5				
Male	5	5	5				
FTD:Hartley							
Female	5	5	5				
Male	5	5	5	15[a]	15	15	15
D & G	×[b]						
Reid & Briggs Chow		×					
Reid & Briggs-Fortified			×		×		×
Rockland Chow				×		×	
Lettuce	Daily	1×/wk	1×/wk	3×/wk	3×/wk	3×/wk	3×/wk

[a] Number of animals per group.
[b] × indicates the diet provided.

TABLE 2

PARTIAL COMPOSITION OF BASIC GUINEA PIG DIETS USED[a]

Component	D & G	Rockland	R & B	Fortified R & B
Ascorbic acid	0.15[a]	60	200	2,000
Protein	19,000	19,000	30,000	30,000
Fat	3,200	3,300	7,300	7,300
Fiber	13,000	14,000	15,000	15,000
Thiamine	0.44	0.47	1.8	1.8
Riboflavin	0.7	0.6	1.6	1.6
Niacin	3.5	2.5	20	20
Vitamin A	1,112 IU	6,077 IU	—	—
Vitamin A acetate	—	—	0.6	0.6
Vitamin D	661 IU	446 IU	—	—
Calciferol	—	—	0.004	0.004
Choline	120	74	170	170
Sodium chloride	800	1,400	400	400
Inositol	—	34	200	200
Pyridoxine	—	0.14	13	13
Vitamin E	—	0.4 IU	—	—
α-Tocopheryl acetate	—	—	2	2
Calcium	1,500	800	1,100	1,100
Phosphorus	400	500	700	700
Pantothenic acid	—	0.2	3.6	3.6

[a] Values are based on data provided by manufacturer; analyses are given in mg/100 g of diet unless specified otherwise. R & B Reid and Briggs.

obtained from an ear vein for pre- and postexposure leukocyte counts, hemoglobin determinations, and packed erythrocyte volume as expressed by the microhematocrit. All animals were weighed prior to the first, and subsequent to the last, day of exposure.

The modified Rochester exposure chambers used in these studies, the methods for generating the vapors of mineral spirits, and the procedures for monitoring the concentrations have been described by Rector *et al.* Air, conditioned to maintain the chambers at 77 ± 2 F and approximately 50% relative humidity, was delivered to the chambers at a rate of 1 m^3 per minute. The contaminant was turned off daily for approximately 2% of the time to feed the animals, change cages, and clean the chamber.

The mineral spirits used in these studies were analyzed by ultraviolet and infrared spectroscopy and were found to be qualitatively identical to the materials used by Rector *et al.*; the aromatic fraction as xylol was 18–20%, while Rector's range was 13–19%. The guinea pigs were exposed continuously for 90 days to the vapors of these mineral spirits at concentrations of approximately 900 mg/m^3. Based on previous work in this laboratory, this concentration would be expected to produce significant toxicity without decimating the animals.

Two experiments were conducted; the experimental parameters of each is shown in Table 1, and the major components of the diets are given in Table 2. In all cases, fresh diets were procured and bulk stores were refrigerated. Under the conditions of the exposure, where it was desired to keep "down-time" to a minimum, it was not considered practicable to determine the quantity of chow consumed by each animal.

Upon completion of the exposure the surviving animals were sacrificed with overdoses of sodium pentobarbital. Necropsies were performed and sections of heart, lung, spleen, and kidney were retained for histologic examination. Before sacrificing the guinea pigs in Experiment II, the animals were housed in individual metabolism cages in order to collect 24-hr urine specimens. These were analyzed for their ascorbic acid content by the method of Vann (1965). Blood specimens were obtained from the animals surviving both experiments and serum urea nitrogen concentration was determined by the automated method described by Marsh *et al.* (1957).

RESULTS

Experiment I

In Experiment I, 5 males and 5 females of each strain were provided with one of 3 diets. One group was placed on the diet used by Rector *et al.*; this consisted of D & G[2] dry chow with 1/4 head of fresh lettuce daily. A second similar group of animals was provided with the guinea pig test diet described by Reid and Briggs (1953), while a third group was given the same diet fortified with a 10-fold excess of ascorbic acid. Both of the groups on the Reid-Briggs[3] diets received 1/4 head of lettuce once a week and all were provided with water ad libitum. The animals were exposed to mineral spirits at a concentration of 892 ± 64 mg/m^3.

Mortality data are shown in Table 3. These results did not indicate any clear-cut evidence of dietary influences on the toxicity of mineral spirits. They did show, however, the influences of sex and strain. When all diet groups were combined, approximately

[2] D & G Guinea Pig Diet, Price-Whithoite Company, Frederick, Maryland.
[3] Reid-Briggs Guinea Pig Test Diet, General Biochemicals, Chagrin Falls, Ohio.

twice as many males as females, from either strain, died as a result of the exposure. Similarly, the NMRI:(ASH) strain of guinea pigs were more susceptive to mineral spirits than the FTD:Hartley strain.

TABLE 3

THE EFFECT OF VITAMIN C ON THE MORTALITY IN GUINEA PIGS EXPOSED FOR 90 DAYS TO MINERAL SPIRITS

	FTD:Hartley strain		NMRI:(ASH) strain		
Diet	Male	Female	Male	Female	Totals
D & G	3/5[a]	2/5	5/5	3/5	13/20
Reid-Briggs	4/5	1/5	4/5	2/5	11/20
Fortified Reid-Briggs	2/5	1/5	4/5	2/5	9/20
	9/15	4/15	13/15	7/15	
	13/30		20/30		

[a] Number of animals that died over the number exposed.

The concentration of serum urea nitrogen was somewhat elevated in all three dietary groups. The mean combined values, plus or minus 1 standard deviation were 33 ± 3, 38 ± 5, and 37 ± 4 mg/100 ml for the animals maintained on the low, medium, and high ascorbic acid diets, respectively; the control value was 27 ± 3 mg/100 ml. The hematologic parameters for all animals were within control limits.

Examination of the organs at necropsy revealed areas of necrosis in several livers and areas of consolidation in several lungs. On histopathologic examination, fatty changes were seen in the livers from approximately half of the animals. These were about evenly divided by sex, but appeared to be slightly more frequent in the animals on the medium and high ascorbic acid diets. Other changes, such as nonspecific inflammation of the lungs and focal areas of tubular calcification of the kidneys, were also noted, but appeared to be unrelated to the experiment.

Experiment II

Since Experiment I showed the influence of sex and strain but did not show any clear-cut evidence of dietary influence, Experiment II was designed to ascertain the influence of ascorbic acid in the diet on the toxicity of mineral spirits given by the respiratory route. In Experiment II, only males of the FTD:Hartley strain were used; 15 were provided with a diet adequate in vitamin C (Rockland[4]) and 15 with a highly fortified diet (Reid-Briggs). These animals were exposed to mineral spirits at a level of 892 ± 56 mg/m^3. Controls of the same sex and strain, maintained on the same diets, were placed in a separate chamber and subjected to the same regimen except that no mineral spirits were introduced into the chamber.

In the animals exposed to the vapors of mineral spirits, the mortality ratio (Table 4) for those on the extremely high ascorbic acid diet (fortified Reid-Briggs) was 2/15; this compared with 10/15 for those on the lower dietary level of ascorbic acid (Rockland). Some diarrhea was noted early in the exposure of the low ascorbic acid animals,

[4] Rockland Guinea Pig Diet, Teklad, Incorporated, Monmouth, Illinois.

but it subsided after several weeks. The surviving animals in both exposed groups gained substantially less body weight than those in the unexposed group; this effect was more pronounced in the animals on the low level of dietary ascorbic acid. The dietary level of ascorbic acid did not affect the body weight gain of the unexposed animals.

TABLE 4

MORTALITY, BODY WEIGHT, AND URINARY ASCORBIC ACID DATA FROM MALE FTD:HARTLEY GUINEA PIGS EXPOSED IN EXPERIMENT II

Statistic	Group	Rockland diet	Fortified Reid-Briggs diet
Mortality (dead/exposed)	Exposed	10/15	2/15
	Unexposed	0/15	0/15
Initial weight (g)	Exposed	363 ± 7[a]	340 ± 30
	Unexposed	362 ± 33	341 ± 23
Terminal weight (g)	Exposed	527 ± 30	591 ± 34
	Unexposed	658 ± 80	648 ± 45
Urinary ascorbic acid (mg/kg/24 hr)	Exposed	16.7 ± 4.9	25.9 ± 8.8
	Unexposed	19.4 ± 9.5	19.2 ± 12.0

[a] Body weight and ascorbic acid data are shown as the mean ± 1 standard deviation.

Urinary ascorbic acid data, obtained from surviving animals, are presented in Table 4. Although there was a wide range within each of the groups, the exposed animals on the lower ascorbic acid diet excreted significantly less ($p < 0.025$) than those on the higher ascorbic acid diet. The unexposed animals did not reveal any such differences. Serum urea nitrogen concentration was again somewhat elevated for the exposed guinea pigs (32.6 ± 8.2 mg/100 ml) while the unexposed animals were within our normal range (27.1 ± 3.6).

Gross examination of the animals at necropsy did not show any marked alterations. On histopathologic examination, interstitial pneumonitis was seen in some of the lungs, focal necrosis in some of the livers, and lymphocytic infiltrates in a few of the kidneys; none of these changes were attributed to the exposure. The fatty changes in the liver, which were noted in Experiment I, were minimal in the animals from Experiment II.

DISCUSSION

The general area of nutrition and diet as they relate to environmental exposure to toxic materials has been reviewed by Friedman (1966) and Shils (1968). Ascorbic acid has been reported as being protective against some toxic materials. Soliman *et al.* (1965) reported a prophylactic effect in rats when the vitamin was injected iv prior to injection of carbon tetrachloride. Ascorbic acid has also been reported to prevent CCl_4-induced testicular inhibition (Chatterjee, 1967) and adrenal hypertrophy (Chatterjee and Bardhan, 1967) in rats. The literature on the protective aspects of ascorbic acid as related to benzene toxicity has been reviewed by Browning (1965). However, reports involving animals in which exogenous ascorbic acid is not required and in which the ascorbic acid was injected in rather large quantities raise the question as to whether the benefits were nutritional or pharmacologic.

Although the results from Experiment I showed a strain and sex difference, they did not reveal a clear-cut influence of diet. In Experiment II a greater number of animals were exposed, and controls with closely matched starting weights were maintained in an identical chamber environment, except for the mineral spirits in the atmosphere. The results of the second experiment did show the influence of diet; mortality was much lower in the animals on the diet with the higher ascorbic acid content. This was corroborated by the body weight data which indicated that the rate of gain in surviving animals on the fortified Reid-Briggs ration more closely paralleled their controls than did the exposed animals on the diet with the lower ascorbic acid content. The urinary ascorbic acid data may also indicate that there was an increased requirement for the vitamin while the animals were being exposed to mineral spirits, since those on the lower diet excreted less than their counterparts on the higher diet; this difference was not seen in controls. These findings emphasize the importance of dietary ascorbic acid for species of animals which are dependent upon exogenous sources of this vitamin.

In interpreting the effect of vitamin C on toxicity several additional factors must be taken into consideration. Terroine (1960) pointed out the interrelationships between vitamin C and other vitamins in the diet. Ershoff (1963) demonstrated toxic effects in rats fed a highly purified diet and challenged with 2,5-di-*tert*-butylhydroquinone; the same effects were not seen in rats maintained on a normal stock ration of the same nutritional adequacy. In our studies it would be difficult to evaluate the influences from other dietary components. Experiment II seemed to indicate that the high ascorbic acid content of the fortified Reid-Briggs test diet overshadowed the disadvantages which might have been present in this semisynthetic diet.

In a previous report from these laboratories Rector *et al.* reported that serum urea nitrogen concentrations in guinea pigs was not affected by exposure to low levels of mineral spirits (114 mg/m^3) but was slightly elevated at intermediate concentrations (514 mg/m^3). The present studies at high levels (892 mg/m^3) confirm and extend these observations.

In summary, the present experiments support and extend the work of Conney and Burns (1962) in which they reported that the metabolism of drugs was significantly influenced by the diet, strain, and sex of the experimental animals; in Experiments I and II chemicals other than drugs were used, and the material was administered by the respiratory route.

ACKNOWLEDGMENTS

The authors are indebted to D. R. Sanders, D. L. Minor, E. M. Swab, R. A. Jones, and R. J. Kane for their assistance in various phases of this study. The constructive review and suggestions of R. C. Wands, Advisory Center on Toxicology, National Academy of Sciences, National Research Council, is gratefully acknowledged.

Dr. J. H. Leathem of Rutgers University, New Brunswick, New Jersey and Dr. M. A. Jackson of Howard University, Washington, D. C. served as consultants in pathology.

REFERENCES

BROWNING, E. (1965). Vitamins in the treatment of chronic benzene poisoning. *J. Occup. Med.* **7**, 554–559.

CHATTERJEE, A. (1967). Role of ascorbic acid in the prevention of gonadal inhibition by carbon tetrachloride, *Endokrinologie* **51**, 319–322.

CHATTERJEE, A., and BARDHAN, N. R. (1967). Prevention of carbon tetrachloride induced renal hypertrophy by the use of ascorbic acid in rats. *Endokrinologie* **51**, 306–307.

CONNEY, A. H., and BURNS, J. J. (1962). Factors influencing drug metabolism. *Advan. Pharmacol.* **1**, 31–58.

ERSHOFF, B. H. (1963). Comparative effects of a purified and stock diet on DBH (2,5-di-*tert*-butylhydroquinone) toxicity in the rat. *Proc. Soc. Exp. Biol. Med.* **112**, 362–365.

FRIEDMAN, L. (1966). Nutritional status and biologic response. *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **25**, 137–144.

MARSH, W. H., FINGERHUT, B., and KIRSH, E. A. (1957). Determination of urea nitrogen with the diacetyl method and an automatic dialyzing apparatus. *Amer. J. Clin. Pathol.* **28**, 681–688.

RECTOR, D. E., STEADMAN, B. L., JONES, R. A., and SIEGEL, J. (1966). Effect on experimental animals of long-term inhalation exposure to mineral spirits. *Toxicol. Appl. Pharmacol.* **9**, 257–268.

REID, M. E. (1962). *Nutrient Requirements of Laboratory Animals*. pp. 11–23. National Academy of Sciences—National Research Council. Publication 990, Washington, D. C.

REID, M. E., and BRIGGS, G. M. (1953). Development of a semi-synthetic diet for young guinea pigs. *J. Nutr.* **51**, 341–354.

SHILS, M. E. (1968). *Modern Nutrition in Health and Diseases* (M. G. Wohl and R. S. Goodhart, eds.), 4th ed., pp. 1159–1182. Lea & Febiger, Philadelphia, Pennsylvania.

SOLIMAN, M. A., ELWI, A. M., EL-KATEB, H., and KAMEL, S. H. (1965). Vitamin C as prophylactic drug against experimental hepatotoxicity. *J. Egypt. Med. Asso.* **48**, 806–812.

TERROINE, T. (1960). *World. Rev. Nutr. Diet.* 102–130.

VANN, L. S. (1965). Rapid micro method for determination of ascorbic acid in urine by ferric reduction. *Clin. Chem.* **11**, 979–985.

Joint FAO/WHO Expert Committee on Food Additives.
1962
Evaluation of the toxicity of a number of antimicrobials and antioxidants
Sixth Report of the Joint FAO/WHO Expert Committee on Food Additives, FAO, WHO, United Nations, pp. 19-22

Joint FAO/WHO Expert Committee on Food Additives. 1966
Specifications for the identity and purity of food additives and their toxicological evaluation: some emulsifiers, stabilizers and certain other substances
Tenth Report of the Joint FAO/WHO Expert Comm. on Food Additives, WHO Tech. Rep. Ser. No. 373, FAO Nutr. Meeting Rep. Ser. No. 43, FAO, WHO, United Nations

Joint FAO/WHO Expert Group. 1970
Requirements of ascorbic acid, vitamin D, vitamin B_{12}, folate and iron
WHO Tech. Rep. Ser. No. 452, FAO Nutr. Meetings Rep. Ser. No. 47, World Health Organization, Food and Agriculture Organization of the United Nations, Geneva

J. Lab. Clin. Med. 25:263-269 1939

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ULTRAVIOLET IRRADIATION AND VITAMIN C METABOLISM*

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WITH the advance of knowledge, interest in vitamin C has shifted from consideration of its importance as an antiscorbutic agent to a fuller appreciation of its function in the physiologic defense mechanism against various infections and intoxications. It is now common experience that most infectious diseases are accompanied by a loss of the vitamin from the body; moreover, it is often possible, experimentally and clinically, to enhance resistance or to hasten recovery by raising the vitamin levels through the administration of ascorbic acid.[1]

The significance of low-grade C deficiencies, or hypovitaminoses, in the epidemiology of infectious diseases is far from being understood; indeed, the very complexity of the problem presents great difficulties for systematic exploration. However, it remains an inviting task to attempt to correlate seasonal variations in susceptibility, as indicated by the periodic oscillations of mortality and morbidity, with cyclic changes of vitamin C metabolism. Unlike the mode of utilization of other vitamins that are stored in considerable reserves in the body, the vitamin C intake and outflow represents a delicately attuned system which undergoes continuous alteration and adjustment. The changes in this system may be measured directly by the degree of C saturation of blood and tissues, on the one hand, and by the rate of C elimination through peripheral secretions and excretions (milk, sweat, tears, saliva, and urine) on the other. That the maintenance of this balance is subject to seasonal influence is suggested by numerous observations which need hardly be related here in detail.[2] It is as yet uncertain to what extent these phenomena are brought about by exogenous or by endogenous factors, individually or collectively; but there are good reasons for assuming that, apart from differences in dietary intake, environmental conditions connected with the operation of climatic constants must play an important part.

Exposure of guinea pigs to high temperatures, for instance, is followed by a diminution of vitamin C in the liver, brain, and adrenals, and the ad-

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ministration of ascorbic acid is said to protect the animals against the lethal effect of heat damage.[3] Similar were the findings of Zook and Sharpless,[4] who not only observed a rapid depletion of C stores in animals with elevated temperatures but also showed that artificial fever reduces the urinary excretion of the vitamin in man. Exposure of guinea pigs to ultraviolet light caused either an increase or a decrease of the ascorbic acid content of the skin, depending upon whether irradiation was brief or prolonged (Castellini[5]); similarly, Wels's[6] experiments demonstrated an actual increase of the sulfhydryl groups in swine skin following ultraviolet raying. These results are in good agreement with the observation of Holtz and Woellpert[7] that the clinical symptoms of scurvy were milder in C deficient guinea pigs, which had received small doses of ultraviolet light, than in nonirradiated controls, even though both sets of animals presented the fully developed picture of scorbutic lesions. The effect of ultraviolet light on vitamin C metabolism in man has been investigated by several authors, but the results are not entirely in accord. While Schade[8] reported that vitamin C administration inhibited both the erythema and pigmentation resulting from ultraviolet irradiation, Urbach and Kral[9] obtained a definite light-protective effect only by combining vitamin C doses with local application of bergamot oil. Unpublished data by Dr. Irving S. Wright,[10] to which we may refer by permission of the author, indicate that the production of an extensive ultraviolet light erythema was followed by an abrupt retention of urinary vitamin C in one out of three subjects; this individual was red-haired, fair-skinned, and could tolerate much less ultraviolet light than the other two subjects. Drigalski,[11] on the other hand, reported no change in the rate of urinary excretion of vitamin C as the result of ultraviolet irradiation. Since ultraviolet light in excessive doses provokes a severe tissue burn, it is not surprising to note that both Stolfi[12] and Einhauser[13] observed a precipitous drop in the vitamin C levels of various organs—especially marked in the adrenal—in rabbits or in guinea pigs whose extremities had been scalded by immersion in hot water. Einhauser also described the lifesaving effect of cortin and vitamin C, either together or alone, when given to animals suffering from experimental burns. The above quotations suffice to delineate the scope of the work that has been done in this field and, at the same time, serve to emphasize the limitations of our present knowledge.

We have been particularly interested in the relationship that seems to exist between vitamin C metabolism and the effect of ultraviolet light in the production of disease, when irradiation transcends the threshold of physiologic stimulation. Clinical experience has amply demonstrated the potential danger of excessive doses of ultraviolet light in the production of critical changes in capillary fragility which may be instrumental in causing hemorrhage in vitamin C deficient patients suffering from pulmonary tuberculosis or gastric ulcers. But apart from such accidents, light damage may possibly play a more fundamental part in the pathogenesis of certain infectious diseases that reach an unexplained seasonal peak of epidemicity during

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the summer months. Thus, the coincidence of sunburn following unrestrained exposure at beaches and swimming pools and the occurrence of cases of poliomyelitis are so frequent and almost pathognomonic that some epidemiologists have seized upon this sequence as indicating the water-borne nature of poliomyelitic infection; in doing so, the obvious fact was overlooked that the individuals bathed not only in the water but also in the sun. The suspected correlation becomes even more compelling when one contrasts the rarity of the disease in southern climes, where the even presence of radiation throughout the year provides for a protective tanning, with its massed incidence in northern latitudes in which the full force of summer sunlight must be borne by an unprepared skin. Conceivably, the apparent racial difference in natural resistance to poliomyelitis that separates the highly susceptible white races from the relatively less susceptible colored races (black and yellow), may in some measure be related to the abundance of pigment in the skin of the latter, acting as a protection against the hypothetical photodynamic injury.

EXPERIMENTAL

It was the object of this study to determine whether or not exposure of laboratory animals to ultraviolet irradiation was followed by a significant change in the vitamin C content of representative tissues. The animals employed in this work were albino guinea pigs, rabbits, and rhesus monkeys; the tissues examined were brain, liver, spleen, and adrenal gland. As the source of ultraviolet light we used a mercury vapor lamp, energized by high frequency currents (Lepel) and emitting a spectrum in which the longer ultraviolet waves, in the range from 2,800 to 3,100 angstrom units, predominate. The same lamp had been used in previous studies on the inactivation of poliomyelitis virus by ultraviolet light.[14] Animals, whose backs had been cleanly shaven, were placed under the lamp at a distance of about 30 cm. from the burner and exposed to the light for from thirty to sixty minutes. After intervals of one and eighteen hours, respectively, the animals were killed, and their organs examined for vitamin C content. For purpose of control, unrayed animals, which had previously been kept on the same dietary regime, were sacrificed simultaneously, and vitamin C titrations of their organs were carried out, side by side, with those of the organs of rayed animals. Preliminary diets were planned to cover the approximate range from inadequacy to adequacy. Duplicate animals were used in each test, pooling the organs of pairs of guinea pigs for vitamin C analysis. The amounts of reduced vitamin C in the tissues were determined by titration with indophenol (sodium 2, 6-dichloro-benzenoneindophenol, Eastman Kodak Co.). Because of its simplicity and accuracy we adopted the modified method of Bessey and King,[15] the experimental error of which is estimated between 5 and 10 per cent; technical details may be found in an earlier paper in which the same method was employed satisfactorily for the determination of vitamin C levels of monkey tissues.[16] The results of the various titrations are brought together in Table I which also gives additional data relating to the experimental setup.

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TABLE I

EFFECT OF ULTRAVIOLET IRRADIATION ON VITAMIN C LEVELS OF TISSUES

EXPERIMENT*	ANIMAL	DIET	DURATION OF IRRADIATION	INTERVAL AFTER RAYING	VITAMIN C CONTENT OF ORGANS (MILLIGRAM PER GRAM)			
					BRAIN	LIVER	SPLEEN	ADRENAL GLAND
1	Guinea pigs	Inadequate	Control	--	0.18	0.07	0.25	0.62
	Guinea pigs	Inadequate	Control	--	0.16	0.08	0.16	0.39
	Guinea pigs	Inadequate	Control	--	0.13	0.08	0.16	0.36
	Guinea pigs	Inadequate	Control	--	0.14	0.08	0.23	0.57
	Guinea pigs	Inadequate	30 min.	1 hour	0.16	0.09	0.22	0.92
	Guinea pigs	Inadequate	30 min.	18 hours	0.15	0.07	0.15	0.36
	Guinea pigs	Inadequate	60 min.	1 hour	0.13	0.09	0.21	0.46
	Guinea pigs	Inadequate	60 min.	18 hours	0.14	0.09	0.24	0.40
2	Guinea pigs	Adequate	Control	--	0.20	0.25	0.54	1.44
	Guinea pigs	Adequate	Control	--	0.17	0.19	0.34	1.02
	Guinea pigs	Adequate	Control	--	0.19	0.26	0.50	1.47
	Guinea pigs	Adequate	Control	--	0.17	0.16	0.41	0.96
	Guinea pigs	Adequate	30 min.	1 hour	0.20	0.25	0.50	1.35
	Guinea pigs	Adequate	30 min.	18 hours	0.16	0.13	0.32	1.09
	Guinea pigs	Adequate	60 min.	1 hour	0.19	0.28	0.53	1.42
	Guinea pigs	Adequate	60 min.	18 hours	0.17	0.16	0.37	0.98
3	Rabbits	Adequate	Control	--	0.19	0.12	0.52	1.76
	Rabbits	Adequate	Control	--	0.20	0.16	0.41	2.29
	Rabbits	Adequate	30 min.	1 hour	0.19	0.13	0.49	1.92
	Rabbits	Adequate	30 min.	18 hours	0.21	0.18	0.53	1.93
	Rabbits	Adequate	60 min.	1 hour	0.19	0.16	0.52	1.77
	Rabbits	Adequate	60 min.	18 hours	0.21	0.18	0.49	2.07
4	Monkeys	C-stored	Control	--	0.16	Not done	0.27	0.51
	Monkeys	C-stored	30 min.	1 hour	0.15	Not done	0.36	0.64

*All experiments were done in June, 1938.

It will be seen from Table I that exposure of guinea pigs, rabbits, and monkeys to ultraviolet light for from thirty to sixty minutes caused no change whatsoever in the vitamin C content of the brain, liver, spleen, and adrenal gland. The average figures for the rayed and unrayed groups, respectively, were almost exactly identical, the range relatively small. This was true for guinea pigs which entered the experiment with partially depleted vitamin C concentrations as well as for guinea pigs which had been permitted to accumulate reserves by previous high level feeding.

The outcome of the above experiments was so clearly negative, irrespective of whether one was dealing with animals capable or incapable of synthesizing vitamin C, that there could be no doubt about the inefficacy of our method of irradiation in disturbing vitamin C metabolism. It was quite evident, however, that our rayed animals at no time exhibited any erythema on the skin despite the fact that the same lamp, even when operated at a greater distance, induced a very intense erythema on human skin after only three minutes' exposure. Marked irregularities in attempts to produce an erythema on the skin of guinea pigs by powerful ultraviolet irradiation have previously been reported by Ellinger.[17] The reason for this discrepancy between the reactivity of human and animal skin is not clear, and we have no explanation to offer for it. However, it occurred to us that the failure of the animals to develop a burn from the ultraviolet light might possibly account for the absence of vitamin C changes; conversely, if a burn were produced

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by another more drastic method, such changes might ensue. We have, therefore, taken another group of guinea pigs, kept one-half for controls, and cauterized the shaven skin of the other half by the brief application of a searing knife over an area of approximately 2 cm. by 2 cm. The lesion thus provoked was entirely superficial, and, while not leading to any severe tissue destruction, proved sufficiently intense to cause discolorization of the epidermis with very slight scab formation. This experiment was carried out three times, once in summer and again in the winter and spring, in each case using duplicate animals, all of which had been kept before on an adequate diet. The results of the three vitamin C assays appear in Table II.

TABLE II

EFFECT OF SKIN CAUTERIZATION ON VITAMIN C LEVELS OF TISSUES

ANIMAL	DIET	CAUTERIZATION	INTERVAL AFTER CAUTERIZATION	VITAMIN C CONTENT OF ORGANS (MILLIGRAM PER GRAM)			
				BRAIN	LIVER	SPLEEN	ADRENAL GLAND
Guinea pigs	Adequate	Control*	--------	0.15	0.16	0.38	1.04
Guinea pigs	Adequate	Cauterized	18 hours	0.13	0.07	0.15	0.38
Guinea pigs	Adequate	Control†	--------	0.11	0.12	0.25	0.51
Guinea pigs	Adequate	Cauterized	18 hours	0.09	0.06	0.18	0.35
Guinea pigs	Adequate	Control‡	--------	0.10	0.04	0.11	0.25
Guinea pigs	Adequate	Cauterized	18 hours	0.09	0.02	0.10	0.37

*Experiment done in June, 1938.
†Experiment done in February, 1939.
‡Experiment done in April, 1939.

It is clear from the data given in Table II that the burned guinea pigs experienced in all organs save one a more or less pronounced drop in vitamin C levels; this drop reached its greatest magnitude in the liver. The change was well marked in all three experiments, although the influence of seasonal variation made itself felt in a tendency for the vitamin C values of all tissues to run noticeably higher in the summer than in the winter and spring. A similar phenomenon was observed before in our vitamin C assay of monkey tissues.[16] The levels found for normal control tissues compare favorably with the range reported in the literature for guinea pigs that have been kept on an adequate diet.[18]

DISCUSSION

The experimental data presented in this paper demonstrate that prolonged ultraviolet irradiation of albino guinea pigs, rabbits, and monkeys, fails to bring about any change in the tissue levels of vitamin C as it also proves singularly ineffective in inducing an erythema on the shaven skin of the rayed animals. However, when a superficial burn is produced on the skin of guinea pigs by the local application of heat, a more or less marked diminution of the vitamin C stores occurs in all the organs tested. The latter observation is in complete harmony with similar findings of Einhauser.[13] It is possible that toxic, histamine-like substances, which have an adverse effect on adrenal function, originate from the burned tissue and require large amounts of vitamin C for their detoxification,[19] a process which may be analogous to the well-known

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depletion of vitamin C following the necrotizing action of diphtheria toxin. The fact that ultraviolet irradiation, without inducing visible tissue damage in laboratory animals, also leaves the vitamin C stores intact is of particular interest in view of Wright's[10] observation that evidence of disturbed vitamin C metabolism occurred only in one individual who showed definite intolerance to ultraviolet light exposure. Evidently, a mechanism operates in some individuals which protects tissues against damage and preserves the vitamin C reserves in the tissues; vice versa, the absence of such a protective mechanism in other individuals, or its breakdown by overdosage of light, may initiate a derangement of vitamin C metabolism. This, in turn, may assume significance as a contributing factor in the lowering of natural resistance against certain infectious diseases.

SUMMARY AND CONCLUSIONS

1. Exposure of guinea pigs, rabbits, and rhesus monkeys to prolonged ultraviolet irradiation caused no change in the tissue levels of vitamin C.

2. Such irradiation failed to produce any evidence of erythema on the skin of the rayed animals.

3. The production of a superficial burn on the skin of guinea pigs by the local application of heat was followed by a more or less marked drop in the content of vitamin C of the brain, liver, spleen, and adrenal gland.

4. Excessive doses of radiant energy apparently cause a derangement of vitamin C metabolism only to the extent that they are capable of producing injury to the tissues.

REFERENCES

1. Perla, D., and Marmorston, J.: Role of Vitamin C in Resistance, Arch. Path. **23**: 543, 683, 1937.
2. Schlemmer, R., Bleyler, B., and Cahnmann, H.: Studien ueber biochemische Aktivatoren der Milch; die Beziehungen zwischen dem Reduktionsvermoegen der Milch auf Vitamin C nach chemischer Methode, Biochem. Ztschr. **254**: 187, 1932.

Rohmer, P., Bezssonoff, N., and Stoerr, E.: L'Influence des facteurs non alimentaires sur la synthèse de la vitamine C par l'organisme animal, Compt. rend. Soc. de biol. **118**: 58, 1935.

Wachholder, K.: Die Versorgung des Säuglings mit Vitamin C, Klin. Wchnschr. **15**: 593, 1936.

Ferdinand, H.: Der Vitamin C—Gehalt der Frauenmilch und der Kuhmilch in den Fruehlingsmonaten, Klin. Wchnschr. **15**: 1311, 1936.

Correns, A. E.: Der Vitamin C—Gehalt der Frauenmilch und der Kuhmilch im Sommer, Klin. Wchnschr. **16**: 81, 1937.

Baumann, T.: Untersuchungen ueber die jahreszeitlichen Schwankungen des Vitamin C—Gehalts der Frauen—und Kuhmilch, ueber den C—Bedarf des Säuglings und ueber die C—Versorgung der staedtischen Bevoelkerung der Schweiz, Schweiz. med. Wchnschr. **67**: 962, 1937.

Kon, S. K., and Watson, M. B.: Vitamin C Content of Cows Milk, Biochem. J. **31**: 223, 1937.

Ippen, R.: Ueber jahreszeitliche Schwankungen des Vitamin C Gehaltes im Organismus, Schweiz. med. Wchnschr. **65**: 431, 1935.

Demole, V.: Fatigue printanière, psychonévroses et précarence en vitamine C, Schweiz. med. Wchnschr. **66**: 685, 1936.

Kasahara, M., and Kawashima, K.: Die jahreszeitliche Schwankung des Vitamin C-Gehaltes in der Muttermilch, Ztschr. f. Kinderh. **58**: 191, 1936.

Sinnko, E. I.: Ueber die Beziehung der Säuglingssterblichkeit und der Mortinatalitaet der Kinder zu den Schwankungen im C-Vitamingehalt der Muttermilch waehrend der verschiedenen Jahreszeiten in Finnland, Acta paediat. **21**: 407, 1937.

4050

Mueller, H.: Variations saisonnières et autres de l'élimination de la vitamine C dans l'urine, Ztschr. f. Vitaminforsch. 7: 311, 1938.
Guggenheim, K.: Investigation on the Seasonal Fluctuation of Vitamin C Excretion in Palestine, J. Hyg. 39: 35, 1939.
Trier, R.: Die jahreszeitlichen Schwankungen der Serumascorbinsäure, Klin. Wchnschr. 17: 976, 1938.
Kuehnau, J.: Die Bedeutung des Fruehjahrs fuer die Entstehung von Avitaminosen, Deutsche med. Wchnschr. 62: 621, 1936.
Melka, J., and Klimo, Z.: Askorbinsäuregehalt in der Cerebrospinal-fluessigkeit des Menschen, Klin. Wchnschr. 17: 302, 1938.
3. Martini, E., and Torda, C.: Die Wirkung der hohen Temperaturen auf den Askorbinsäuregehalt der Meerschweinchenorgane, Klin. Wchnschr. 16: 1763, 1937.
4. Zook, J., and Sharpless, G. R.: Vitamin C Nutrition in Artificial Fever, Proc. Soc. Exper. Biol. & Med. 39: 233, 1938.
5. Castellini, P. G.: Sul comportamento dell'acido ascorbico nella cute normale e trattata con raggi ultravioleti, Gior. ital. di dermat. e sif. 77: 967, 1936.
6. Wels, P.: Weitere Untersuchungen ueber Reduktionen in der bestrahlten Haut, Arch. f. exper. Path. u. Pharmakol. 188: 265, 1938.
7. Holtz, P., and Woellpert, K.: Ueber den Einfluss der Ultravioletbestrahlung auf das Krankheitsbild des experimentellen Skorbuts, Arch. f. exper. Path. u. Pharmakol. 182: 164, 1936.
8. Schade, H. A.: Beitrag zur Frage des Einflusses von Vitamin C (1-Ascorbinsäure) auf Pigmentierungsvorgänge, Klin. Wchnschr. 14: 60, 1935.
9. Urbach, E., and Kral, F.: Lichtschutz durch Kombination von Vitamin C und Bergamottoel, Klin. Wchnschr. 16: 960, 1937.
10. Wright, I. S.: Personal communication.
11. von Drigalski, W.: Ueber den Stoffwechsel der Vitamine, Ergebn. d. inn. Med. u. Kinderh. 55: 29, 1938.
12. Stolfi, S.: Studi sulla biochimica della ustioni e dello shock traumatico. 1. Contenuto en acido ascorbico della glandola surrenale, Boll. Soc. ital. di biol. sper. 11: 1918, 1936.
13. Einhauser, M.: Behandlung schwerer Verbrennungen mit Nebennierenrindenhormon und Vitamin C im Tierversuch, Klin. Wchnschr. 17: 127, 1938.
14. Jungeblut, C. W.: Effect of Ultraviolet Irradiation on Poliomyelitis Virus in Vitro, Proc. Soc. Exper. Biol. & Med. 37: 160, 1937.
15. Bessey, O. A., and King, C. G.: Distribution of Vitamin C in Plant and Animal Tissues and Its Determination, J. Biol. Chem. 103: 687, 1933.
Musulin, R. R., and King, C. G.: Metaphosphoric Acid in Extraction and Titration of Vitamin C, J. Biol. Chem. 116: 409, 1936.
16. Jungeblut, C. W., and Feiner, R. R.: Vitamin C Content of Monkey Tissues in Experimental Poliomyelitis, J. Exper. Med. 66: 479, 1937.
17. Ellinger, F.: Ueber die Entstehung eines den Blutdruck senkenden und den Darm erregenden Stoffes aus Histidin durch Ultraviolet-strahlung, Arch. f. Exper. Path. u. Pharmakol. 136: 129, 1928.
Ellinger, F.: Weitere Untersuchungen ueber die Entstehung des Lichterythems, Arch. f. Exper. Path. u. Pharmakol. 149: 343, 1930.
18. Hawley, E. E., Daggs, R. G., and Stephens, D. J.: Effect of Administration of Acid and Alkaline Salts Upon Ascorbic Acid Contents of Guinea Pig Tissues, J. Nutrition 14: 1, 1937.
19. Leibowitz, J., and Guggenheim, K.: On Detoxicating Effects of Ascorbic Acid, Ztschr. f. Vitaminforsch. 8: 8, 1938-39.

Amer J. Med 40: 119-26, Jan 66

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Case Reports

Vitamin B_{12}, Ascorbic Acid and Iron Metabolism in Scurvy*

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An interrelationship between ascorbic acid and vitamin B_{12} metabolism has been demonstrated in man [*1,2*]. In a series of patients with pernicious anemia, adult celiac disease and regional ileitis, Cox et al. [*1*] found low serum vitamin B_{12} levels associated with significantly lower than normal levels of plasma ascorbic acid. Simultaneously, there was increased clearance of injected sodium ascorbate from the plasma. Vitamin B_{12} therapy was necessary before the abnormal ascorbic acid metabolism was corrected. Recently Vilter et al. [*2*] have confirmed these findings.

In the following study the interrelationship between ascorbic acid and vitamin B_{12} was studied in a patient who presented with scurvy. The serum vitamin B_{12} was normal, but the detection of methylmalonic acid in the urine indicated suboptimal vitamin B_{12} stores [*3,4*]. Despite massive vitamin C replacement therapy, repletion of the vitamin B_{12} stores, as indicated by abolition of methylmalonic acid excretion, was necessary before the serum ascorbic acid level returned to normal. The anemia in this patient was also investigated and an iron overload pattern with altered iron metabolism was noted. The demonstration and clinical implications of these findings are presented herein.

CASE REPORT

A seventy-two year old bachelor (H. H. No. 21076) was admitted to the hospital on May 23, 1964, with complaints of weakness, dyspnea, recurrent epistaxis and painful bruises on the thighs. Six months before admission the patient had noticed several small ecchymoses on the inner aspect of his left thigh. Subsequently, easy bruisability and recurrent epistaxis developed. Two weeks prior to admission painful ecchymoses occurred, involving the entire inner aspect of the right and left thighs. During this period weakness and progressive exertional dyspnea developed. The patient denied having a previous history of chronic illness and there was no family history of hemorrhagic disease. He was a retired truck driver and janitor, and lived alone. He drank about five bottles of beer a week, and his diet consisted of doughnuts, hamburgers, soups, coffee, milk occasionally, and assorted sandwiches. He had difficulty in eating solids because he was edentulous and had no prosthesis. He had lost 25 pounds over a six month period.

Physical examination revealed an emaciated white man who was alert and in no acute distress. The blood pressure was 128/60 mm. Hg; the pulse 96 per minute; temperature 100.6°F., rectal; and respirations 22 per minute. Pertinent findings on physical examination included large ecchymoses covering the medial aspects of both thighs that extended to the anterior and posterior surfaces from knee to groin. The lesions were warm, indurated and tender. There were many smaller purpuric lesions over the lower legs and a perifollicular petechial eruption over both hands, wrists and calves. The body hair was coarse and showed corkscrew changes, most prominently on the chest and extensor surfaces of the arms. There was no evidence of a bleeding point in the nose; except for pallor of the mucosa and complete edentia, the remainder of the head and neck examination was within normal limits. There was no significant lymphadenopathy, and the liver and spleen were not palpable.

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Neurologic examination revealed no abnormalities. Position and vibration sense were intact.

On admission the hemoglobin concentration was 6.8 gm. per cent, the hematocrit 22 per cent, the white blood cell count 5,400 per cu. mm., the reticulocyte count 9.8 per cent and the platelet count 150,000 per cu. mm. Throughout the period of observation the white blood cell count ranged from 5,100 to 8,700 per cu. mm. and the platelet count from 150,000 to 300,000 per cu. mm. The initial white blood cell differential was 75 per cent polymorphonuclear leukocytes, 1 per cent band forms, 22 per cent lymphocytes and 2 per cent monocytes. The circulating granulocytes were hypersegmented. The red blood cells on peripheral smear inspection demonstrated some hypochromia, marked anisocytosis and poikilocytosis, with macrocytosis and polychromasia.

Osmotic fragility of the red blood cells before and after incubation was normal. The Coombs' test, direct and indirect, yielded negative results. The serum protein electrophoresis was normal. The bone marrow was hypercellular with pronounced normoblastic hyperplasia. Red cell maturation was normoblastic. The myeloid:erythroid ratio was 1:2. The iron stores in the bone marrow were increased and graded as 6+. (Fig. 1.) The results of a coagulation survey, including fibrinolytic studies, were normal. The result of a tourniquet test was positive, but the bleeding time was normal. Several stool examinations for occult blood were negative.

Routine urinalysis, blood sugar, blood urea nitrogen, serum creatinine and serum electrolytes, including calcium and phosphorus determinations were within normal limits. The initial serum bilirubin was 0.5 mg. per cent direct and 1.8 mg. per cent total. The results of cephalin flocculation and thymol turbidity tests were normal. The bromsulfalein retention was 10 per cent in 45 minutes (normal <7 per cent retention in 45 minutes).

Roentgenograms of the upper gastrointestinal tract and the small bowel did not reveal any abnormalities. A peroral small bowel biopsy specimen was normal as was a three day fecal fat analysis. These studies excluded significant malabsorption. Pernicious anemia was excluded by the finding of free acid on gastric analysis and a Schilling test result of 12 per cent excretion in 24 hours.

MATERIALS AND METHODS

Serum for vitamin assay and serum iron determination was collected in sterile vacutainers. After clotting the serum was separated by centrifugation and frozen at −20°C. until the assays were performed. Serum folate activity was measured by the microbiological method of Herbert using Lactobacillus casei [5]. Normal values range from 7.0 to 25 mμg. per ml.; borderline results are 4.0 to 6.9 mμg. per ml.; and low values are 3.9 mμg. per ml. or less. Serum vitamin B_{12} activity was assayed by the microbiological method of Hutner et al. using Euglena gracilis *Z* [6]. The normal range in our laboratory is 200 to 1,000 μμg. per ml. Serum ascorbic acid was assayed using dinitrophenylhydrazine [7]. The normal range is 0.3 to 1.4 mg. per cent. Vitamin C clearance was performed by the method of Cox et al. [7].

Urine was collected for formiminoglutamic acid determination over an 8 hour period following a 10 gm. oral histidine load. Formiminoglutamic acid was measured by conventional voltage electrophoresis on cellulose acetate membranes according to the method of Kohn et al. [8]. Under these testing conditions and using this technic normal subjects do not excrete enough formiminoglutamic acid to be detected. When formiminoglutamic acid is detected it is visually graded on a scale of *trace* to 4+.

Urinary methylmalonic acid excretion was measured in 24 hour specimens preserved with xylene. The technic employed was that of Barness et al., utilizing two-dimensional paper chromatography [4]. Normal subjects do not excrete detectable amounts of methylmalonic acid in the urine in 24 hours. When methylmalonic acid is found it is visually estimated and the results reported as trace or positive.

Blood counts and bone marrow examinations were performed by routine methods. The platelets were enumerated by the method of Brecher and Cronkite [9]. The reticulocytes were counted utilizing new methylene blue stain [10].

Serum iron was measured by the method of Ramsey [11]. The normal range is 70 to 150 μg. per cent. Marrow iron stores were graded visually by the method of Gale et al. [12]. The scale ranges from 0 to 6+, a score of 2+ to 3+ being considered normal. Ferrokinetic studies were performed according to methods described previously [13]. External counting of the injected radioactive iron (Fe^{59}) was performed by the method of Pollycove and Mortimer [14]. A cobalt 60 (Co^{60})-labeled vitamin B_{12} absorption test was performed using 0.5 μc. of labeled vitamin B_{12}. Normal results are > 10 per cent urinary excretion in 24 hours.

RESULTS

The patient's course and therapy can be divided into four periods. (Fig. 2.)

Period 1 was a control period (days 1 to 8). The diet, 1,800 to 2,000 calories, was low in vitamin C but not deficient in animal protein.

Period 2 was a period in which massive vitamin C replacement therapy was given in the hospital (days 9 to 49). The diet, 1,800 to 2,000 calories, was entirely unrestricted.

Period 3 was spent in a nursing home (days 50 to 94). Animal protein in the diet was increased (liver was served three times a week). Supplemental vitamin C therapy was continued.

Period 4 was during the second hospital ad-

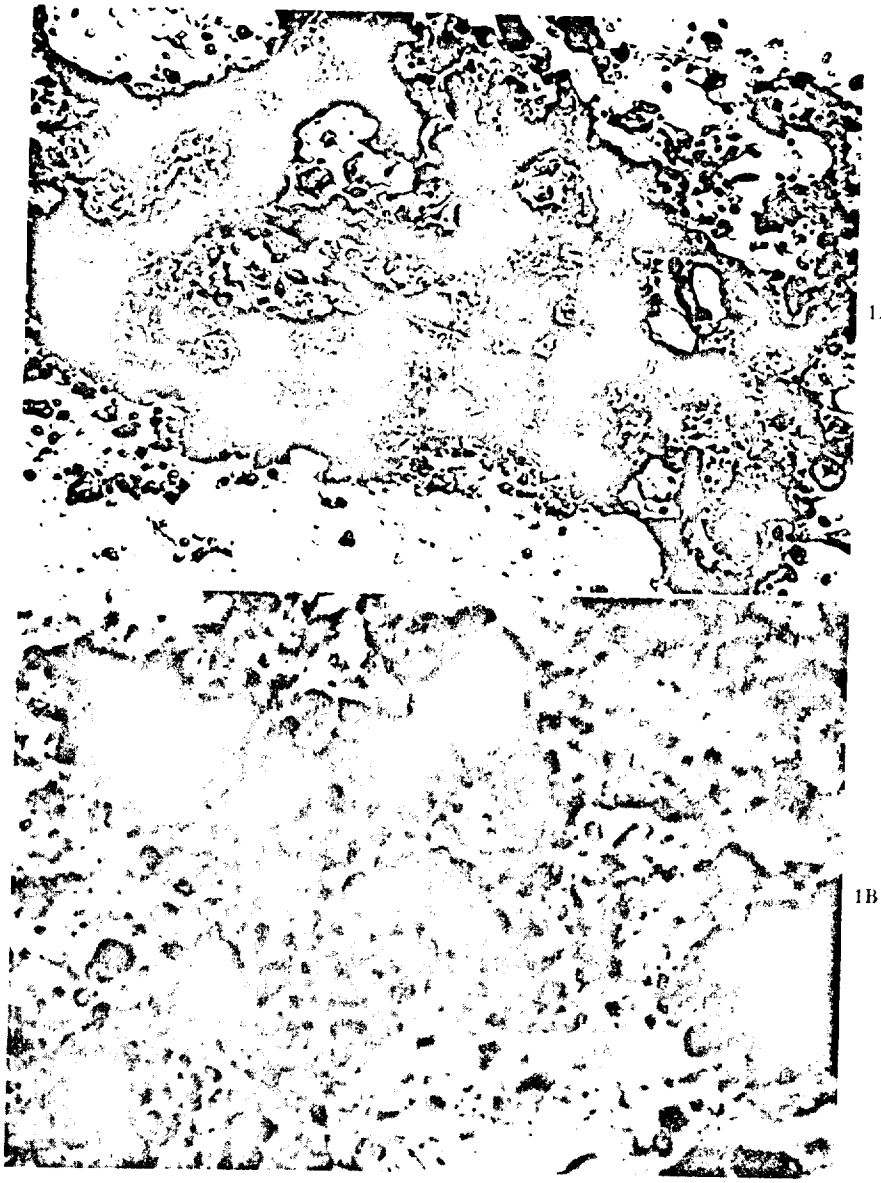


FIG. 1. A, bone marrow stained for iron (black masses). Original magnification × 100. B, bone marrow stained for iron. Original magnification × 970.

mission (days 95 to 110). The diet was the same as that in period 2 and vitamin C supplements were continued.

Serial hematologic studies including hemoglobin concentration, reticulocyte count, determination of serum vitamin B_{12} activity, serum folate activity, serum ascorbic acid levels, urinary excretion of formiminoglutamic acid after a histidine load had been given methylmalonic acid excretion, are depicted in Figure 2.

Period 1 (Days 1 to 8). During this time the diagnosis of scurvy was established on the basis of the history of dietary inadequacy, the characteristic physical findings and the low serum vitamin C levels. No vitamin therapy was given but the patient was maintained on bed rest. The hemorrhagic areas remained unchanged, and the reticulocyte count began to fall. However, progressive weakness, anorexia, mental confusion and hypotension necessitated the start

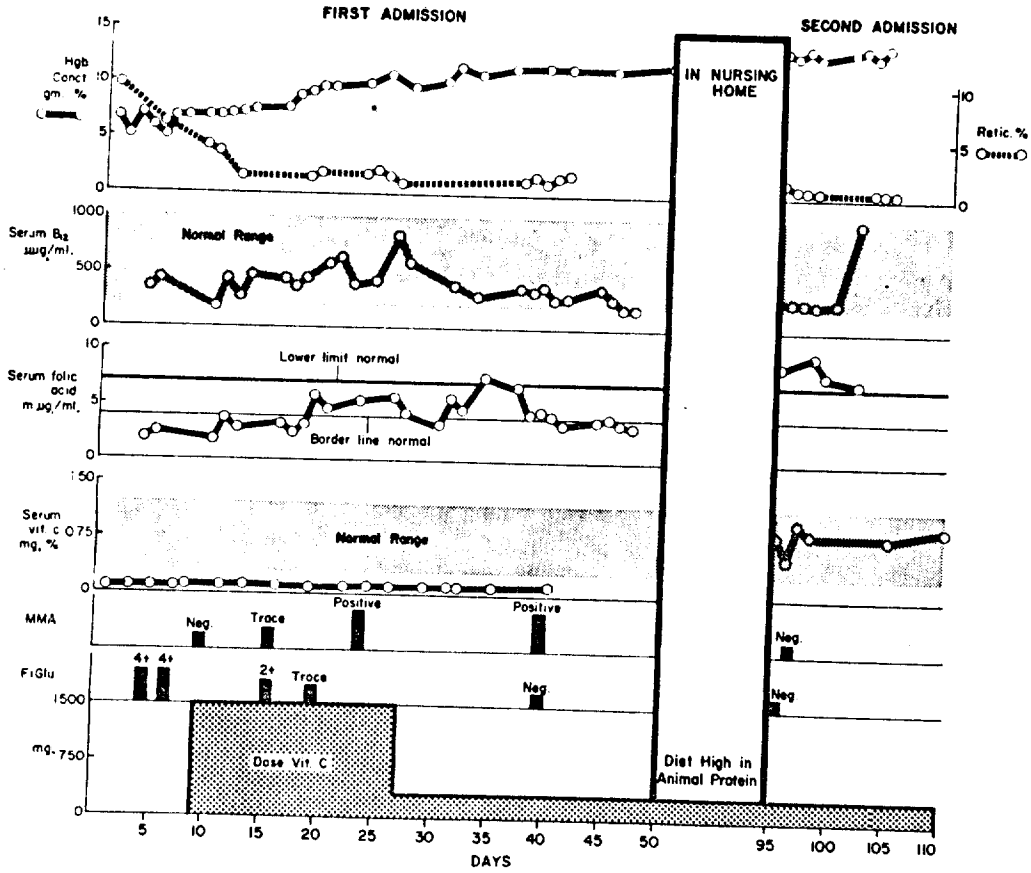


FIG. 2. Results of hematologic studies and therapy. FiGlu = formiminoglutamic acid. MMA = methylmalonic acid.

of vitamin C therapy on the ninth day of observation.

On day 5 ferrokinetic studies and radioactive chromium (Cr^{51}) survival studies were started. Bone marrow iron stores were greatly increased and graded as 6+. (Fig. 1.) Fe^{59} clearance ($T^1/_2$) was 20 minutes; serum iron was 52 μg. per cent and plasma iron turnover was 1.2 mg. per 24 hours per 100 ml. of whole blood (normal, 0.45 to 0.60 mg. per 24 hours per 100 ml. whole blood). External body counting demonstrated an abnormal pattern in that there was excessive accumulation of iron in the liver and spleen as compared to the sacrum. (Fig. 3.) Seven days later (day 12) there was 100 per cent reappearance of iron in the peripheral blood. However, by this time therapy with large doses of vitamin C had been started (day 9).

Serum folate activity ranged from 2.0 to 2.6 mμg. per ml. After oral histidine loading a large amount of formiminoglutamic acid was detected in the urine. The serum vitamin B_{12} activity was repeatedly above 200 μμg. per ml. and there was no methylmalonic acid excretion (day 9).

The serum vitamin C level repeatedly was less than 0.10 mg. per cent.

Period 2 (Days 9 to 49). On the ninth hospital day 500 mg. of sodium ascorbate was given intravenously followed by the oral administration of 500 mg., three times a day, from day 9 through day 27. Oral doses of vitamin C, 100 mg. three times a day, were given throughout the remainder of the study. With the start of vitamin C therapy there was prompt subjective improvement as manifested by increased appetite and a decrease in weakness. The hemorrhagic areas were absorbed gradually over the next two months.

The reticulocyte count returned to normal levels and there was a progressive increase in the

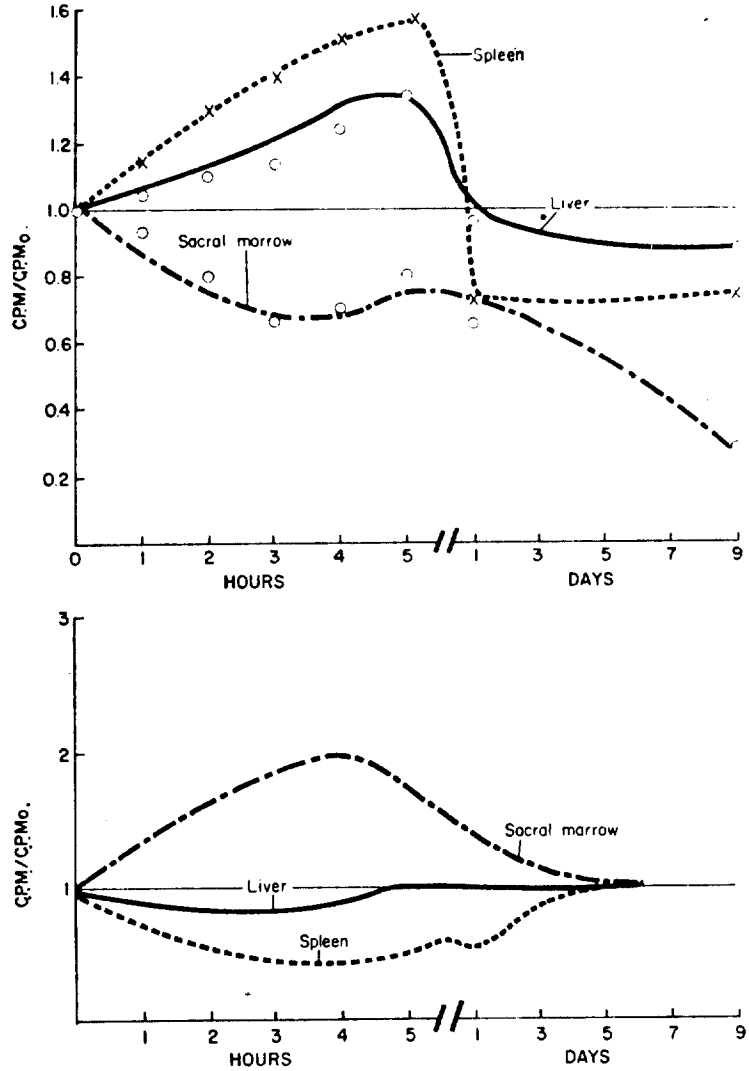


FIG. 3. Results of external iron counting over the liver, spleen and sacrum in the patient (upper curves) and in normal subjects (lower curves).

hemoglobin concentration. The initially decreased serum folate activity gradually increased to the borderline low range, and formiminoglutamic acid disappeared from the urine. The serum vitamin B_{12} activity remained within the normal range. Methylmalonic acid was first detected in trace amounts on day 15 and in large amounts on day 23 and day 40. Despite vitamin C ingestion there was no significant change in the low serum vitamin C level during this period. However, the clinical response to vitamin C therapy confirmed the diagnosis of scurvy.

Period 3 (Days 50 to 94). On the forty-ninth day of observation the patient was discharged to a nursing home. He was given a diet high in animal protein and oral vitamin C supplementation, 100 mg. three times a day, was continued. He remained asymptomatic and gained 10 pounds.

Period 4 (Days 95 to 110). The patient was readmitted to the hospital for further study on the ninety-fifth day. He felt well and the physical examination was within normal limits. The hemoglobin concentration was 14 gm. per cent, and the reticulocyte count was 0.8 per cent. The

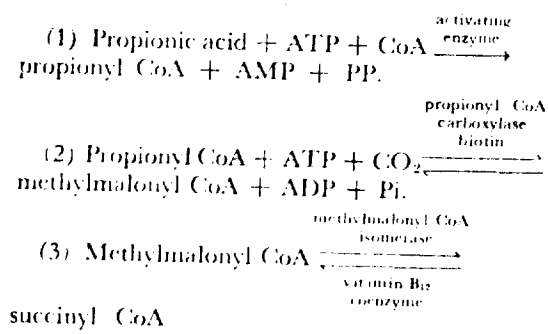
serum vitamin B_{12} level was within the normal range, and the serum folate activity was greater than 7 mμg. per ml. There was no formiminoglutamic acid excretion and no methylmalonic acid was detected in the urine. Serum ascorbic acid levels were within the normal range, and the vitamin C clearance was entirely normal. The bone marrow aspiration was within normal limits. However, iron stores were still increased and were graded 5+. The bromsulfalein retention was 3 per cent in 45 minutes and the serum bilirubin was 0.12 mg. per cent direct, and 0.38 mg. per cent total. The patient was discharged on the one hundred and tenth day and has since remained well.

During all periods of observation the serum iron concentration ranged from 48 to 92 μg. per cent. Unfortunately, ferrokinetic studies could not be repeated after the hemoglobin concentration had returned to normal.

COMMENTS

The symptomatic improvement, the increase in hemoglobin concentration and the decrease in reticulocyte count in this patient is characteristic of the response of a scorbutic subject to therapy with vitamin C [15]. However, the persistently low serum vitamin C levels, despite adequate vitamin C therapy, and the detection of methylmalonic acid in the urine suggested an interrelationship between total body vitamin B_{12} stores and serum ascorbic acid activity.

Recent work has demonstrated that methylmalonic acid excretion in the urine is a sensitive indicator of vitamin B_{12} deficiency in man [3,4]. In biological systems the conversion of propionic acid to succinic acid has been shown to proceed through the following reactions [16–19]*:



* ATP = adenosine triphosphate; CoA = coenzyme A; AMP = adenosine monophosphate; PP = pyrophosphate; ADP = adenosine diphosphate; Pi = inorganic phosphate.

Cox and White [3] and Barness et al. [4] have demonstrated that methylmalonic acid is found in the urine of patients deficient in vitamin B_{12} and that it disappears following vitamin B_{12} therapy. Recently Kahn et al. have demonstrated that methylmalonic acid excretion is a more sensitive indicator of the adequacy of vitamin B_{12} stores than is the serum vitamin B_{12} activity [20]. These workers demonstrated in four different patients with vitamin B_{12} deficiency who had been given vitamin B_{12} replacement therapy that methylmalonic acid was excreted at a time when the serum vitamin B_{12} activity, the clinical picture and the hematologic parameters were normal. In addition, in rats with experimentally produced vitamin B_{12} deficiency methylmalonic acid appears in the urine before the serum vitamin B_{12} level falls [21]. These data indicate that more vitamin B_{12} is needed to abolish methylmalonic excretion than is needed to normalize the serum vitamin B_{12} activity.

In the patient herein described the serum vitamin B_{12} activity was within the normal range throughout the entire period of observation. Furthermore, methylmalonic acid was not detected during the control period, but trace amounts were noted on day 15 and larger amounts on day 25 and day 40. This implies that the metabolic defect associated with inadequate vitamin B_{12} stores developed during the period of intensive vitamin C therapy and at a time of maximum hemoglobin regeneration. Undoubtedly the patient's vitamin B_{12} stores were somewhat reduced initially, probably because of poor diet. We hypothesize that the added stress of blood formation further increased his vitamin B_{12} needs and created a deficiency in body stores as indicated by the methylmalonic acid excretion. However, sufficient total body vitamin B_{12} was present to keep the serum activity within the normal range. Parenthetically, the progressive increase in hemoglobin concentration despite the detection of methylmalonic acid excretion confirms previous observations that complete hematologic recovery may occur even though methylmalonic acid excretion continues [4].

Cox et al. [1] and Vilter et al. [2] have reported subnormal plasma ascorbic acid levels and rapid plasma vitamin C clearance in patients deficient in vitamin B_{12} despite adequate or increased vitamin C intake. Their parameters of vitamin B_{12} deficiency included abnormal bone marrow and peripheral blood morphology,

and decreased serum vitamin B_{12} activity. Prior to vitamin B_{12} replacement, massive vitamin C therapy did not fully correct the low plasma ascorbic acid levels or the rapid vitamin C clearance. After vitamin B_{12} replacement therapy, however, the plasma ascorbic acid levels returned to normal and vitamin C clearance improved. In the patient described here the same sequence of events occurred except that the deficiency of vitamin B_{12} was detected only on a metabolic basis. The serum ascorbic acid level did not return to normal until full replenishment of vitamin B_{12} stores, as indicated by an absence of methylmalonic acid excretion, had occurred. The low serum vitamin C level during period 2 could not be ascribed to other known causes of depression of the serum activity such as surgery or inflammation. It can be concluded, therefore, that the persistently low serum ascorbate level after vitamin C therapy was produced by deficient vitamin B_{12} stores. Unfortunately, the exact time for methylmalonic acid disappearance is not known precisely but presumably it occurred during period 3.

The anemia of scurvy is not completely understood. Factors considered in its pathogenesis include hemorrhage, hemolysis, megaloblastic arrest due to associated folic acid deficiency, and iron deficiency [*22*]. In this patient hemorrhage into the tissues and folic acid deficiency existed and probably contributed to the anemia. Attempts to evaluate a hemolytic state by Cr^{51} study were inconclusive because ascorbic acid therapy had to be started two days after the chromium study was initiated.

Prior to therapy peripheral blood studies revealed some hypochromia and ferrokinetic studies demonstrated an increased plasma iron turnover. In addition, the bone marrow iron stain revealed markedly increased iron stores. However, external body counting of the injected Fe^{59} demonstrated that much of the iron was being deposited in the liver and spleen. This pattern of ferrokinetics is similar to that seen in hemochromatosis. Goldberg has shown that vitamin C is a cofactor for heme synthetase [*23*]. Conceivably, deficient body stores of vitamin C might have impaired the activity of this enzyme and could have led to the development of a sideroblastic anemia and iron overload. The ferrokinetic studies in this patient tend to support this hypothesis. These studies suggest that iron overload is an additional important factor in the anemia of scurvy. Ineffective erythropoiesis was not demonstrated on the basis of the normal reappearance of iron seven days after the start of the kinetic study. However, vitamin C had been given for three days before iron reincorporation was measured.

Finally, this case demonstrates the multiplicity of nutritional deficiencies that may be observed in scurvy, some of which are detected only by sensitive metabolic tests. In view of the complexity of the situation, as evidenced by this study, firm conclusions concerning the pathogenesis of the anemia of scurvy cannot be made on a broad basis. Rather each case must be extensively studied before any generalizations can be proposed.

SUMMARY

A patient with scurvy was studied with particular reference to vitamin C, vitamin B_{12}, folic acid and iron metabolism. Methylmalonic acid excretion indicated the development of deficient vitamin B_{12} stores during replacement therapy with vitamin C. Massive doses of vitamin C produced prompt clinical and hematologic improvement, but the serum vitamin C activity did not return to normal until vitamin B_{12} stores were replenished. Ferrokinetic studies and bone marrow iron stain before therapy demonstrated a pattern of iron overload with accumulation of iron in the liver and spleen.

This case documents the interdependence of the serum vitamin C level and the vitamin B_{12} stores. Total repletion of vitamin B_{12} stores is necessary for maintenance of a normal serum ascorbic acid level. Abnormal iron metabolism may be one of the important factors in the anemia of scurvy.

Acknowledgment: We are indebted to Dr. Lewis Barness of the Hospital of the University of Pennsylvania for performing the assay for methylmalonic acid reported in this study.

REFERENCES

1. Cox, E. V., Gaddie, R., Matthews, D., Cooke, W. T. and Meynell, M. J. An inter-relationship between ascorbic acid and cyanocobalamin. *Clin. Sc.*, 17: 681, 1958.
2. Vilter, R. W., Will, J. J., Wright, T. and Rullman, D. Inter-relationships of vitamin B_{12}, folic acid, and ascorbic acid in the megaloblastic anemias. *Am. J. Clin. Nutrition*, 12: 130, 1963.
3. Cox, E. V. and White, A. M. Methylmalonic acid excretion: an index of vitamin B_{12} deficiency. *Lancet*, 2: 853, 1962.
4. Barness, L. A., Young, D., Mellman, W. J., Kahn, S. B. and Williams, W. J. Methylmalonate excre-

tion in a patient with pernicious anemia. *New England J. Med.*, 268: 144, 1963.

5. Herbert, V. The assay and nature of folic acid activity in human serum. *J. Clin. Invest.*, 40: 81 1961.

6. Hutner, S. H., Bach, M. K. and Ross, G. I. M. A sugar containing basal medium for vitamin B_{12} assay with Euglena. Application to body fluids. *J. Protozool.*, 3: 101, 1956.

7. Natelson, S. Microtechniques of clinical chemistry, 2nd ed., p. 121. Springfield, Ill., 1963. Charles C Thomas.

8. Kohn, J., Mollin, D. L. and Rosenbach, L. M. Conventional voltage electrophoresis for formiminoglutamic acid determination in folic acid deficiency. *J. Clin. Path.*, 14: 345, 1961.

9. Brecher, G. and Cronkite, E. P. Morphology and enumeration of human blood platelets. *J. Appl. Physiol.*, 3: 365, 1950.

10. Brecher, G. New methylene blue as a reticulocyte stain. *Am. J. Clin. Path.*, 19: 895, 1949.

11. Ramsey, W. N. M. The determination of iron in blood, plasma or serum. *Clin. chim. acta*, 2: 214, 1957.

12. Gale, E., Torrance, J. and Bothwell, T. The quantitative estimation of total iron stores in human bone marrow. *J. Clin. Invest.*, 42: 1076, 1963.

13. Brodsky, I. The use of ferrokinetics in the evaluation of Busulphan therapy in polycythemia vera. *Brit. J. Haemat.*, 10: 291, 1964.

14. Pollycove, M. and Mortimer, R. The quantitative determination of iron kinetics and hemoglobin synthesis in human subjects. *J. Clin. Invest.*, 40: 753, 1961.

15. Vilter, R. W., Woolford, R. M. and Spies, T. D. Severe scurvy. A clinical and hematologic study. *J. Lab. & Clin. Med.*, 31: 609, 1946.

16. Flavin, M., Obtiz, P. J. and Ochoa, S. Metabolism of propionic acid in animal tissues. *Nature*, 176: 823, 1955.

17. Lengyel, B., Mazumder, R. and Ochoa, S. Metabolism of propionic acid in animal tissues. VI. Mammalian methylmalonyl isomerase and vitamin B_{12} coenzymes. *Proc. Nat. Acad. Sc.*, 46: 1312, 1960.

18. Stadtman, E. R., Overath, P., Eggerer, H. and Lynen, F. Role of biotin and vitamin B_{12} coenzyme in propionate metabolism. *Biochem. & Biophys. Res. Commun.*, 2: 1, 1960.

19. Stjernholm, R. and Wood, H. G., Methylmalonyl isomerase. II. Purification and properties of enzyme from propion-bacteria. *Proc. Nat. Acad. Sc.*, 47: 303, 1961.

20. Kahn, S. B., Williams, W. J., Barness, L. A., Young, D., Schafer, B., Vivacqua, R. J. and Beaupre, E. W. Methylmalonic acid excretion—sensitive indicator of vitamin B_{12} deficiency in man. *J. Lab. & Clin. Med.*, 66: 75, 1965.

21. Barness, L. A., Young, D., Kahn, S. B., Shafer, R. and Williams, W. J. Unpublished experiments.

22. Goldberg, A. The anaemia of scurvy. *Quart. J. Med.*, 32: 51, 1963.

23. Goldberg, A. The enzymic formation of haem by the incorporation of iron into protoporphyrin; importance of ascorbic acid, ergothioneine, and glutathione. *Brit. J. Haemat.*, 5: 150, 1959.

Blood 31(1): 55-56, 1968

Metabolic Interrelationship between Vitamin B_{12} and Ascorbic Acid in Pernicious Anemia

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SUBNORMAL PLASMA ascorbic acid concentration and rapid plasma ascorbate clearance have been found in patients with vitamin B_{12} deficiency despite adequate intake of vitamin C.[1,2] Cox et al.[2] noted in their group of vitamin B_{12}-deficient patients that these abnormalities of ascorbate metabolism were still present 7–14 days following therapy with sufficient vitamin B_{12} to cause clinical and hematological response. Prolonged vitamin B_{12} therapy was necessary before ascorbate metabolism returned to normal.[2]

Recently we described a patient with scurvy and anemia in whom, during therapy with vitamin C, methylmalonate (MMA) excretion was detected.[3] At this time, the hemoglobin concentration was rapidly rising but the plasma vitamin B_{12} activity was normal. It was speculated that MMA excretion suggested depletion of body vitamin B_{12} stores, since therapy with vitamin B_{12} caused abolition of MMA excretion.[4,5] In this patient despite long term administration of high doses of vitamin C, the plasma ascorbate concentration did not become normal until MMA excretion was abolished.[3] These data suggested that the plasma ascorbate concentration is related to the excretion of MMA and is directly or indirectly related to vitamin B_{12} stores.

In the study reported herein, observations were made on three patients with vitamin B_{12} deficiency associated with pernicious anemia with the purpose of correlating plasma ascorbic acid concentration with MMA excretion. Red blood cell (RBC) vitamin B_{12} activity was measured serially in order to evaluate its sensitivity as an index of vitamin B_{12} body stores when compared to MMA excretion. The data suggest an interrelationship between vitamin B_{12} stores and plasma levels of ascorbic acid.

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Presented before the meeting of the Subcommittee on Vitamin B_{12} and Folic acid at the 8th annual meeting of the American Society of Hematology at New Orleans, Louisiana, December 1966.

Supported in part by USPHS Grant CA 06832-04.

First submitted April 25, 1967; accepted for publication June 9, 1967.

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Materials and Methods

Blood was collected in sterile heparinized vacutainers (B-D. Company, Rutherford, N. J.) from patients in the fasting state. Within one hour of collection under sterile precautions an aliquot of whole blood was removed for determination of routine blood counts, another aliquot was frozen (−20 C) for whole blood folate assay, and the plasma and RBC of the remainder separated and frozen for subsequent vitamin assays.

Blood counts were performed by routine methods. Platelets were counted by the method of Brecher and Cronkite[6] and reticulocytes counted after staining with new Methylene blue.[7] Bone marrow was aspirated from the posterior iliac crest and the specimen stained with Wrights stain. Specimens were stained for iron by the method of Dry.[8] Iron stores were graded by the method of Gale, Torrance and Bothwell.[9]

All vitamin assays were performed within a week of collection of the blood specimen. Plasma folate was assayed microbiologically utilizing *L. Casei* as described by Herbert.[1] Whole blood (W.B.) folate was measured by adding 4 ml. of vitamin free distilled water to 1 ml. whole blood, centrifuging to remove red cell membranes and then diluting 1 ml. of the supernatant with 9 ml. phosphate buffer, 0.05 M. pH 6.1 containing 150 mg. per cent ascorbic acid and incubating for 90 minutes at 37.[11] The mixture was then autoclaved at 108 C for 30 minutes. Following centrifugation, 0.1 ml. of the clear protein free supernatant was assayed for folate activity in a manner similar to plasma folate. Red blood cell (RBC) folate was then calculated by the following formula:

$$\text{RBC fol. ng./ml.} = \frac{\text{W.B. fol. ng./ml.} - \text{Pl. fol. ng./ml.}}{\text{ml. RBC/ml. W.B.}} + \text{Pl. fol. ng./ml.}$$

Normal values for plasma folate are 7–18 ng./ml. with borderline values of 4–6.9 ng./ml. Normal RBC folate ranges from 175–900 ng./ml.

Plasma vitamin B_{12} activity was assayed utilizing *E. Gracilis-Z* by the method of Hutner, Bach and Ross.[12] RBC vitamin B_{12} activity was measured by adding to 1.0 ml. of packed RBC, 1.0 ml. of 0.4M acetate buffer pH 5.23, 0.2 ml., 0.1 per cent (W/V) KCN., and 8.0 ml. of vitamin free distilled water. Following autoclaving at 108 C for 30 minutes, the samples were centrifuged and an aliquot of the supernatant was assayed for vitamin B_{12} activity in a manner similar to plasma.[13] The normal plasma vitamin B_{12} activity is 200–1000 pg./ml. with borderline values of 150–200 pg./ml. Normal RBC vitamin B_{12} activity ranges from 200–600 pg./ml.

Plasma ascorbate concentration was measured by the method described by Natelson.[14] This method utilizes dinitrophenylhydrazine and measures total plasma ascorbate. Normal values are 0.4–1.4 mg. per cent. In order to rule out possible interference in the measurement of plasma ascorbate by MMA or some metabolite in the plasma, a recovery experiment was performed using plasma obtained from W.E. An aliquot of plasma obtained during the pretreatment period was obtained and sufficient ascorbic acid added to raise the concentration of vitamin C to 0.5 mg. per cent. The results were as follows:

Plasma alone	< 0.1 mg. per cent
Plasma and ascorbic acid	0.52 mg. per cent
Water and ascorbic acid	0.5 mg. per cent

MMA was measured in 24 hour urine specimens collected under xylene. MMA was assayed* by the method of Barness et al.[5] In this method, 10 ml. of the 24 hour urine specimen is continuously extracted with ether. The ether is then evaporated and the residue redissolved in a small amount of ether and the total spotted on paper for chromatography. Results are expressed semiquantitatively. Normal urine does not contain detectable MMA by this method. A +1 result implies 6 mg./L. concentration with MMA with ± indicating

Assays performed in the laboratory of Dr. L. Barness, Hospital of the University of Pennsylvania, Phila., Penn.

Supported by PHS Grant AM02231.

Table 1a.—*Hematological Data before Therapy*

Patient Age/Sex	HGB g. %	HCT %	WBC/mm³	Platelet ×10⁻³/mm³	Retic %	Marrow Cell.‡	Marrow Iron	Schilling Test without I.F.†	Schilling Test with I.F.
W. E. 52 Male	6.8-7.5	20-23	3700-5250	235-260	0.3-0.6	ME=1:1	3+	<1.0%	6.4%
Y. Q. 68 Fem.	5.5-6.4	17-20	3450-3750	27-34	1.1-1.3	ME=1:1	2+	<1.0%	10.0%
E. H. 68 Fem.	6.0-9.2*	17-29*	3070-3500	101-104	0.5-1.3	ME=1:1	3+	<1.0%	17.0%
Normal	12-17	42-52	4500-10,000	150-450	0.5-1.5	3-7:1	2-4+	>5.0%	>5.0%

*3 Units Packed Cells.
‡All Marrows Megaloblastic.
†I.F.=Intrinsic Factor.

Table 1b.—*Biochemical and Dietary Data before Therapy*

Patient	Vitamin B_{12} Plasma pg./ml.	Vitamin B_{12} RBC pg./ml.	Folate Plasma ng./ml.	Folate RBC ng./ml.	Figlu	M.M.A. (24 hr.)	Plasma Ascorbate mg. %	Dietary History
W.E.	58-102	170-200	8.0-13.5	100-140	4+	1+-3+	0.1	On Multi-Vit.& Iron Prep. Containing 75 mg. Vit. C
Y.Q.	42-52	130-135	14-15	214-226	NEG.	8+-10+	0.1-0.15	Anorexia, Wt. Loss on no Vit. Supplement
E.H.	54-60	98-260*	7.8-15	334-675*	NEG.	4+	0.18-0.26	On Multi-Vit. Containing 75 mg. Vit. C
Normal	200-1,000	200-600	7-20	175-900	NEG.	0	0.4-1.4	

*After Transfusion.

3–5.9 mg./l. Trace indicates some MMA present but less than 3 mg./L. In the present study, virtually all urine collections exceeded 1000 ml. Therefore, results of +1 or greater indicate excretion of MMA of greater than 6 mg. per day.

Formimino-1-glutamic acid excretion (FiGlu) in 6 hour urine specimens was measured on cellulose acetate membranes[15] following 10 g. oral histidine loading. Normal individuals do not excrete detectable FiGlu by this method. Results are expressed semiquantitatively (0–4+) with values of 200 μg./ml. being read as +2, 300 μg./ml. 3+ and greater than 300 μg./ml. 4+.

Gastric analysis was performed by standard techniques employing tube drainage of stomach contents before and after Ewald test meal. Free acid was measured by titration with Topfers reagent and pH with Hydrion paper.

Schilling tests were performed utilizing 0.5 μc. doses of Co60 labeled vitamin B_{12} (specific activity 0.5 μc/μg.) These studies were performed after the study was completed.

Results

Pretreatment

Pertinent hematological data prior to therapy are listed in Table 1a. Patients Y.Q. and E.H. had pancytopenia while W.E. had leukopenia and anemia. All peripheral smears revealed ovalomacrocytosis and hypersegmented polymorphonuclear neutrophiles. Bone marrow examination demonstrated hypercellu-

Table 2.—*Hematological and Biochemical Data following Therapy*

PATIENT Y. Q.

Day of B_{12} Therapy	HGB g. %	Retic %	Plasma B_{12} pg./ml.	RBC B_{12} pg./ml.	Plasma Vit. C mg. %	Urine MMA (24 hr.)	Therapy Vit. B_{12} µg./day	Therapy Vit. C mg./day
Pre (3 days)	5.5-6.4	1.1-1.3	42-52	130-145	<0.1-0.15	8+-10+	0	75 daily
2	5.4	2.0	78	135	—	2+	1* (day 1-11)	75
4	5.7	2.1	104	110	0.11	8+	1	75
9	6.1	6.5	206	135	—	3+	1	75
11	6.7	5.0	230	110	0.23	2+	1 (end 1 µg)	75
12	—	3.3	190	100	—	2+	10* (day 12-17)	200 (day 1
15	6.5	—	232	280	0.14	2+	10	200
17	6.2	4.7	392	250	—	—	10 (end 10 µg)	200
18	7.1	5.5	240	260	0.11	1+	900+ (day 18-49)	200
25	8.6	4.4	192	310	0.18	1+	900	200
30	9.6	5.7	196	340	0.20	1+	900	75
49	13.2	0.6	212	290	0.37	0	900	75
Normal	12-17	0.5-1.5	200-1000	200-600	0.4-1.4	0	—	—

* Intramuscularly.
\+ Oral.

larity with decreased myeloid-erythroid ratio, frankly megaloblastic erythropoiesis and adequate marrow iron stores in all three.

In Table 1b are listed the biochemical and dietary data obtained in these patients. Prior to therapy, all patients had low plasma and RBC vitamin B_{12} levels, while plasma folate concentration was normal. RBC folate was low in W.E. and normal in the other patients. Interestingly, only W.E. excreted FiGlu.

All patients excreted MMA in their urine. The daily amount of MMA excreted was variable from day to day in individual patients and was dissimilar when each patient was compared to the other. This variation in daily excretion of MMA and the lack of correlation between the magnitude of MMA excretion and the clinical or hematological parameters of vitamin B_{12} deficiency have been documented in prior studies.[4,16]

Plasma ascorbate concentration was below the normal range in all patients even though W.E. and E.H. were ingesting sufficient vitamin C to ensure normal plasma ascorbate levels.

Patient Y.Q. had anorexia and weight loss prior to admission and her initially low plasma ascorbate activity might have been related to lack of intake of vitamin C.

All patients had absolute achlorhydria even after Ewald stimulation. Other laboratory studies obtained included upper gastrointestinal x-rays, chest x-rays, liver function and renal function studies, which were normal in all three patients.

Schilling tests revealed malabsorption of vitamin B_{12} due to the absence of intrinsic factor in all three patients. These data indicate that the cause of the vitamin B_{12} deficiency demonstrated in these patients was pernicious anemia.

Table 3a.—*Hematological and Biochemical Data following Therapy*
PATIENT W. E.

Day of B_{12} Therapy	HGB g. %	Retic %	Plasma B_{12} pg./ml.	RBC B_{12} pg./ml.	Plasma Vit. C mg. %	Urine MMA (24 hr.)	Therapy Vit. B_{12} µg./day	Therapy Vit. C mg./day
Pre (5 days)	6.8-7.5	0.3-0.6	58-102	170-200	0.1	1+-3+	0	75
3	7.0	0.7	130	190	0.1	2+	900 (day 1-103)	75
4	7.0	1.1	148	200	—	—	900	75
5	6.8	2.7	148	180	—	—	900	75
7	6.7	5.6	212	190	0.18	2+	900	75
21	10.4	1.4	190	380	0.14	1+	900	75
42	11.7	0.6	196	360	0.25	1+	900	75
68	12.4	—	196	280	—	±	900	75
103	13.7	—	380	290	0.56	0	—	—
Normal	12-17	0.5-1.5	200-1000	200-600	0.4-1.4	0	—	75

Table 3b.—*Plasma and RBC Folate Activity following Therapy*
PATIENT W. E.

Day of B_{12} Therapy	Plasma Folate ng./ml.	RBC Folate ng./ml.	Urine MMA	Plasma Vit. C mg %
Pre	8.0-13.5	100-140	1+-3+	<0.1
3	6.2	161	2+	<0.1
7	7.0	211	2+	0.18
21	7.4	264	1+	0.14
42	5.4	213	1+	0.25
68	8.8	344	±	—
103	12.0	—	0	0.56

Post Treatment

Results obtained following therapy are given in Tables 2, 3, 3b, 4 and Fig. 1. Table 2 lists data obtained on patient Y.Q. Initially this woman was given laily 1 µg. injections of vitamin B_{12}. Reticulocytosis occurred and by the 9th lay the plasma vitamin B_{12} activity became normal. On day 12, the daily dose f parenteral vitamin B_{12} was increased to 10 µg. By this time, the RBC itamin B_{12} activity had not become normal. With continued therapy, it rose normal by day 15. During this period the plasma ascorbate concentration emained sub-normal despite adequate intake of vitamin C and significant mounts of MMA were detected in 24 hour urine specimens. On the 18th ay, daily oral vitamin B_{12} therapy was begun. In patients with pernicious nemia given large oral doses of vitamin B_{12}, one may assume that 1 per cent f the ingested dose is absorbed.[17] Therefore, in this and subsequent patients, he daily absorbed dose of vitamin B_{12} is assumed to be approximately 9 µg.

As therapy continued, the hemoglobin concentration became normal. However, MMA excretion continued and plasma ascorbate concentration remained low despite the fact that daily 200 mg. doses of vitamin C were given from day 12 through day 29. Plasma ascorbate reached the low normal range by day 49 and at this time MMA excretion was not detected.

In Table 3a are listed data obtained on W.E. This man was treated with

Table 4.—*Hematological and Biochemical Data following Therapy*

PATIENT E. H.

Day of B_{12} Therapy	HGB g. %	Retic %	Plasma B_{12} pg./ml.	RBC B_{12} pg./ml.	Plasma Vit. C mg. %	Urine MMA (24 hr.)	Vit. B_{12} mg./day	Vit. C mg./day
Pre (5 days)	6.0-9.2*	0.5-1.3	54-60	98-260*	0.18-0.26	4+	—	75
3	7.9	3.0	138	290	0.4	4+	900 (day 1-89)	75
6	9.0	13.6	130	400	0.24	2+	900	75
13	11.9	4.6	200	400	0.4	1+	900	75
19	12.8	1.4	200	310	0.3	1+	900	75
33	15.0	—	340	400	0.48	±	900	75
47	13.0	—	291	410	0.11	±	900	75
68	14.9	—	540	—	0.18	±	900	75
89	—	—	291	—	—	±	900 End Oral Rx.	75
96	14.3	—	230	310	—	1+	1000 μg. im.	75
124	14.1	—	258	240	—	1+	1000 μg. im.	75
152	13.9	—	250	260	0.18	1+	1000 μg. im.	75
180	15.5	—	—	—	1.1	Faint	1000 μg. im.	75
						Trace	daily × 14 d.	—
187	—	—	—	—	—	0		75
194	14.8	—	900	—	1.2	0		75
Normal	12-17	0.5-1.5	200-1000	200-600	0.4-1.4	0	—	75

*Given Three Units Packed Cells.

daily oral 900 μg. doses of vitamin B_{12}. The data demonstrate that the plasma ascorbate concentration remained low, despite adequate vitamin C intake, until sufficient vitamin B_{12} had been absorbed to abolish MMA excretion. In a manner similar to the first patient, plasma and RBC vitamin B_{12} activity returned to normal before MMA excretion was abolished. Table 3b lists changes in plasma and RBC folate concentration in this patient. Following institution

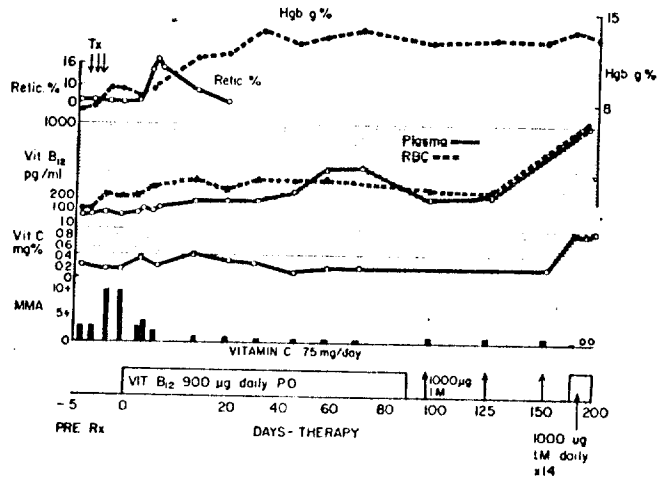

Fig. 1.—Hematologic and biochemical changes in patient E. H. following vitamin B_{12} therapy.

of vitamin B_{12} therapy, plasma folate levels fell and RBC folate activity rose. Both plasma and RBC folate activity returned to normal before vitamin C activity became normal.

Results of therapy in patient E.H. are listed in Table 4 and graphically presented in Fig. 1. Because of congestive heart failure secondary to hypertension and anemia, this patient was given 3 units (750 ml. total) of packed RBC prior to vitamin B_{12} therapy. This caused the RBC vitamin B_{12} activity to become normal although there was no significant change in plasma vitamin B_{12} level. Following institution of oral vitamin B_{12} therapy, reticulocytosis occurred and subsequently plasma vitamin B_{12} activity became normal. By day 19, hemoglobin concentration, plasma and RBC vitamin B_{12} activity were normal although MMA excretion persisted and plasma ascorbate concentration remained generally below normal. Significant excretion of MMA persisted throughout the 89 days of oral vitamin B_{12} therapy. When oral vitamin B_{12} therapy was stopped and the excretion of MMA checked 7 days later (day 96), significant quantities of MMA were detected. At this time, hemoglobin concentration, plasma and RBC vitamin B_{12} activity were normal. One thousand μg. of I.M. vitamin B_{12} were given on day 96 and 124. Despite these injections of vitamin B_{12}, MMA excretion persisted and on day 152 plasma ascorbate concentration was still below normal despite an adequate intake of vitamin C. On day 152, another parenteral injection of vitamin B_{12} was given and when MMA excretion was checked 4 weeks later (day 180), only a faint trace of MMA was detected. At this time plasma ascorbate concentration was normal. Subsequently, daily 1000 μg. parenteral injections of vitamin B_{12} were given for 14 days. No further MMA excretion was found and plasma ascorbate concentration remained within the normal range.

Discussion

The data obtained in this study document an interrelationship between the adequacy of vitamin B_{12} stores and the maintenance of a normal plasma ascorbate concentration. Other investigators have demonstrated similar abnormalities of vitamin C metabolism in vitamin B_{12} deficient patients.[1,2] Cox et al.[2] noted low plasma ascorbate levels and rapid ascorbate clearance in vitamin B_{12}-deficient patients, most of whom were ingesting sufficient vitamin C to ensure normal plasma ascorbate levels and normal ascorbic acid clearance. These investigators also noted a delay in the return to normal of ascorbate metabolism following vitamin B_{12} repletion therapy. The present study confirms the above observations and demonstrates that abnormalities of ascorbate metabolism persist in vitamin B_{12}-deficient patients until MMA excretion is abolished.

MMA excretion has been detected in vitamin B_{12}-deficient human beings[4,5,16,18] and is the last abnormality to disappear in patients undergoing replacement therapy.[4] Specifically, following institution of gradual repletion therapy, reticulocytosis, rise in hemoglobin concentration and plasma vitamin B_{12} level are seen. Depending upon the rapidity of replacement therapy, MMA excre-

tion will persist for a period of time until further vitamin B_{12} therapy causes its abolition.[4]

The data obtained in this study again document these findings. This suggests that the continued excretion of MMA indicates suboptimal replenishment of vitamin B_{12} stores. Evidence in favor of this hypothesis is the fact that the most specific method of abolishing MMA excretion is the administration of vitamin B_{12}.[4,16] It has also been shown that the repetitive administration of large doses of vitamin B_{12} will abolish MMA excretion much more rapidly than will small dose gradual repletion therapy.[4] However, further data are required which correlate MMA excretion with hepatic stores of vitamin B_{12} before it can be definitively concluded that continued MMA excretion indicates suboptimal repletion of vitamin B_{12} stores.

The study reported herein offers a possible explanation for the previously mentioned findings of Cox et al.[2] These investigators noted a delay of 7–14 days in the return of the ascorbate abnormalities following large I.M. doses of vitamin B_{12}. Since MMA excretion persists for periods of 2–15 days following administration of large doses of vitamin B_{12},[4] the data suggest that the ascorbate abnormalities would remain abnormal until MMA excretion is abolished by prolonged vitamin B_{12} therapy. Our study indicates that subnormal plasma ascorbate persists until sufficient vitamin B_{12} has been given to abolish MMA excretion.

There is no adequate explanation for the ascorbate abnormalities described. In otherwise normal human beings, a normal plasma ascorbate concentration is dependent upon an adequate intake and absorption of vitamin C.[19] Two of our patients were ingesting enough vitamin C prior to and following vitamin B_{12} therapy to maintain an adequate plasma ascorbate concentration. The only patient (Y.Q.) who had not been ingesting sufficient vitamin C prior to therapy was given a two week course of 200 mg. of vitamin C daily. Despite this therapy, her plasma ascorbate did not return to normal until vitamin B_{12} therapy abolished MMA excretion. Inflammation, malignancy, stress, surgery and pregnancy may depress the plasma ascorbate level.[20] None of these phenomena were found in our patients. It might be suggested also that the presence of MMA in the plasma (which is inferred by its excretion in the urine) might interfere with the assay of vitamin C. Recovery experiments were performed and no interference detected. Impaired absorption of vitamin C or excessive excretion of the vitamin are also possible explanations for the findings. However, studies of absorption and excretion were not performed at the time of the original study. In no patient was there clinical evidence of any malabsorption. The recovery of the vitamin C level following vitamin B_{12} therapy suggests that if excessive excretion of vitamin C were the mechanism of depression of plasma ascorbate level, then this is somehow related to MMA excretion. Further study of this possibility seems indicated.

Smith[21] suggested an antioxidant property for vitamin B_{12}, postulating that deficiency of vitamin B_{12} might lead to a rapid conversion of reduced ascorbate to oxidized ascorbate (dehydroascorbate). Since in our study total plasma ascorbate (oxidized and reduced) was measured and low levels detected, the

antioxidant effect of vitamin B_{12} does not appear to explain the abnormal vitamin C metabolism noted in these patients. Cox et al.[2] measured reduced and oxidized ascorbate separately and found no abnormal conversion of reduced to oxidized ascorbate in vitamin B_{12}-deficient patients.

Rats made deficient in vitamin E and some premature infants with vitamin E deficiency excrete MMA. Vitamin B_{12} administration abolished the MMA excretion although administration of vitamin E reduces the excretion but does not completely abolish it.[22] It is interesting that vitamin E and vitamin C are active in redox reactions. It is conceivable that the low plasma levels of vitamin C found in the patients herein reported may be related to excessive tissue utilization of vitamin C created in part by excessive production of MMA.

Despite the low levels of plasma ascorbate found in these patients, no evidence of scurvy was noted. In this study and the one of Cox et al.,[2] tissue levels of vitamin C were not measured. However, the plasma ascorbate level has been used by many investigators as an index of adequacy of vitamin C nutrition. This study suggests that plasma vitamin C levels might not accurately reflect the adequacy of vitamin C nutrition in patients with vitamin B_{12} deficiency. It may well be that these patients are actually vitamin C-deficient but this problem cannot be resolved until tissue levels of vitamin C are measured in vitamin B_{12}-deficient patients.

Abnormalities in vitamin C metabolism have been demonstrated in patients with folate deficiency.[23] Folate abnormalities were detected in one patient in our study (W.E.). This patient had a high normal plasma folate level, a RBC folate level below the lower limits of normal and excreted FiGlu following a histidine load. Similar abnormalities have been noted in vitamin B_{12}-deficient patients by others.[11,24] The actual cause of these folate abnormalities is speculative and debated.[24,25] However, of three patients studied, only W.E. had a RBC folate level below the lower limit of normal and this patient excreted FiGlu while the others did not. Since not all patients with vitamin B_{12} deficiency excrete FiGlu,[24] it might be interesting to correlate RBC folate activity with FiGlu excretion. However, in this patient absolute deficiency of folate did not exist in view of the normal plasma folate activity. Serial measurement of plasma and RBC folate concentration following vitamin B_{12} therapy in this patient and in the others revealed a fall in plasma folate and a rise in RBC folate before MMA excretion was abolished and plasma vitamin C levels became normal. These data imply that the low plasma ascorbate concentration was not directly related to abnormal folate metabolism.

One of the purposes of this study was the evaluation of RBC vitamin B_{12} activity as an index of body stores of vitamin B_{12} in patients undergoing replacement therapy. Herbert[26] has demonstrated that as vitamin B_{12} deficiency develops, serum vitamin B_{12} activity falls first and is slowly followed by RBC vitamin B_{12} level. Kelly and Herbert[27] also reported finding two patients with low serum vitamin B_{12} levels and normal RBC vitamin B_{12} activity. They suggest that RBC vitamin B_{12} activity in man may more accurately reflect tissue stores of vitamin B_{12} than does serum vitamin B_{12} activity. Similar conclusions are suggested by the data reported herein in the two patients in whom this

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measurement was possible. In both Y.Q. and W.E., initially low plasma vitamin B_{12} activity returned to normal before RBC vitamin B_{12} levels. However, MMA excretion persisted despite normal RBC vitamin B_{12} activity in these patients. These and other data[4] lead to the suggestion that MMA excretion may be a more sensitive index of the adequacy of vitamin B_{12} stores than the RBC or plasma vitamin B_{12} levels. It may be that the deficiency of vitamin B_{12} per se leads to apoenzyme deficiency of methylmalonate isomerase. Under these circumstances, continued MMA excretion would reflect apoenzyme deficiency and not deficiency of body stores of vitamin B_{12}. Until MMA excretion is correlated with hepatic vitamin B_{12} content, this question must remain unanswered.

Summary

An interrelationship between vitamin C and vitamin B_{12} was studied in three patients with vitamin B_{12} deficiency associated with pernicious anemia. Subnormal plasma ascorbate concentrations were found prior to therapy confirming previous observations. Following vitamin B_{12} administration and utilizing methylmalonate (MMA) excretion as a biochemical index of vitamin B_{12} deficiency, low plasma ascorbate concentrations persisted until MMA excretion was abolished. In two patients, RBC vitamin B_{12} activity was also serially measured in order to evaluate its sensitivity as an index of vitamin B_{12} stores when compared to MMA excretion. The data demonstrate that in these two vitamin B_{12}-deficient patients undergoing slow repletion therapy, RBC vitamin B_{12} activity returns to normal before MMA excretion is abolished.

Whether continued MMA excretion in these patients indicates a greater sensitivity of MMA excretion as an index of deficiency of vitamin B_{12} stores than does RBC vitamin B_{12} activity remains to be answered by future work.

SUMMARIO IN INTERLINGUA

Le relation inter vitamina C e vitamina B_{12} esseva studiate in tres patientes con carentia de vitamina B_{12} associate con anemia perniciose. Concentrationes infranormal de ascorbato plasmatic esseva trovate ante le therapia in confirmation de previe observationes. Post le administration de vitamina B_{12}, e con le uso del excretion de methylmalonato (MMA) como indice biochimic del carentia de vitamina B_{12}, basse concentrationes plasmatic de ascorbato persisteva usque le excretion de MMA esseva abolite. In duo patientes, le activitate erythrocytic de vitamina B_{12} esseva, in plus, mesurate serialmente, con le objectivo de evaluar su sensibilitate como indice del reservas de vitamina B_{12} in comparation con le excretion de MMA. Le datos demonstra que in iste duo patientes a carentia de vitamina B_{12} subjicite a un therapia a lente repletion, le activitate erythrocytic de vitamina B_{12} retorna al norma ante que le excretion de MMA es abolite.

Si o non le excretion de MMA in iste patientes indica que le sensibilitate del excretion de MMA es plus grande que illo del activitate erythrocytic de vitamina B_{12} como indice del carentia del reservas de vitamina B_{12} es un question que debe esser resolvite per investigationes futur.

REFERENCES

1. Will, J. J., Mueller, J. F., Glazer, H. S., Friedman, B. I., Vilter, R. W.: The alteration of ascorbic acid oxidation in pernicious anemia. J. Lab. Clin. Med. 42:967, 1953.
2. Cox, E. V., Gaddie, R., Matthews, D., Cooke, W. T., and Meynell, M. J.: An interrelationship between ascorbic acid and cyanocobalmin. Clin. Sci. 17:681, 1958.

3. Kahn, S. B., and Brodsky, I.: Vitamin B_{12} ascorbic acid and iron metabolism in scurvy. Am. J. Med. 40:119, 1966.
4. Kahn, S. B., Williams, W. J., Barness, L. A., Young, D., Shafer, B., Vivaqua, R., Beaupre, E.: Methylmalonic acid excretion, a sensitive indicator of vitamin B_{12} deficiency in man. J. Lab. Clin. Med. 66:75, 1965.
5. Barness, L. A., Young, D., Mellman, W. J., Kahn, S. B., and Williams, W. J.: Methylmalonate excretion in a patient with pernicious anemia. N.E.J.M. 268: 144, 1963.
6. Brecher, G., and Cronkite, E. P.: Morphology and enumeration of human blood platelets. J. Appl. Phys. 3:365, 1950.
7. Brecher, G.: New methylene blue as a reticulocyte stain. Am. J. Clin. Path. 19:895, 1949.
8. Dry, D. S.: Improved methods for the demonstration of mitochondria, glycogen, fat and iron in animal cells. South Afr. J. Sci. 41:298, 1945.
9. Gale, E., Torrance, J., Bothwell, T.: The quantitative estimation of total iron stores in human bone marrow. J. Clin. Invest. 42:1076, 1963.
10. Herbert, V.: The assay and nature of folic acid activity in human serum. J. Clin. Invest. 40:81, 1961.
11. Cooper, B. A., and Löwenstein, L.: Relative folate deficiency of erythrocytes in pernicious anemia and its correction with cyanocobalamin. Blood 24:502, 1964.
12. Hutner, S. H., Bach, M. K., Ross, G. I. M.: A sugar containing basal medium, for vitamin B_{12} assay with Euglena Application to body fluids. J. Protozool 3:101, 1956.
13. Biggs, J. C., Mason, S. L. A., Spray, G. H.: Vitamin B_{12} activity in red cells. Brit. J. Haem. 10:36, 1964.
14. Natelson, S.: Microtechnics of clinical chemistry, p. 121. Charles Thomas, Springfield, Illinois, 1963.
15. Kohn, J., Mollin, D. L., Rosenbach, L. M.: Conventional voltage electrophoresis for formiminoglutamic acid determination in folic acid deficiency. J. Clin. Path. 14:345, 1961.
16. Cox, E. V., and White, A. M.: Methylmalonic acid excretion: an index of vitamin B_{12} deficiency. Lancet 28:53 1962.
17. Waife, S. O., Jansen, C. J., Jr., Crabtree, R. E., Grinnan, E. L., Fouts, P. J.: Oral vitamin B_{12} without intrinsic factor in the treatment of pernicious anemia. Ann. Int. Med. 58:810, 1963.
18. Bashir, H. V., Hinterberger, H., Jones, B. P.: Methylmalonic acid excretion in vitamin B_{12} deficiency. Brit. J. Haem. 12:704, 1966.
19. Burch, H. B.: Methods for detecting and evaluating ascorbic acid deficiency in man and animals. Ann. N.Y. Acad. Sci. 92:268, 1961.
20. Goldsmith, G. A.: Human requirements for vitamin C and its use in clinical medicine. Ann. N.Y. Acad. Sci. 92: 230, 1961.
21. Smith E. L.: Vitamin B_{12}. Brit. Med. Bull. 12:56, 1956.
22. Barness, L. A., and Oski, F.: Personal communication.
23. Vilter, R. W., Will, J. J., Wright, T., Rullman, D.: Interrelationships of vitamin B_{12} folic acid and ascorbic acid in the megaloblastic anemias. Am. J. Clin. Nutr. 12:130, 1963.
24. Herbert, V., and Zalusky, R.: Interrelationships of vitamin B_{12} and folic acid metabolism. Folic acid clearance studies. J. Clin. Invest. 41:1263, 1962.
25. Lubby, A. L., Cooperman, J. M., Teller, D. N.: Urinary excretion of formiminoglutamic acid. Application in diagnosis of clinical folic acid deficiency. Am. J. Clin. Nutr. 7:397, 1959.
26. Herbert, V.: The megaloblastic anemias. New York, Grune & Stratton, 1959, pp. 84–85.
27. Kelly, A., and Herbert, V.: Coated charcoal assay of erythrocyte vitamin B_{12} levels. Blood; 29:139, 1967.

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Ascorbic acid metabolism in human cancer. By S. KAKAR and C. W. M. WILSON, *Department of Pharmacology, University of Dublin, Trinity College, Dublin 2, Ireland*

It has been demonstrated that tumour tissue in experimental animals has a high level of ascorbic acid during the period of rapid growth (Musalin, Silverblatt, King & Woodward, 1936), and blood ascorbic acid concentrations are low in patients with gastric carcinoma, though they are normal in patients with benign gastric tumours (Freeman & Hafkesbring, 1957). Leucocyte and plasma ascorbic acid values have been compared in normal children (4–14 years) and those with acute lymphatic leukemia (Table 1). Ascorbic acid intake was similar, and white cell counts

Table 1. *Plasma, leucocyte, skin and secondary cancer ascorbic acid values in children and geriatric patients*

	No. patients	Leucocyte (μg/10^8 cells)	Plasma (μ mol/l)
Children			
Control blood	10	56·4 ± 21·9	22·7 ± 11·4
Leukemic blood	10	35·9 ± 15·1	54·0 ± 14·2
p		<0·05	<0·001
Geriatric			
Control blood	7	26·0 ± 17·1	26·7 ± 11·4
Lung cancer blood	1	12·5	7·4
		Normal (μg/g)	Tumour (μg/g)
Skin biopsy	1	25	46

were normal, in the control and in the leukemic children. Duration of the leukemia was 1½–3 years. Ascorbic acid concentrations were significantly lower in the leukemic children. In one male patient aged 60 suffering from pulmonary carcinoma for 5 months, leucocyte and plasma values were considerably lower than in normal geriatric subjects. The patient had numerous skin secondaries. Biopsy of a skin secondary showed that it contained almost twice as much ascorbic acid as unaffected adjacent skin. The content of adjacent unaffected skin was 31% of the normal skin concentration (81 μg/g; Barton, Laing & Barisoni, 1972). Plasma and tissue ascorbic acid concentrations are reduced in these two forms of cancer. Leucocyte levels are also diminished, suggesting that leucocyte ascorbic acid stores have been depleted. The ascorbic acid content of the secondary was higher than that of adjacent normal tissue. This suggests that growing tumour tissue in humans selectively concentrates ascorbic acid, thus accounting for the depleted leucocyte stores and reduced plasma levels of the vitamin in human cancer.

REFERENCES

Barton, G. M. G., Laing, J. E. & Barisoni, D. (1972). *Int. Z. VitanForsch* **42**, 524.
Freeman, J. T., Hafkersbring, R. (1957). *Gastroenterology* **32**, 878.
Musalin, R. R., Silverblatt, E., King, C. G. & Woodward, G. E. (1936). *Am. J. Cancer* **27**, 707.

MacFarlane, W. V. & Howard, B. (1970). In *Physiology of Digestion and Metabolism in the Ruminant* p. 362 [A. T. Phillipson, editor]. Newcastle upon Tyne: Oriel Press.
Silver, S. (1969). *Proc. Natn. Acad. Sci. U.S.A.* **62**, 764.
Strange, R. E. & Shon, M. (1964). *J. gen. Microbiol.* **34**, 99.

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CONTRACEPTION

EFFECT OF ESTROGEN-CONTAINING ORAL CONTRACEPTIVES ON PLATELET AND PLASMA ASCORBIC ACID CONCENTRATIONS

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A B S T R A C T

Women taking oral contraceptives exhibited significantly lower platelet ascorbic acid levels than controls at two weeks after both groups had been placed on a low Vitamin C diet. Theoretically, this may alter the negative electrical potential of platelets, which could contribute to the increased incidence of thromboembolic disease.

Accepted for publication August 18, 1971.

INTRODUCTION

Thromboembolism associated with oral contraceptives has been the focus of much attention. Estrogens have been primarily implicated(1,2), but the mode of action remains to be elucidated.

The observation in our laboratory that estrogen lowered plasma and blood vessel ascorbic acid in guinea pigs(3) led to the present study to determine the effect of estrogen, in the dosage currently being employed in oral contraceptives, upon the depletion of plasma and platelet ascorbic acid levels in humans.

METHODS

Twenty volunteer women from the nursing and secretarial staff, age 22 to 38 years, were chosen. Eleven of the women served as controls; they had not taken oral contraceptives for at least six months. Nine women served as the test group; they had been taking oral contraceptives for an average of 12 months (range: 3-24 months). The estrogen content of the contraceptive pill was 50 µg for seven of the women, and 80 µg for two of them. Four of the test group and two of the control group were non-smokers; the remaining volunteers were moderate to heavy smokers.

All twenty women were placed on a diet containing 10 mg Vitamin C per day. This diet was supplemented with a 100 mg Vitamin C tablet each morning for the first seven days, in order to bring each subject to a comparable high starting level of plasma and platelet ascorbic acid. The first blood sample was drawn 24 hours after the last Vitamin C tablet, and this sample was taken as the initial value. The subjects then remained on the 10 mg per day Vitamin C diet without further supplementation until the completion of the study, and blood samples were drawn at weekly intervals. Blood samples of some of the volunteers were also analyzed prior to commencement of the study.

Four of the eleven controls did not complete the study. One developed deep vein thrombophlebitis on the day the initial blood sample was drawn. A second had a plasma ascorbic acid level of only 0.13 mg per 100 ml after being supplemented for the week, which we felt was too low to sustain a depletion study. A third left the study after the first week of depletion because of illness, and a fourth was eliminated because platelets could not be isolated from her initial blood sample. In the test group, one woman was removed from the study after the initial sample because of flu syndrome, and was restarted three weeks after symptoms abated. The results of only those who participated fully in the study have been reported.

Analysis was carried out as follows. A 29 ml blood sample was taken from an antecubital vein into a 30 ml plastic syringe containing 1 ml heparin (1000 units). The blood was transferred into a 30 ml tube and centrifuged at 250 g for 30 minutes. This yielded a red cell layer, a white cell film, and turbid platelet-rich plasma (PRP). The PRP was pipetted off into a 15 ml centrifuge tube and spun for 20 minutes at 1000 g. in a refrigerated centrifuge. This yielded clear platelet-free plasma (PFP) and a platelet button, shown by Wright staining to be composed almost entirely of platelets. A plasma extract was made by adding 2 ml of PFP to 3 ml of 3% metaphosphoric acid and centrifuging for 10 minutes at 1000 g.

The platelet button was blotted dry, transferred to a tared micro beaker, and weighed in a Mettler H20 semi-micro balance. It was then ground in a 15 ml glass tissue grinder with 3 ml of 1.3% metaphosphoric acid. Grinding was carried out for two minutes, using an electric stirrer rotating at 1500 rpm. The resultant extract was decanted into a plastic tube and centrifuged for 10 minutes at 1000 g.

The extracts of both plasma and platelets were assayed for ascorbic acid by the 2, 6-dichlorophenolindophenol method(4) using a Beckman DBG recording spectrophotometer which allowed for extrapolation of readings to zero time and corrections for turbidity. Each determination was performed in duplicate.

Plasma copper was estimated on at least two of the four samples from each subject, using the diethyldithiocarbamate method(5). Account was taken of the dilution effect of the 1 ml of heparin in calculating the plasma copper and ascorbic acid levels.

Redox potentials (at room temperature 25° C), with reference to a standard calomel electrode (SCE) of aqueous solutions of physiological concentrations of ascorbic acid at pH 7.4 (in chelex treated M/10 sodium phosphate buffer) as defined and described in a previous publication(3), were measured in order to calculate that component of the electrical potential across the platelet wall which might be due to differences in concentration of ascorbate between platelets and plasma. At pH 7.4 ascorbic acid in solution is chiefly in the form of its monosodium salt.

RESULTS

The results of the chemical analyses are summarized in Table I. The control group shows a minimal decline in platelet ascorbic acid during the first two weeks of depletion, and then falls sharply during the third week. The test group exhibits a rapid initial decline followed by leveling off of the platelet ascorbic acid concentration. This difference becomes statistically

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significant ($p < 0.05$) after two weeks of depletion. The plasma ascorbic acid levels fell at comparable rates in the test and control groups (Fig. 1).

TABLE I

Mean values of plasma and platelet ascorbic acid concentrations, during ascorbic acid depletion study.

			PRE-STUDY	INITIAL	FIRST WEEK	SECOND WEEK	THIRD WEEK
Plasma Ascorbic Acid in mg/100 ml	Controls	$\bar{X}$	0.51	0.83	0.35	0.44	0.19
		(n)	(4)	(7)	(7)	(7)	(6)
		S.D.	± 0.39	± 0.13	± 0.21	± 0.03	± 0.13
	Pill-Users	$\bar{X}$	0.85	0.92	0.55	0.39	0.25
		(n)	(3)	(9)	(9)	(9)	(6)
		S.D.	± 0.02	± 0.14	± 0.13	± 0.12	± 0.13
Platelet Ascorbic Acid in mg/100 g	Controls	$\bar{X}$	23.3	34.9	33.9	32.9	25.5
		(n)	(4)	(6)	(7)	(7)	(5)
		S.D.	± 5.35	± 7.66	± 8.56	± 6.03	± 6.38
	Pill-Users	$\bar{X}$	24.3	34.7	29.6	26.6	25.2
		(n)	(3)	(9)	(9)	(9)	(6)
		S.D.	± 5.47	± 8.11	± 6.55	± 4.65	± 4.07

Plasma copper levels showed a mean of 171.9 ± 24.1 mg per 100 ml for the test group (n=23) and 95.9 ± 22.6 mg per 100 ml for the control group (n=16), being statistically significant at $p < 10^{-9}$.

Redox potentials (E_{SCE}) of ascorbate solutions in M/10 phosphate buffer at pH 7.4 showed an inverse linear correlation with ascorbic acid concentrations ranging from 16 to 46 mg of

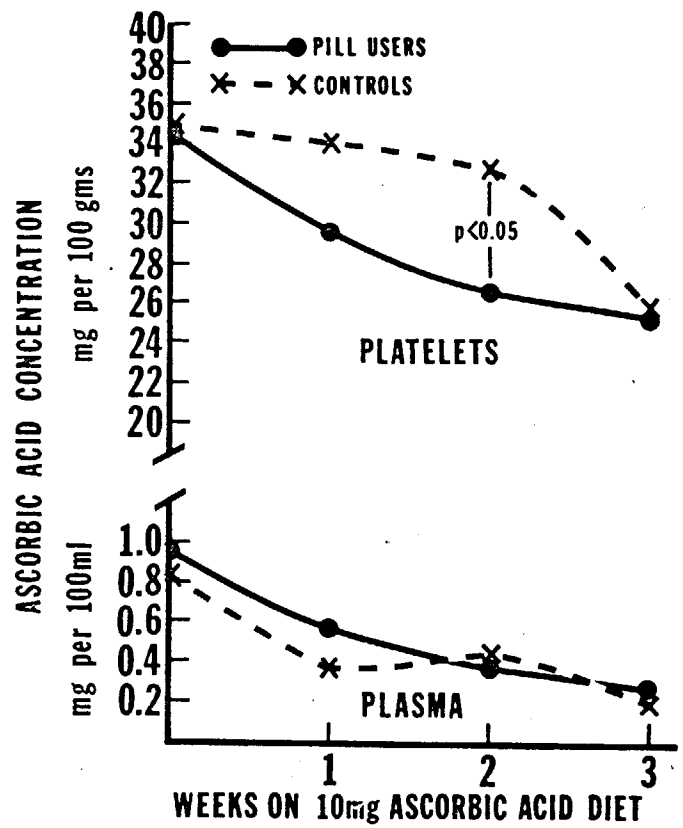

Figure 1: Mean platelet and plasma ascorbic acid levels of subjects on depletion diet.

ascorbic acid (AA) per 100 ml, which can be expressed by the equation:

$$E_{SCE}\ (mv) = -0.6 \times AA\ (mg\ per\ 100\ ml) + 0.1$$
(regression coefficient = -0.98)

This represented the range of AA concentration in platelets. For the range of plasma AA, the formula

$$E_{SCE}\ (mv) = -13.8 \times AA\ (mg\ per\ 100\ ml) + 59.7$$
(regression coefficient = -0.95)

had been arrived at earlier[3].

Both sets of values in the range 0.35 mg to 46 mg AA/100 ml solution may be jointly expressed by the logarithmic function,

$$E_{SCE}\ (mv) = -41.2 \log_{10} AA\ (mg/100\ ml) + 41.5$$
(regression coefficient for E_{SCE} vs $\log_{10}$ AA = -0.99)

Actual mean values obtained in both the ranges are shown in Figure 2.

Mean electrical potential differences between platelets and plasma due to ascorbic acid for each sample, arrived at by subtracting the E_{SCE} of platelet AA from the corresponding E_{SCE} of plasma AA, are shown in Table II. The test group mean is lower in each week than that of the control group, with a maximum difference of 5 mv occurring after one week of depletion. This difference, however, is not statistically significant.

TABLE II

Estimated ascorbate contribution to platelet-plasma potential differences.

		PRE-STUDY	INITIAL	FIRST WEEK	SECOND WEEK	THIRD WEEK
Controls	$\bar{X}$	67.5	70.1	75.9	74.2	73.5
	(n)	(4)	(7)	(7)	(7)	(5)
	S.D.	± 3.92	± 4.57	± 6.45	± 3.54	± 3.38
Pill-Users	$\bar{X}$	63.8	69.0	70.9	71.2	72.1
	(n)	(3)	(9)	(9)	(9)	(6)
	S.D.	± 3.99	± 4.16	± 3.83	± 3.74	± 1.61

Mean values for all subjects who completed the study are shown above.

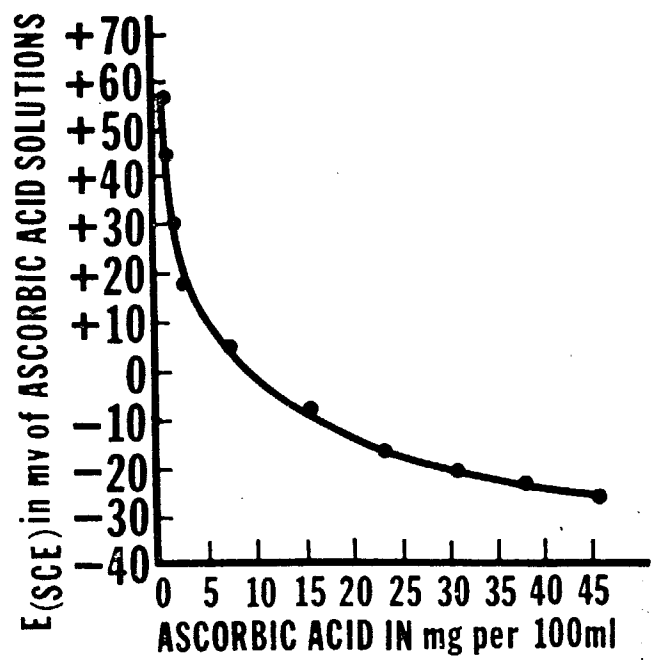

Figure 2: Redox potentials of solutions of ascorbic acid in M/10 phosphate buffer at pH 7.4 made with calomel and platinum electrodes in concentrations equivalent to those found in platelets and plasma at 25° C., expressed as $E_{(SCE)}$, the potential above or below that of calomel.

DISCUSSION

It has been shown that estrogens cause appreciable reduction in the ascorbic acid concentrations of the liver, adrenals, and uteri of guinea pigs, rats and mice[6,7,8]. In addition, estrogens reduce plasma and blood vessel ascorbic acid in guinea pigs[3]. The present study extends these findings to include the influence of estrogen on platelet ascorbic acid after two weeks on a Vitamin C poor diet in human subjects. Whereas the control group platelet AA fell by only 6.1% after two weeks of depletion, the corresponding fall in the test group was 20%. This altered rate of decline could be due to an increased excretion of ascorbic acid or to the well-known increased concentration of the ascorbate oxidase ceruloplasmin, as evidenced by the greatly elevated plasma copper values among the pill users in this study; alternatively, there may be an increase in some other ascorbate oxidase, hastening

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conversion of ascorbic acid to its unstable dehydro compound.

The plasma AA levels declined at approximately the same rate for both the control and test groups. If platelet ascorbic acid levels are representative of ascorbic acid stores in other tissues, then it would seem that dietary supplementation of "women on the pill" with Vitamin C in a dose of 100 mg a day would be sufficient to overcome the ascorbic acid depletion caused by estrogens.

Platelets are rich in ascorbic acid. It has been suggested that the high ascorbic acid in platelets is important for their hemostatic properties of adhesion and aggregation, and that the estrogen effect upon them may be due to altered ADP metabolism(9,10). Sahud and Aggeler(11) found a reduction of two-thirds in the ascorbic acid concentration of platelets following thrombin and ADP-induced aggregation. Platelets have also been shown to exhibit altered electrophoretic behavior in patients of both sexes when estrogen is administered(1,12).

Calculations based on our observations reveal a lowered, though not statistically significant, negative electrical ascorbate potential of platelets relative to plasma in the test group as compared to the control group. If ascorbic acid contributes significantly to the overall negative potential of platelets, a decrease in platelet AA would be expected to lessen this negative charge and hence diminish the primary electromotive force repelling platelets from one another and also from endothelial cells. If, as may be inferred, the endothelial cells also have lessened negative charges due to ascorbic acid depletion, this effect will become more pronounced and the possibility of occasional spontaneous thrombosis will be increased. It is interesting that the woman (C.R.) in the control group who developed thrombophlebitis had platelet and plasma ascorbic acid levels of 23.5 and 1.09 mg per 100 ml respectively, giving a platelet-plasma ascorbate potential difference of 59 mv in her initial blood sample: this is 11 mv (or 2.4 x SD) below the corresponding control group mean of 70 mv, and was, in fact, lower than that of any of the other 69 samples studied. It may be that alteration in the electrical potential of platelets is an important factor in the increased incidence of thromboembolic phenomena associated with oral contraceptives, and that the effect of estrogens in lowering platelet ascorbic acid contributes significantly to this.

This work needs to be repeated with much larger groups of volunteers, preferably on a controlled diet perhaps in a metabolic ward.

ACKNOWLEDGEMENTS

The authors wish to thank Mrs. Thomasina McQueen for the planning of the diet used in the study, and Miss Jo Ann Taranto for her assistance in preparing the manuscript.

REFERENCES

1. Inman, W. H. W. and Vessey, M. P. Investigation of deaths from pulmonary, coronary, and cerebral thrombosis and embolism in women of child-bearing age. Brit Med J 2: 193-199 (1968).

2. Veterans Administration Cooperative Urological Research Group. Treatment and survival of patients with cancer of the prostate. Surg Gynec Obstet 124: 1011-1017 (1967).

3. Saroja, N., Mallikarjuneswara, V. R. and Clemetson, C. A. B. Effect of estrogens on ascorbic acid in the plasma and blood vessels of guinea pigs. Contraception 3: 269-277 (1971).

4. Owen, J. A. and Iggo, B. Use of p-chloromercuribenzoic acid in determination of ascorbic acid with 2:6-dichlorophenolindophenol. Biochem J 62: 675-680 (1956). Also in: Clinical Chemistry Principles and Technics pp 715-719, by R. J. Henry. New York: Harper and Row Publishers (1964).

5. Gubler, C. J., Lahey, M. E., Ashenbrucker, H., Cartwright, G. and Wintrobe, M. M. Studies on copper metabolism. I. A method for the determination of copper in whole blood, red cells, and plasma. J Biol Chem 196: 209-220 (1952).

6. Mosonyi, J. Einfluss der Sexualhormone auf den C-Vitamingehalt der Nebennieren und der Leber von Meerschweinchen. Hoppe-Seylers Z Physiol 242: 158-164 (1936).

7. Leathem, J. Some biochemical aspects of the uterus. Ann N Y Acad Sci 75: 463-471, Article 2 (1959).

8. Clemetson, C. A. B. Caeruloplasmin and green plasma. Lancet 2: 1037 (1968).

9. Elkeles, R. S., Hampton, J. R. and Mitchell, J. R. A. Effect of estrogens on human platelet behavior. Lancet 2: 315-318 (1968).

10. Barkhan, P. Ascorbic acid in leukocytes and platelets. Brit Med J 1: 1357 (1966).

CONTRACEPTION

11. Sahud, M. A. and Aggeler, P. M. Utilization of ascorbic acid during platelet aggregation. Proc Soc Exp Biol Med 134: 13-16 (1970).

12. Bolton, C. H., Hampton, J. R. and Mitchell, J. R. A. Effect of oral contraceptive agents on platelets and plasma-phospholipids. Lancet 1: 1336-1341 (1968).

J. PHARMACOL AND EXP. THERAP 82(1): 86-88, 1944

THE EFFECT OF ASCORBIC ACID ON THE ACUTE TOXICITY OF SULFANILAMIDE FOR GUINEA PIGS

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Received for publication June 28, 1941

The toxic effect of sulfanilamide in humans appears to be reduced by ascorbic acid (3, 4). In order to determine the relationship between sulfanilamide and vitamin C in another species unable to synthesize C, the guinea pig was chosen as an ideal animal. This paper is concerned with the effect of ascorbic acid on the toxicity of sulfanilamide in guinea pigs.

METHODS. The amount of sulfanilamide which kills fifty per cent of the animals (LD_{50}) was determined according to the methods of Gaddum (6) and of Bliss (1, 2).

The sulfanilamide was administered by stomach tube as a twenty per cent suspension in water, and the dose was calculated in terms of grams per kilogram of body weight. All animals were observed for seven days.

Guinea pigs used in these experiments belonged to both sexes and weighed approximately 300 grams. All animals were observed for at least three days before being used.

The LD_{50} was determined for both fasting and non-fasting guinea pigs. Non-fasting animals were fed on a diet of mixed grains, cod liver oil (U.S.P. XI), brewer's yeast (Fleischman), and greens *ad lib.* Food was removed from the cages of fasting animals about fifteen hours before treatment was begun.

The non-fasting animals were divided into two groups: a) normal and b) high vitamin C. The fasting animals were divided into three groups: a) normal, b) high vitamin C, and c) depleted.

Ascorbic acid (Merck's Cebione) was administered intraperitoneally in aqueous solution freshly prepared prior to each series of injections, and the pH was adjusted to approximately 7.4.

Guinea pigs receiving ascorbic acid were injected several times during the experiment to maintain a high blood vitamin C. Some were injected with 10.0 mgm. ascorbic acid ½ hour prior to the administration of the single dose of sulfanilamide, 10.0 mgm. 1 hour after, and 10.0 mgm. again 2½ hours after the sulfanilamide had been given. Others were given, in addition to these three doses, a fourth injection of 10.0 mgm. on the day of the sulfanilamide dosage and three 10.0 mgm. injections daily for two successive days. As there was no significant difference between these two series of guinea pigs receiving ascorbic acid, they were combined and are referred to as the high vitamin C animals.

Animals were depleted by depriving them of greens for ten days.

RESULTS. The LD_{50} of sulfanilamide for normal, non-fasting guinea pigs is 3.13 grams per kg; for fasting, normal guinea pigs, 2.16 grams per kg.

Neither massive repeated injections of ascorbic acid nor ten day depletion of vitamin C affects the acute toxicity of sulfanilamide for guinea pigs.

TABLE 1

*LD_{50}'s * of sulfanilamide for fasting and non-fasting guinea pigs in different states of vitamin C nutrition*

NON-FASTING GUINEA PIGS (MAY–JULY, 1940)			FASTING GUINEA PIGS (AUGUST–NOVEMBER, 1940)		
Dose	Mortality	Per cent mortality	Dose	Mortality	Per cent mortality
Normal animals					
gms. per kg.			*gms. per kg.*		
3.90	3/4	75	3.87	10/11	91
3.56	1/4	25	3.35	13/14	93
3.45	4/7	57	2.92	12/14	86
3.38	19/23	83	2.58	24/31	77
3.31	3/8	38	2.41	16/20	80
3.21	1/4	25	2.24	4/11	36
			1.90	2/11	18
LD_{50} = 3.13 (2.78–3.51)			LD_{50} = 2.16 (1.97–2.36)		
High vitamin C					
3.45	4/5	80	3.87	15/16	94
3.38	15/30	50	3.35	16/19	84
			2.92	17/19	89
			2.24	4/22	18
			1.90	7/22	32
LD_{50} = 3.29 (2.90–3.75)			LD_{50} = 2.40 (2.19–2.63)		
Vitamin C depleted					
			2.58	6/7	86
			2.24	8/12	67
			1.90	4/13	31
			LD_{50} = 2.05 (1.78–2.35)		

* Since slopes (b) for these groups do not differ significantly, all LD_{50}'s were calculated with a composite "b", which is equal to 7.937 ± 1.154.

Figures in parentheses following the LD_{50}'s refer to Range of LD_{50} 95 times in 100.

TABLE 2

Significant difference of means between LD_{50}'s of sulfanilamide for fasting and non-fasting guinea pigs in different states of vitamin C nutrition*

	NON-FASTING		FASTING		
	Normals	Vitamin C	Normals	Vitamin C	Vitamin C depleted
Non-Fasting					
Normals		0.56	4.46	3.28	4.40
Vitamin C	0.56		5.37	3.62	4.67
Fasting					
Normals	4.46	5.37		1.51	0.61
Vitamin C	3.28	3.62	1.51		1.88
Vitamin C depleted	4.40	4.67	0.61	1.88	

* Any number greater than 2.75 indicates that the difference between the compared LD_{50}'s is significant (Probability = .01 (t = 2.75) for 30 or more animals (5).

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SUMMARY

1. The LD_{50} of sulfanilamide for fasting guinea pigs is significantly less than that for non-fasting animals.

2. Vitamin C has no influence on the acute toxicity of sulfanilamide for guinea pigs.

REFERENCES

1. Bliss, C. I., Ann. Applied Biol., **22**: 134, 1935.
2. Bliss, C. I., Ann. Applied Biol., **22**: 307, 1935.
3. Dainow, I., Ann. malad. vénér., **34**: 513, 1939.
4. Dainow, I. and Zimmet, D., Compt. rend. soc. de biol., **130**: 846, 1939.
5. Fisher, R. A., Statistical Methods for Research Workers (Oliver and Boyd), 1934.
6. Gaddum, J. H., Medical Research Council, Special Report Series, #183, 1933.

Jap J Pharmacol 17(1): 64-75. 1967

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Jap. J. Pharmacol. **17**, 64-75 (1967)

CONTRASTIC EFFECTS OF ETHIONINE AND CARBONTETRACHLORIDE ON ASCORBIC ACID METABOLISM AND ON THE ACTIVITY OF MICROSOMAL DRUG-METABOLIZING ENZYMES IN RATS*

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Received for publication August 27, 1966

It is well known that the administration of chloretone, phenobarbital or methylcholanthrene induces an increase in the urinary excretion of ascorbic acid and they also induce an increase in the activities of drug-metabolizing enzyme of liver microsomes (1-3). These results indicate that there may be some relationships in the increase in the activities of microsomal drug-metabolizing enzymes and the increase in the urinary excretion of ascorbic acid. The increased activities of microsomal drug-metabolizing enzymes are prevented by the joint administration of ethionine (4-6).

However, in a previous work, Kato *et al.* presented the evidence that the increase in the urinary excretion of ascorbic acid after the administration of chloretone or phenobarbital was not prevented by the joint administration of ethionine which completely prevented the increase in the activities of microsomal drug-metabolizing enzymes (7). These results were not in accord with the results presented by Tuoster *et al.* which the increased urinary excretion by chloretone or barbital is prevented by the joint administration of ethionine (8). One of the purpose of the present studies, therefore, is to reinvestigate the effect of ethionine on the increase in the urinary excretion of ascorbic acid induced by chloretone, phenobarbital or methylcholanthrene in comparison with the effect of carbontetrachloride.

On the other hand, in these studies Kato *et al.* observed an increase in the urinary excretion of ascorbic acid after the administration of ethionine, while the administration of carbontetrachloride markedly depress the urinary excretion of ascorbic acid. The both drugs are well known hepatotoxic agents, therefore the administration of ethionine must decrease the biosynthesis of ascorbic acid and, thus it should decrease the urinary excretion of ascorbic acid. The second purpose of the present studies, therefore, is to elucidate the mechanism by which ethionine produces the increased excretion of ascorbic acid into urine.

* A part of preliminary report of this work was published in *Experientia* **18**, 9 (1962).

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The evidence was given that ethionine could not prevent the increase in the urinary excretion of ascorbic acid induced by chloretone, phenobarbital or methylcholanthrene with the doses which almost completely prevented the increase in the activities of microsomal drug-metabolizing enzymes.

On the other hand, the administration of carbontetrachloride markedly prevented the increase in the urinary excretion of ascorbic acid induced by the drugs with the doses which only partially prevents the increase in the activities of microsomal drug-metabolizing enzymes. Futheremore, ethionine decreased the liver content of ascorbic acid and thus increased the urinary excretion, while carbontetrachloride decreased the urinary excretion probably through a decrease in the ascorbic acid biosynthesis.

MATERIALS AND METHODS

Female rats of Long-Evans strain, weighing about 160-180 g were used. In some experiments, female rats of Wistar strain, weighing about 160-180 g were used. All rats were fed on an ascorbic acid free synthetic diet, the protein content was about 25%. Carbontetrachloride were diluted in corn oil and given intraperitoneally in volume of 2 ml/kg. Ethionine is dissolved in 0.9% NaCl solution and given interaperitoneally in volume of 10 ml/kg. Phenobarbital and chloretone were dissolved in distilled water and given intraperitoneally in volume of 2 ml/kg.

Determination of ascorbic acid: Ascorbic acid contents in tissue and serum were measured by the method of Roe (9), and amount of urinary ascorbic acid was determined by the method of dichlorphenolindophenol.

Enzyme assay: The rats were decapitated and 25% liver homogenate prepared with 1.15% isotonic KCl solution at 2°-4°C. The homogenates were centrifuged at 9,000×g for 15 minutes. The microsomal oxidations of drugs were carried out by incubation of 9,000×g supernatant fraction (3 ml) with 1.5 μmole of pentobarbital, meprobamate or zoxazolamine for 60 minutes at 37°C under air, in the presence of cofactor mixture containing 0.1 ml of glucose-6-phosphate (20 μmole), 0.1 ml of NADP (0.4 μmole), 0.1 ml of $MgCl_2$ (50 μmole), 0.1 ml of nicotinamide (50 μmole) and 1.4 ml of 0.1 M sodium phosphate buffer pH 7.4. The final volume was 5.0 ml.

Chemical methods: The metabolisms of pentobarbital, meprobamate and zoxazolamine were determined by disapperance of the substrates according to the method of Brodie *et al.*, Hoffman and Ludwig and Conney *et al.*, respectively (10-12).

RESULTS

1. Effects of ethionine and carbontetrachloride on tissue content and urinary excretion of ascorbic acid

As shown in Table 1, the administration of ethionine markedly decreased the content of ascorbic acid in the liver, while it decreased only slightly the content in the kidney, thus the ratio between liver and kidney markedly was decreased. In contrast, the administration of carbontetrachloride slightly decreased the content of ascorbic acid in the liver, while it more clearly decreased the content in the kidney, thus the ratio between liver

approximately the same constant; there is, however, no evidence that either of these substances, or dihydroxymaleic acid, occurs in natural products or in foods as normally processed. Thus the finding of a reduction rate similar to that of ascorbic acid in these

Table 3. *Rates of reaction of gluco-reductone and amino-reductone with indophenol dye*

Reactant	Time after start of reaction (sec.)	Percentage of reaction completed	
		pH 1·4	pH 3·5
Gluco-reductone*	0·28	28	26
	0·55	37	34
	0·78	38	37·5
	1·1	42	38–39†
	2·5	52	49
	6	63	61
	11	72	71
	16	76	76
	31	82	83
	45	85	86
	60	89	90
	120	94	95
	180	97	97
Amino-reductone*	0·28	38	29
	0·55	46	38
	0·78	50	41·5
	1·1	51·5	44
	6	72	63
	11	79	72
	16	83	76·5
	31	89	83
	60	93	89
	120	99	95
	180	100	98

* Concentrations of reactant and of 2:6-dichlorophenolindophenol equivalent to 0·04 mg. of ascorbic acid/ml.; temperature 20°.

† Range of values in multiple observations; remaining values are averages of two or three observations.

Table 4. *Velocity constants of reactions of various reactants with indophenol dye,* 20°, pH 1·4 *and* 3·5

	K, calculated, in all cases, for 10^{-3}N × sec., and as for a bimolecular reaction	
	pH 1·4	pH 3·5
Ascorbic acid	30 ± 2	30 ± 2
*iso*Ascorbic acid	30 ± 2	30 ± 2
Hydroxytetronic acid	35 ± 2	35 ± 2
Reductic acid	17 ± 1	17 ± 1
Dihydroxymaleic acid	167 ± 2	25 ± 1
Reductone (Euler)	0·54 ± 0·08	0·54 ± 0·08
Cysteine	0·048 ± 0·06	0·048 ± 0·06
Sulphite	0·20 ± 0·02	0·20 ± 0·02
Thiosulphate	25 ± 1	1·5 ± 0·04
Ferrous ions	86 ± 2	7·4 ± 0·8

instances is of little or no consequence in relation to the practical analytical problem of the quantitative estimation of ascorbic acid in foodstuffs and food preparations.

The experimental conditions, and the more detailed conclusions, for each of these substances, were as follows.

TABLE 2. Tissue content of ascorbic acid after the administration of ascorbic acid in rats pretreated with ethionine or carbontetrachloride.

Treatment	Liver (μg/g)	Kidney (μg/g)	Liver/Kidney ratio	Serum (μg/ml)
1) Controls	313 ± 8 (8)	143 ± 5 (8)	2.15	12 ± 2 (8)
2) Ethionine	172 ± 9 (8)	139 ± 8 (8)	1.24	14 ± 2 (8)
3) CCl_4	219 ± 9 (8)	89 ± 3 (8)	2.46	9 ± 1 (8)
4) Ascorbic acid	428 ± 7 (12)	197 ± 4 (12)	2.17	17 ± 3 (12)
5) Ethionine + Ascorbic acid	235 ± 11 (12)	193 ± 12 (12)	1.22	17 ± 2 (12)
6) CCl_4 + Ascorbic acid	352 ± 12 (11)	145 ± 9 (12)	2.43	15 ± 2 (11)

Difference and significance :

	Liver Diff. (%)	Signif.	Kidney Diff. (%)	Signif.	Liver/Kidney ratio
1)——2)	−55	$p<0.01$	6	N.S.	42
1)——3)	−30	$p<0.01$	40	$p<0.01$	14
1)——4)	+37	$p<0.01$	+33	$p<0.01$	1
1)——5)	−25	$p<0.01$	+30	$p<0.01$	43
1)——6)	+12	$p<0.05$	+ 2	N.S.	13
2)——5)	+37	$p<0.01$	+39	$p<0.01$	2
3)——6)	+61	$p<0.01$	63	$p<0.01$	1

Each value represents the average ± standard error from 8-12 rats. The numerals in the brackets indicate numbers of animals used.

The rats were treated for 3 days with 200 mg/kg of ethionine or 600 mg/kg of carbontetrachloride and moreover on the fourth day half of non-treated and treated rat were given intraperitoneally 200 mg/kg of ascorbic acid and all rats were killed 4 hours later.

As shown in Table 2, the amount of increase in liver ascorbic acid after the administration of ascorbic acid was 115 μg/g, 63 μg/g and 133 μg/g, respectively, in control rats, ethionine treated rats and carbontetrachloride treated rats. On the other hand, the amount of in crease in kidney ascorbic acid after the administration of ascorbic acid was 39 μg/g, 54 μg/g and 46 μg/g, respectively, in control rats, ethionine treated rats and carbontetrachloride treated rats.

These results may support the proposed hypothesis that the administration of ethionine may interfere a storage mechanism of ascorbic acid in liver, while carbontetrachloride may decrease the biosynthesis of ascorbic acid.

3. *Effect of ethionine on the increase in the tissue content and urinary excretion of ascorbic acid induced by the treatment with chloretone, phenobarbital or methylcholanthrene*

The administration of chloretone, phenobarbital and methylcholanthrene markedly increased the urinary excretion of ascorbic acid and it increased liver and kidney contents of ascorbic acid.

As shown in Table 3, the urinary excretion of ascorbic acid was increased by 970%, 300% or 573%, respectively, in the rats pretreated with chloretone, phenobarbital or methylcholanthrene.

TABLE 3. Effect of ethionine on the increase in the tissue content and urinary excretion of ascorbic acid induced by the treatment with chloretone, phenobarbital or methylcholanthrene.

Treatment	Tissue content (μg/g) Liver	Kidney	Liver/Kidney ratio	Urinary excretion (μg/100g/day)
1) Controls	280 ± 8(12)	147 ± 5(12)	1.92	424 ± 46(12)
2) Ethionine (ET) 50	192 ± 9(8)	145 ± 7(8)	1.32	895 ± 98(8)
3) Ethionine (ET) 200	138 ± 6(8)	139 ± 4(8)	0.99	1314 ± 121(8)
4) Chloretone (CT)	354 ± 13(12)	185 ± 8(12)	1.91	4535 ± 345(11)
5) ET 50 + CT	221 ± 12(11)	196 ± 13(12)	1.13	4941 ± 431(12)
6) ET 200 + CT	165 ± 8(12)	191 ± 10(11)	0.86	4605 ± 455(12)
7) Phenobarbital (PB)	330 ± 12(12)	175 ± 9(12)	1.89	1695 ± 210(12)
8) ET 50 + PB	213 ± 9(12)	170 ± 11(12)	1.25	1705 ± 235(11)
9) ET 200 + PB	148 ± 7(11)	182 ± 10(12)	0.81	1825 ± 310(12)
10) Methylcholanthrene (MC)	337 ± 14(12)	176 ± 12(12)	1.92	2855 ± 295(12)
11) ET 50 + MC	223 ± 7(11)	167 ± 8(11)	1.34	2690 ± 345(11)
12) ET 200 + MC	150 ± 8(12)	165 ± 10(12)	0.91	2355 ± 260(12)

Difference and significance :

	Tissue content Liver Diff. (%)	Liver Signif.	Kidney Diff. (%)	Kidney Signif.	Liver/Kidney ratio Diff. (%)	Urinary excretion Diff. (%)	Signif.
1)—2)	−31	p<0.01	−2	N.S.	−31	+111	p<0.01
1)—3)	−59	p<0.01	−6	N.S.	−48	+210	p<0.01
1)—4)	−26	p<0.01	−26	p<0.01	−1	+970	p<0.01
1)—5)	−21	p<0.01	−33	p<0.01	−41	+1064	p<0.01
1)—6)	−41	p<0.01	−29	p<0.01	−55	+986	p<0.01
1)—7)	−18	p<0.01	−19	p<0.05	−2	+300	p<0.01
1)—8)	−24	p<0.01	−16	N.S.	−37	+302	p<0.01
1)—9)	−47	p<0.01	−24	p<0.01	−58	+330	p<0.01
1)—10)	−20	p<0.01	−20	p<0.05	0	+573	p<0.01
1)—11)	−22	p<0.01	−14	N.S.	−30	+534	p<0.01
1)—12)	−46	p<0.01	−12	N.S.	−53	+456	p<0.01
4)—5)	−38	p<0.01	−6	N.S.	−41	+9	N.S.
4)—6)	−46	p<0.01	3	N.S.	−55	+2	N.S.
7)—8)	−36	p<0.01	−3	N.S.	−34	0	N.S.
7)—9)	−55	p<0.01	−4	N.S.	−57	+7	N.S.
10)—11)	−34	p<0.01	−5	N.S.	−30	−6	N.S.
10—12)	−55	p<0.01	7	N.S.	−53	−17	N.S.

Each value represents the average ± standard error from 8–12 rats.
The numerals in the brackets indicate numbers of animals used.
Chloretone (50 mg/kg, i.p.), phenobarbital (70 mg/kg, i.p.) or methylcholanthrene (25 mg/kg, i.p.) were given for 4 days and ethionine was given 30 minutes before the injection of the drugs.
The rats were killed 24 hours after last injection.
The urines were collected for 24 hours started immediately after the last injection.

However, ethionine decreased the liver ascorbic acid content in the rats treated with chloretone, phenobarbital or methylcholanthrene as well as in control rats. In contrast, ethionine did not significantly alter the kidney ascorbic acid in control rats and the drug-treated rats.

Moreover, the administration of ethionine did not prevent the increase in the urinary excretion of ascorbic acid induced by the chloretone, phenobarbital or methylcholanthrene.

4. *Effect of ethionine on the increase in the activities of drug-metabolizing enzymes of liver microsomes induced by the treatment with chloretone, phenobarbital or methylcholanthrene*

The administration of chloretone or phenobarbital increased the metabolism of pentobarbital by 94% and 220%, respectively, while they increased the metabolism of meprobamate by 113% and 230%, respectively. Moreover, the administration of methylcholanthrene increased the metabolism of zoxazolamine by 375%. As reported in previous papers (5, 6, 13), the joint injection of ethionine partially (50 mg/kg) or almost completely (200 mg/kg) prevented the induced increase in the metabolism of drugs (Table 4). However, as given in Table 3 these doses of ethionine could not block the stimulatory action of chloretone, phenobarbital or methylcholanthrene on urinary excretion of ascorbic acid.

These results suggested that the mechanism of stimulation of the activities of microsomal drug-metabolizing enzymes and of urinary excretion of ascorbic acid may be different.

5. *Effect of carbontetrachloride on the increase in the tissue content and urinary excretion of ascorbic acid induced by the treatment with chloretone, phenobarbital or methylcholanthrene*

As shown in Table 5, the administration of carbontetrachloride, in contrast to the effect of ethionine, partially blocked the increased urinary excretion of ascorbic acid induced by chloretone, phenobarbital or methylcholanthrene. For example, urinary excretion of ascorbic acid in chloretone treated rats decreased by 57% and 76%, respectively, by the joint treatment of 80 mg/kg and 300 mg/kg of carbontetrachloride.

Moreover, urinary excretion of ascorbic acid in methylcholanthrene treated rats decreased by 52% and 69% respectively by the joint treatment of 80 mg/kg and 300 mg/kg of carbontetrachloride. Since the decrease in kidney content of ascorbic acid in chloretone, phenobarbital or methylcholanthrene treated rats by the joint injection of carbontetrachloride was less markedly than that in normal rats, liver/kidney ratio of ascorbic acid was not significantly changed, while the ratio was increased in the rats treated only with carbontetrachloride.

These results, therefore, suggest that the administration of carbontetrachloride decrease the synthesis of ascorbic acid in liver and decrease kidney content through decrease urinary excretion of ascorbic acid.

6. *Effect of carbontetrachloride on the increase in the activities of drug-metabolizing enzymes of liver microsomes induced by the treatment with chloretone, phenobarbital or methylcholanthrene*

The administration of 80 mg/kg or 300 mg/kg of carbontetrachloride markedly decrease the metabolism of pentobarbital, meprobamate and zoxazolamine (Table 6). In contrast to ethionine which completely inhibited the induction of the drug-metabolizing enzyme, the administration of carbontetrachloride only partially inhibited. For example, the administration of 300 mg/kg of carbontetrachloride decreased the metabolism of pentobarbital, meprobamate and zoxazolamine by 67%, 67% and 58%, respectively, while, the

TABLE 4. Effect of ethionine on the increase in the activities of liver microsomal drug-metabolizing enzymes induced by the treatment with chloretone, phenobarbital or methylcholanthrene.

Treatment	Enzyme activity (μg/g/hr) Pentobarbital	Meprobamate	Zoxazolamine
1) Controls	49 ± 4(6)	53 ± 5(6)	85 ± 6(6)
2) Ethionine (ET) 50	48 ± 3(6)	55 ± 4(6)	89 ± 7(6)
3) Ethionine (ET) 200	46 ± 5(6)	52 ± 3(6)	81 ± 5(6)
4) Chloretone (CT)	95 ± 8(6)	113 ± 12(6)	—
5) ET 50 + CT	61 ± 7(5)	67 ± 7(6)	—
6) ET 200 + CT	44 ± 4(6)	54 ± 5(6)	—
7) Phenobarbital (PB)	157 ± 12(6)	175 ± 11(6)	—
8) ET 50 + PB	81 ± 9(5)	86 ± 8(5)	—
9) ET 200 + PB	56 ± 5(6)	71 ± 10(6)	—
10) Methylcholanthrene (MC)	—	—	403 ± 29(6)
11) ET 50 + MC	—	—	179 ± 23(6)
12) ET200 + MC	—	—	121 ± 16(6)

Difference and significance :

	Pentobarbital Diff. (%)	Signif.	Meprobamate Diff. (%)	Signif.	Zoxazolamine Diff. (%)	Signif.
1)—2)	− 2	N.S.	+ 4	N.S.	+ 5	N.S.
1)—3)	− 6	N.S.	− 2	N.S.	− 5	N.S.
1)—4)	+ 94	p<0.01	+113	p<0.01	—	—
1)—5)	+ 25	N.S.	+ 26	N.S.	—	—
1)—6)	− 10	N.S.	+ 1	N.S.	—	—
1)—7)	+220	p<0.01	+230	p<0.01	—	—
1)—8)	+ 65	p<0.01	+ 62	p<0.01	—	—
1)—9)	− 14	N.S.	+ 34	N.S.	—	—
1)—10)	—	—	—	—	—	+374	p<0.01
1)—11)	—	—	—	—	—	+110	p<0.01
1)—12)	—	—	—	—	—	+ 42	N.S.
4)—5)	− 36	p<0.05	− 41	p<0.05	—	—
4)—6)	− 54	p<0.01	− 52	p<0.01	—	—
7)—8)	48	p<0.01	51	p<0.01	—	—
7)—9)	64	p<0.01	− 60	p<0.01	—	—
10)—11)	—	—	—	—	− 56	p<0.01
10)—12)	—	—	—	—	70	p<0.01
2)—5)	27	N.S.	22	N.S.	—	—
2)—8)	69	p<0.01	56	p<0.01	—	—
2)—11)	—	—	—	—	+ 101	p<0.01
3)—6)	4	N.S.	1	N.S.	—	—
3)—9)	27	N.S.	37	N.S.	—	—
3)—12)	—	—	—	—	49	p<0.05

Each value represents the average ± standard error from 8–12 rats.
The numerals in the brackets indicate numbers of animals used.
Chloretone (50 mg/kg, i.p.), phenobarbital (70 mg/kg, i.p.) or methylcholanthrene (25 mg/kg, i.p.) were given for 4 days and ethionine was given 30 minutes before the injection of the drugs. The rats were killed 24 hours after the last injection.

TABLE 5. Effect of carbontetrachloride on the increase in the tissue content and urinary excretion of ascorbic acid induced by the treatment with chloretone, phenobarbital or methylcholanthrene.

Treatment	Tissue content (μg/g) Liver	Kidney	Liver/Kidney ratio	Urinary excretion (μg/100g/day)
1) Controls	285 ± 10(12)	158 ± 4(12)	1.80	398 ± 35(12)
2) CCl_4 80	268 ± 12(8)	121 ± 3(8)	2.22	250 ± 48(8)
3) CCl_4 300	230 ± 17(8)	93 ± 4(8)	2.47	141 ± 41(8)
4) Chloretone (CT)	367 ± 14(12)	198 ± 7(12)	1.86	4452 ± 658(12)
5) CCl_4 80 + CT	315 ± 15(12)	168 ± 5(12)	1.87	1913 ± 395(11)
6) CCl_4 300 + CT	281 ± 9(11)	165 ± 6(12)	1.70	1055 ± 255(12)
7) Phenobarbital (PB)	325 ± 12(12)	185 ± 8(11)	1.76	2259 ± 425(11)
8) CCl_4 80 + PB	306 ± 13(12)	165 ± 4(12)	1.85	1283 ± 206(12)
9) CCl_4 300 + PB	275 ± 12(11)	156 ± 7(12)	1.76	975 ± 283(11)
10) Methylcholanthrene (MC)	332 ± 13(12)	207 ± 9(12)	1.60	3213 ± 493(12)
11) CCl_4 80 + MC	294 ± 10(12)	178 ± 5(11)	1.65	1544 ± 216(11)
12) CCl_4 300 + MC	271 ± 12(12)	159 ± 4(12)	1.70	1008 ± 148(12)

Difference and significance :

	Tissue content: Liver Diff. (%)	Liver Signif.	Kidney Diff. (%)	Kidney Signif.	Liver/Kidney ratio Diff. (%)	Urinary excretion Diff. (%)	Urinary excretion Signif.
1)—— 2)	− 6	N.S.	− 24	$p<0.01$	− 23	− 37	$p<0.05$
1)—— 3)	− 19	$p<0.05$	− 41	$p<0.01$	− 42	− 65	$p<0.01$
1)—— 4)	+ 29	$p<0.01$	+ 25	$p<0.01$	+ 3	− 1018	$p<0.01$
1)—— 5)	+ 11	N.S.	+ 6	N.S.	+ 4	− 381	$p<0.01$
1)—— 6)	− 2	N.S.	+ 4	N.S.	− 6	+ 165	$p<0.01$
1)—— 7)	+ 14	$p<0.05$	+ 17	$p<0.01$	− 2	− 468	$p<0.01$
1)—— 8)	+ 7	N.S.	+ 4	N.S.	+ 3	+ 222	$p<0.01$
1)—— 9)	− 4	N.S.	− 1	N.S.	− 2	+ 145	$p<0.01$
1)——10)	+ 17	$p<0.05$	+ 31	$p<0.01$	− 11	+ 790	$p<0.01$
1)——11)	+ 3	N.S.	+ 13	N.S.	− 8	+ 329	$p<0.01$
1)——12)	− 5	N.S.	0	N.S.	− 6	− 126	$p<0.01$
4)—— 5)	− 14	$p<0.05$	− 15	$p<0.01$	0	57	$p<0.01$
4)—— 6)	− 23	$p<0.01$	− 17	$p<0.01$	− 6	− 76	$p<0.01$
7)—— 8)	− 6	N.S.	− 11	$p<0.05$	+ 5	− 43	$p<0.05$
7)—— 9)	− 15	$p<0.01$	− 16	$p<0.05$	0	57	$p<0.01$
10)——11)	− 11	$p<0.05$	− 14	$p<0.01$	+ 3	52	$p<0.01$
10)——12)	− 18	$p<0.01$	23	$p<0.01$	7	69	$p<0.01$

Each value represents the average ± standard error from 8–12 rats.

The numerals in the brackets indicate numbers of animals used.

Chloretone (50 mg/kg, i.p.), phenobarbital (70 mg/kg, i.p.) or methylcholanthrene (25 mg/kg, i.p.) were given for 4 days and carbontetrachloride was given 30 minutes before the injection of the drugs.

The rats were killed 24 hours after the last injection.

The urines were collected for 24 hours started immediately after the last injection.

joint administration of carbontetrachloride decreased phenobarbital induced increase in the metabolism of pentobarbital and meprobamate by 54 and 49%, respectively, and methylcholanthrene induced increase in the metabolism of zoxazolamine by 57%.

The effect of carbontetrachloride on the microsomal enzymes was similar to the effect on urinary excretion of ascorbic acid.

DISCUSSION

The administration of chloretone, phenobarbital or methylcholanthrene and other drugs an increase in the urinary excretion of ascorbic acid through an increased biosynthesis in liver (1). On the other hand, the administration of these drugs induces an increase in the activities of liver microsomal enzymes which metabolize foreign compounds (14, 15). The induction of the drug-metabolizing enzymes of liver microsomes was inhibited by joint injection of ethionine or puromycin (4 6, 16).

However, as demonstrated in the present paper, the administration of 200 mg kg of ethionine completely prevent the stimulatory effect of chloretone, phenobarbital or methylcholanthrene on the activities of the drug-metabolizing enzymes, in contrast, the administration of same dose of ethionine did not prevent the stimulatory effect of chloretone, phenobarbital or methylcholanthrene on the urinary excretion of ascorbic acid.

Moreover, the administration of ethionine markedly decreased the ascorbic acid content in liver, but it markedly increased the urinary excretion of ascorbic acid and it did not decrease the activities of drug-metabolizing enzymes of liver microsomes. These results suggested that the administration of ethionine increase in the release of ascorbic acid from liver and thus increase the urinary excretion. This suggestion was supported by the evidence reported in Table 2 that the administration of ascorbic acid increased liver content less markedly in ethionine treated rats than in control rats. Since the administration of ethionine markedly depressed the content of ATP in the liver for more than 24 hours, thereby loss of ATP may resulted in a release of ascorbic acid from a storage pool (17, 18).

However, the lack of preventive effect of ethionine to the increase in the urinary excretion of acid by chloretone, phenobarbital or methylcholanthrene probably can not be attributed only to the increased urinary excretion by ethionine. The lack of preventive effect of ethionine therefore suggest that the mechanism of stimulation in the biosynthesis of ascorbic acid by the drugs may differ from that of the induction of the activities of drug-metabolizing enzymes and more the stimulation in the biosynthesis is not related to "de nuovo" synthesis of protein.

On the other hand, Touster reported that administration of chloretone or barbital increased the activities of uridinediphosphateglucose dehydrogenase (8), and a joint administration of ethionine completely prevented the increase in the excretion of ascorbic acid and in the activity of the enzyme.

These results are completely not in accord with the results of our preliminary and present investigations. Contrary to the present investigation, Touster employed male

TABLE 6. Effect of carbontetrachloride on the increase in the activities of liver microsomal drug-metabolizing enzymes induced by the treatment with chloretone, phenobarbital or methylcholanthrene.

Treatment	Enzyme activity (μg/g/hr) Pentobarbital	Meprobamate	Zoxazolamine
1) Controls	45 ± 5(6)	52 ± 6(6)	81 ± 5(6)
2) CCl_4 80	29 ± 3(6)	29 ± 5(6)	56 ± 7(6)
3) CCl_4 300	15 ± 3(6)	17 ± 3(6)	34 ± 6(5)
4) Chloretone (CT)	91 ± 6(6)	96 ± 7(5)	—
5) CCl_4 80 + CT	60 ± 5(6)	69 ± 4(6)	—
6) CCl_4 300 + CT	42 ± 3(5)	49 ± 5(5)	—
7) Phenobarbital (PB)	144 ± 9(6)	163 ± 11(6)	—
8) CCl_4 80 + PB	93 ± 7(6)	106 ± 8(6)	—
9) CCl_4 300 + PB	66 ± 6(6)	83 ± 3(6)	—
10) Methylcholanthrene (MC)	—	—	451 ± 34(6)
11) CCl_4 80 + MC	—	—	289 ± 32(6)
12) CCl_4 300 + MC	—	—	193 ± 21(6)

Difference and significance :

	Pentobarbital Diff. (%)	Signif.	Meprobamate Diff. (%)	Signif.	Zoxazolamine Diff. (%)	Signif.
1)—2)	− 36	p<0.05	− 45	p<0.05	− 31	—
1)—3)	− 67	p<0.01	− 67	p<0.01	− 58	—
1)—4)	+ 102	p<0.01	+ 87	p<0.01	—	—
1)—5)	− 33	p<0.05	− 33	p<0.05	—	—
1)—6)	− 7	N.S.	− 5	N.S.	—	—
1)—7)	+ 220	p<0.01	+ 213	p<0.01	—	—
1)—8)	+ 107	p<0.01	+ 104	p<0.01	—	—
1)—9)	− 47	p<0.05	− 59	p<0.01	—	—
1)—10)	—	—	—	—	+ 457	p<0.01
1)—11)	—	—	—	—	+ 257	p<0.01
1)—12)	—	—	—	—	+ 138	p<0.01
4)—5)	− 34	p<0.01	− 28	p<0.01	—	—
4)—6)	− 54	p<0.01	− 49	p<0.01	—	—
7)—8)	− 36	p<0.01	− 35	p<0.01	—	—
7)—9)	− 54	p<0.01	− 49	p<0.01	—	—
10)—11)	—	—	—	—	− 36	—
10)—12)	—	—	—	—	− 57	—
2)—5)	+ 106	p<0.01	+ 138	p<0.01	—	—
2)—8)	+ 220	p<0.01	+ 265	p<0.01	—	—
2)—11)	—	—	—	—	+ 416	p<0.01
3)—6)	+ 180	p<0.01	+ 188	p<0.01	—	—
3)—9)	+ 310	p<0.01	+ 388	p<0.01	—	—
3)—12)	—	—	—	—	+ 468	p<0.01

Each value represents the average ± standard error from 8–12 rats.
The numerals in the brackets indicate numbers of animals used.
Chloretone (50 mg/kg, i.p.), phenobarbital (70 mg/kg, i.p.) or methylcholanthrene (25 mg/kg, i.p.) were given 4 days and carbontetrachloride was given 30 minutes before the injection of the drugs. The rats were killed 24 hours after the last injection.

rats and given ethionine in a diet. However, the effect of ethionine on the decrease in the liver ATP content and protein synthesis is markedly in female rats than in male (19). And single injection of ethionine markedly decreased the liver ATP content for more than 24 hours.

In further experiments, we failed to demonstrate the prevention of the increased urinary excretion in female rat by feeding on ethionine containing diet. On the other hand, the administration of carbontetrachloride decreased the ascorbic acid content in the liver and kidney and the urinary excretion of ascorbic acid. Moreover, it decreased the activities of drug-metabolizing enzymes of liver microsomes. However, the administration of ascorbic acid increased the liver ascorbic acid content more markedly in the carbontetrachloride treated rats than in control rats.

These results suggested that the administration of carbontetrachloride decreased biosynthesis of ascorbic acid in liver and then decreased the ascorbic acid content in the liver and kidney and the urinary excretion of ascorbic acid. Moreover, carbontetrachloride partially prevent the stimulatory action of the chloretone, phenobarbital or methylcholanthrene on the urinary excretion of ascorbic acid as well as on the activities of microsomal enzymes.

Thus, the action of ethionine may be mainly due to the decrease of liver content of ATP and consequently decrease the capacity of storage of ascorbic acid and block the induction of the drug-metabolizing enzymes of liver microsomes, while the action of carbontetrachloride is mainly due to nonspecific damage of liver cell and consequently decrease the biosynthesis of ascorbic acid and the activities of the microsomal drug-metabolizing enzymes.

SUMMARY

Administration of ethionine to rats markedly decreased the ascorbic acid content in liver and increased the urinary excretion of ascorbic acid. In contrast, administration of carbontetrachloride decreased the ascorbic acid content in liver and kidney and the urinary excretion. Thus, the ratio of ascorbic acid content between liver and kidney decreased in the rats treated with ethionine, contrary it increased in the rats treated with carbontetrachloride.

On the other hand, the joint administration of ethionine prevented the stimulation of urinary excretion of ascorbic acid as well as the induction of drug-metabolizing enzymes of liver microsomes by the administration of chloretone, phenobarbital or methylcholanthrene.

In contrast, the joint administration of carbontetrachloride only partially prevented the stimulation of urinary excretion of ascorbic acid and the induction of drug-metabolizing enzymes.

It was postulated that ethionine may interefere the mechanism of storage of ascorbic acid in liver and then increased the release from storage site and consequently increased the urinary excretion of ascorbic acid, while carbontetrachloride resulted a specific damage

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of the liver and decreased biosynthesis of ascorbic acid and consequently increased the urinary excretion of ascorbic acid.

REFERENCES

1) Burns, J.J., Conney, A.H., Dayton, P.G., Evance, C., Martin, G.R. and Taller, D. : *J. Pharmac. exp. Ther.* **129**, 132 (1960)
2) Conney, A.H., Davison, C., Gastel, B. and Burns, J.J. : *Ibid.* **130**, 1 (1960)
3) Kato, R. and Chiesara, E. : *Atti Acad. Med. Lombarda* **15**, 443 (1960)
4) Conney, A.H., Miller, J.A. : *J. biol. Chem.* **228**, 753 (1957)
5) Kato, R. : *Med. Exp.* **3**, 95 (1960)
6) Conney, A.H., Michelson, I.A. and Burns, J.J. : *J. Pharmac. exp. Ther.* **132**, 202 (1961)
7) Kato, R., Vassanelli, P. and Frontino, G. : *Experientia* **18**, 9 (1962)
8) Touster, O. and Hollman, S. : *Ann. N.Y. Acad. Sci.* **92**, 318 (1961)
9) Roe, J.H. : *Methods of Biochemical Analysis*, Vol. **1**, Edited by Glick, D., p. 115, Interscience, New York (1954)
10) Brodie, B.B., Burns, J.J., Mark, L.C., Lief, P.A., Bernstein, E. and Papper, E.M. : *J. Pharmac. exp. Ther.* **109**, 266 (1953)
11) Hoffman, A.J. and Ludwig, B.J. : *Am. Pharm. Ass.* **68**, 740 (1959)
12) Conney, A.H., Trousof, N. and Burns, J.J. : *J. Pharmac. exp. Ther.* **128**, 333 (1960)
13) Kato, R., Chiesara, E. and Vassanelli, P. : *Biochem. Pharmac.* **11**, 211 (1962)
14) Remmer, H. : *Metabolic Factors Controlling Duration of Drug Action*, Edited by Brodie, B.B., p. 235, Pergamon Press, London (1962)
15) Conney, A.H. and Burns, J.J. : *Adv. in Pharmac.* Vol. **1**, London (1962)
16) Conney, A.H. and Gilman, A.G. : *J. biol. Chem.* **283**, 3682 (1963)
17) Shull, K.H. : *Ibid.* **237**, PC 1734 (1962)
18) Villa-Trevino, S., Shull, K.H. and Farber, E. : *Ibid.* **238**, 1757 (1963)
19) Ntori, Y. : *Ibid.* **238**, 2075 (1963)

J. AMER. MED. ASSOC., 224: 268, 1973.

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Ascorbic Acid Effects on Serum Glucose Values

To the Editor.—Recently, people have been taking large doses of ascorbic acid for prevention or cure of myriad illnesses. Pauling recommended that 250 mg to 15 gm (and in special cases as much as 40 gm) be taken orally each day for prevention and cure of the common cold.[1] Large doses of ascorbic acid have also been advocated for therapy of surgical wounds, arteriosclerosis, rheumatic heart disease, various bacterial infections, infantile pneumonia, surgical shock, and burns. Since ascorbic acid is a potent reducing agent, high serum levels of this vitamin may potentially interfere with any colorimetric assay based on reduction. In a commercially available automatic analyzer system (colorimetric), serum glucose assay is based on reduction by glucose of colorless cupric neocuprione to colored cuprous neocuprione.[2] In this method, sodium carbonate is added to minimize the effects of normal levels of serum reducing agents. To determine whether high-serum ascorbic acid levels can cause false elevations in serum glucose values, we measured glucose on pooled sera with proved normal range glucose concentration, but varied the concentration of the ascorbic acid.

Test samples.—The following concentrations of ascorbic acid: 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 mg/100 ml were found. Low concentrations of ascorbic acid are known to occur in vivo on a normal diet (physiologic range from 0.6 to 1.5 mg/100 ml)[3]; values above 1.5 mg/100 ml can occur in vivo following large oral doses of ascorbic acid.[4] The rapid renal excretion of ascorbic acid makes it unlikely that serum values above 16 mg/100 ml occur.[3] Dilutions were prepared with pure crystalline ascorbic acid dissolved in a commercially prepared pooled serum with a mean glucose concentration of 91 mg/100 ml, well within the normal range, and measured daily for three months on an automatic analyzer system. Each day, one set, consisting of one control serum sample without added ascorbic acid, plus one sample of each of the seven dilutions of ascorbic acid listed above, was assayed for glucose. A total of 14 sets (112 specimens) were assayed. The data were subjected to single classification analysis of variance. A posteriori testing of means was performed.

The variation among the groups of different serum concentrations of ascorbic acid was significantly greater than the variation within the groups ($P<0.01$). Only the highest concentration, the group of specimens containing 16.0 mg/100 ml of ascorbic acid, had a statistically significant elevation of the mean glucose value above the control group, ie, 4.2 mg/100 ml above the group that had no added ascorbic acid. Although this mean glucose elevation is statistically significant, it is probably not clinically significant.

We conclude that the automatic analyzer method for serum glucose may be used in the presence of elevated serum ascorbic acid levels, without adversely affecting the diagnostic value of the test.

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1. Pauling L: *The Common Cold.* San Francisco, W H Freeman & Company Publishers, 1970.
2. *Technicon SMA Manual* T69 122A. Technicon Corporation, Tarrytown, NY, September 1969.
3. Cantaro, Scheparatz: *Biochemistry,* ed 4. Philadelphia, WB Saunders Company, 1967.
4. Allen ES: Effect of timed release on bioavailability of ascorbic acid: Ascorbicap vs. non-timed dosage f. *Cur Ther Res* 11:745-749, 1969.

Arch. Environ. Health 19: 74-82 1969

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Effect of Ascorbic Acid on Pulmonary Silicosis of Guinea Pigs

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The role of ascorbic acid on developing pulmonary silicosis of normal and scorbutic guinea pigs was studied for 115 days. In normal silicotic guinea pigs an increasing concentration of ascorbic acid in lungs with the advancement of silicosis was observed. The increase of ascorbic acid was directly related to collagen percentage. Adrenal ascorbic acid showed an increase with lapse of time, and blood ascorbic acid did not show any alteration. In scorbutic silicotic guinea pigs the collagen fiber formation was retarded but the hydroxyproline contents and acid mucopolysaccharide remained the same as in normal silicotics.

ASCORBIC acid has been shown to play an important role in collagen biosynthesis in wound healing, but there is a considerable lacuna in our knowledge concerning its action in the fibrosis of silicosis. In the present investigation, therefore, an attempt has been made to study the effect of subclinical ascorbic acid deficiency on the developing pulmonary silicosis in guinea pigs.

Materials and Methods

Description of Dust Sample.—Quartz dust of particle size below 52μ was obtained. Dust of particle size less than 5μ was prepared by sedimentation in distilled water.[1] The details of size distribution and chemical analysis of the quartz dust used in these investigations are given in Table 1.

Preparation of Dust Suspension.—Into a 150-ml screw-capped bottle 7.5 gm of quartz dust was placed, well shaken with 100 ml of sterile physiological saline, and sterilized by autoclaving (20 minutes at 15-lb pressure). A few drops of aseptically separated guinea pig serum were added to keep the dust well suspended, and the mixture was agitated on an electric shaking machine until the moment of use. One milliliter of the suspension contained 75 mg of the quartz dust.

Animals.—Guinea pigs were chosen as experimental animals because they do not have a mechanism for endogenous synthesis of ascorbic acid. One hundred and forty female guinea pigs weighing 300 to 400 gm were used.

Synthetic Diet.—All the animals were fed a diet consisting of crushed barley, 64 parts; crushed gram (*Cicer arietinum*), 20 parts; casein, 12 parts; calcium carbonate, 3 parts; sodium chloride, 1 part; and 25 mg of ascorbic acid per animal per day. Vitamin A and D were supplied biweekly (2,400 and 400 international units, respectively). The scorbutic diet consisted of the same constituents with only 0.1 mg of ascorbic acid per animal per day.

Production of Silicotic Lesions.—The animals were lightly anesthetized with ether and the dust suspension injected intratracheally.[2,3]

Plan of Experiment

Experiment 1.—*To Determine the Relationship of Ascorbic Acid to Developing Silicosis in Normal Guinea Pigs.*—Fifty-two animals fed on normal diet were divided into two groups. Group 1, 32 animals, were inoculated intratracheally with 1 ml dust suspension, and group 2, 20 animals, with 1 ml sterilized normal saline.

Experiment 2.—*To Study the Scorbutic Effect on Collagen Synthesis in Pulmonary Silicosis in Guinea Pigs.*—When the scorbutic animals had lost 20% to 25% of ascorbic acid from their body depot, they were divided into two groups. Group 1, 46 animals, were inoculated intratracheally with 1 ml dust suspension. Group 2, 30 animals, with 1 ml sterilized normal saline.

Submitted for publication April 23, 1968; accepted May 27.

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Table 1.—Size Distribution and Chemical Analysis of Quartz Dust

Particle Size in Microns	% of Particles By Number	% of Particles By Mass
0.46-0.68	3.22	0.12
0.68-0.95	11.39	1.25
0.95-1.35	38.47	11.93
1.35-1.90	29.89	26.22
1.90-2.70	13.00	32.26
2.70-3.80	4.00	28.21
3.80-5.40	...	...
Particles counted	746	
Estimated surface area, sq m/gm	1.85	
Silicon dioxide	98.70%	
Ferric oxide	0.10%	
Total oxides (iron oxide, aluminum oxide, titanium oxide, phosphorus pentoxide)	0.97%	
Loss on ignition	0.09%	

Techniques

Pathological.—Routine autopsy was carried out on all animals which died or were killed. The lungs of the killed animals were gently distended by injecting 10 ml of 10% formol saline through the trachea which was exposed at the neck, without opening the thoracic cavity. The trachea was then tied off, the thoracic cavity opened, and the lungs with tied-off portion of the trachea removed were put in 10% formol saline. The lungs of guinea pigs which died were first removed from the thoracic cavity, examined, and distended gently to the normal size. After preliminary fixation, blocks were selected along the long axis of both the lungs at the level of the hilum to include the maximum representative area from each lung. Fixation was completed in fresh fixative. Blocks of lungs were embedded in paraffin and cut at 5μ. Multiple sections from each block were stained with hematoxylin and eosin, silver impregnation for reticulin,[4] acid *fuchsin aniline blue trichrome for collagen*,[5] and alcian blue for acid mucopolysaccharides.[6] Histological changes in acid mucopolysaccharides were quantitated, faint trace of stain +, intense reaction +++. Histochemical demonstration of ascorbic acid was done in the lungs and adrenals by the silver nitrate method.[7] Pathological grading of the lesions was assessed according to Belt and King.[3]

Biochemical.—*Collagen Analysis.*—All the lung tissue except that used for histopathological studies was dried at 105 C. Paraffin-embedded blocks and tissue shavings were added to it and the whole treated three times with xylene at 37 C. The mixture was again dried and the dry weight recorded. This represented the total dry weight of lungs without eight to ten tissue sections of 5μ thickness used for sections. Collagen analysis was performed by the method of Stegemann.[8]

Ascorbic Acid Estimation.—Total ascorbic acid was estimated in lungs, adrenals, and blood from the heart by the method of Roe and Kuether[9] as modified by Schaffert and Kingsley.[10]

Results

Mortality of Animals.—Three normal silicotic animals died during intubation and two subsequently, the cause of their death being impossible to ascertain. There were no deaths among the normal controls. Five animals of the scorbutic silicotic group died during intubation and nine subsequently. There were eight deaths among scorbutic control animals (Table 2).

Pathological Findings

Macroscopic Examination of the Lungs.—In experiment 1 the lungs which were inoculated with dust showed small, whitish, pinpoint, dust collections, distributed diffusely at 15 and 30 days; but later the distribution was focal. The lesions were more on the posterior than the anterior surface. The lungs of group 2 were normal. No gross lesion in any other organ was seen.

Gross examination of the animals of experiment 2 revealed subcutaneous hemorrhages. Hemorrhages in the form of small petechiae were observed on the surface of the liver, and the pleural and peritoneal cavities contained plenty of serosanguineous fluid. Besides, lungs inoculated with dust revealed small foci of dust deposits.

Microscopic Examination of the Lungs.—The pathological grading is shown in Table 2.

Microscopic examination of the silicotic animals of experiment 1 at 15 days revealed a moderate, diffuse cellular infiltration of macrophages and thickening of alveolar walls. The macrophages were seen both in the thickened alveolar septa as well as in the lumen of the alveoli. The majority of silica particles were found in the cytoplasm of macrophages, though a few could be seen either free in the alveolar lumen or in the

Table 2.—*Pathological Changes Produced by Quartz in Lungs of Normal Silicotic (Experiment 1) and Scorbutic Silicotic (Experiment 2) Guinea Pigs*

	Normal Silicotic		Normal Control		Scorbutic Silicotic		Scorbutic Control	
Days of Survival	Mode of Death	Grade of Fibrosis	Mode of Death	Grade of Fibrosis	Mode of Death	Grade of Fibrosis	Mode of Death	Grade of Fibrosis
1-15	K*	...	K	...	D‡	...	K	...
	K	...	K	...	K	...	K	...
	K	...			K	...		
16-30	D†	1	K	...	K	1	D	...
	K	1	K	...	K	1	K	...
	K	1	K	...	K	1	K	...
	K	1	K	...				
31-60	K	1	K	...	D‡	...	D‡	...
	K	1	K	...	D	1	D	...
	K	2	K	...	D	1	K	...
			K	...	K	1	K	...
					K	1		
					K	1		
61-90	D	1	K	...	D	1	D	...
	K	2	K	...	D	1	D	...
	K	2	K	...	K	1	K	...
	K	2	K	...	K	1	K	...
					K	1		
91-115	K	2	K	...	D‡	...	D‡	...
	K	3	K	...	D	2	D	...
	K	3	K	...	D	2	D	...
			K	...	K	1	K	...
					K	2	K	...
					K	2		

*Killed.
†Died.
‡Autolysed.

Fig 1.—Loose and fine reticulin at 30 days, in sections of lungs from normal-diet animals injected with 75 mg of quartz dust silver impregnation (grade 1 min, × 75).

Fig 2.—Compact reticulin and definite nodule formation at 60 days, in sections of lungs from normal-diet animals injected with 75 mg of quartz dust silver impregnation (grade 1 max, × 75).

Fig 3.—Thick reticulin and some collagen in nodules at 90 days, in sections of lungs from normal-diet animals injected with 75 mg of quartz dust silver impregnation (grade 2 max, × 75).

Fig 4.—Large amount of collagen with a few coarse and tightly packed reticulin fibers at 115 days, in sections of lungs from normal-diet animals injected with 75 mg of quartz dust silver impregnation (grade 3, × 75).

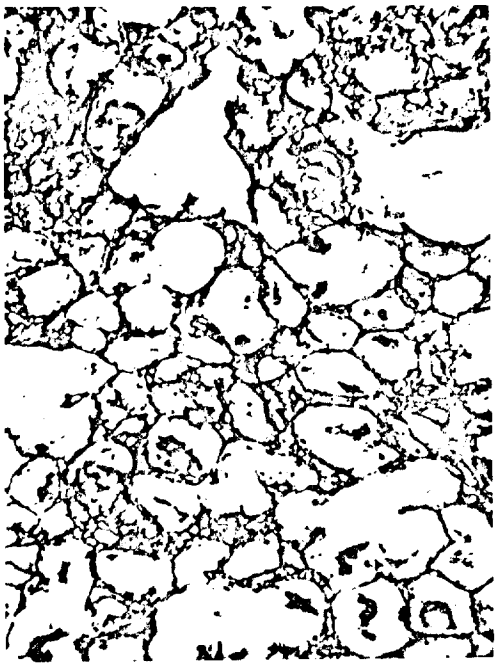


Fig 5.—Fine and diffuse reticulin formation at 30 days, in sections of lungs from scorbutic-diet animals injected with 75 mg of quartz dust silver impregnation (× 185).

Fig 6.—Thicker reticulin fibers at 60 days in sections of lungs from scorbutic-diet animals injected with 75 mg of quartz dust silver impregnation, with no cellular aggregates or reticulin in alveolar lumen (× 185).

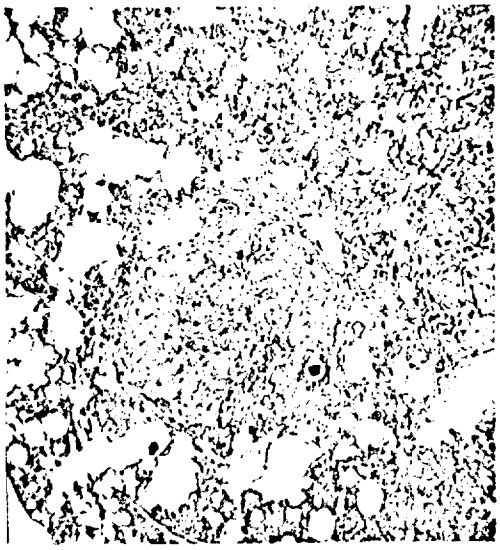
Table 3.—Relation of Total Lung Ascorbic Acid to Developing Silicosis in Normal Guinea Pigs

	Group 1 (Normal Silicotic)		Group 2 (Normal Control)	
Days of Exposure	Ascorbic Acid, mg/100 gm (Wet Weight)	% Collagen	Ascorbic Acid, mg/100 gm (Wet Weight)	% Collagen
0	24.26 ± 0.56 (6)*	7.80 ± 0.12 (6)*	24.26 ± 0.56 (6)*	7.80 ± 0.12 (6)*
15	24.83 ± 1.33 (3)	8.22 ± 0.17 (3)	22.08 ± 1.71 (4)	7.47 ± 1.16 (2)
30	21.50 ± 1.10 (3)	8.92 ± 0.18 (3)	24.79 ± 2.84 (4)	7.62 ± 0.31 (4)
60	26.54 ± 2.06 (3)	9.42 ± 0.48 (3)	27.40 ± 0.70 (3)	7.97 ± 0.26 (4)
90	32.66 ± 1.41 (6)	9.72 ± 0.95 (3)	25.68 ± 2.83 (4)	8.29 ± 0.30 (4)
115	36.47 ± 1.65 (3)	10.70 ± 0.56 (3)	26.57 ± 1.39 (4)	8.01 ± 0.39 (4)

*No. of animals used.

alveolar septa. At 30 days the dust cell reaction had further increased, and the cells were usually found in groups or clusters around blood vessels and bronchioles. In places the dust cell aggregations were found in the lumen of the alveoli which were completely occluded. Slight reticulinosis had commenced in the nodules of dust cells which were seen as tiny fibrils arranged in a loose network (grade 1) (Fig 1). At 60 days the macrophage response of the pulmonary tissue had slightly diminished. The nodules at this stage showed maximum number of dust cells, and their size was comparatively bigger than at 30 days. The reticulin fibers in these nodules had become thicker and tended to be arranged in compact masses (grade 1 maximum) (Fig 2). Collagen fibers made their appearance first at 90 days, and at this stage the nodules were composed of thick reticulin and a few collagen fibers (grade 2) (Fig 3). At 115 days the histopathological picture did not markedly differ from that seen at 90 days except that the number of collagen fibers showed an increase in number, though thick reticulin still persisted (grade 3) (Fig 4). Completely collagenous nodules could not be seen in any of the animals.

Fig 7.—Compact nodules of reticulin fibers at 115 days, in sections of lungs from scorbutic-diet animals injected with 75 mg of quartz dust silver impregnation, with very few collagen fibers (grade 2 min, × 75).



Histochemically ascorbic acid could be seen as a few very minute black granules in the alveolar septa and areas of fibrosis. Ascorbic acid granules were larger and plentiful in the cells of adrenal cortex. Alcian blue positive ground substance was found in the areas of silicotic nodule formation until 90 days, after which it was seen only in traces.

Histopathological examination of silicotic lungs in experiment 2 revealed hemorrhages and extravasation of erythrocytes in the lung parenchyma as early as 15 days, and its intensity increased with progression of the disease. Macrophage response at 15 days was more pronounced and diffuse than in the normal silicotic animals. The dust was distributed evenly throughout the lung parenchyma. The macrophages did not form clumps or aggregates, and, consequently, the reticulin formation first seen at 30 days was

Table 4.—Relation of Total Lung Ascorbic Acid to Developing Silicosis in Scorbutic Guinea Pigs

Days of Exposure	Group 1 (Scorbutic Silicotic) Ascorbic Acid, mg/100 gm (Wet Weight)	Group 1 % Collagen	Group 2 (Scorbutic Control) Ascorbic Acid, mg/100 gm (Wet Weight)	Group 2 % Collagen
0	18.90 ± 0.68 (6)*	8.20 ± 0.17 (6)*	18.90 ± 0.68 (6)*	8.20 ± 0.17 (6)*
15	16.94 ± 1.71 (3)	8.03 ± 0.23 (3)	13.48 ± 1.30 (2)	7.82 ± 0.37 (2)
30	12.56 ± 1.96 (3)	8.35 ± 0.38 (3)	11.35 ± 1.92 (2)	7.43 ± 0.82 (3)
60	12.14 ± 1.03 (3)	8.87 ± 0.14 (3)	9.04 ± 0.83 (2)	8.45 ± 0.60 (2)
90	6.60 ± 0.48 (6)	9.34 ± 0.49 (3)	4.32 ± 0.23 (6)	7.40 ± 0.52 (2)
115	4.92 ± 0.41 (3)	10.89 ± 0.48 (3)	2.26 ± 0.53 (3)	8.32 ± 0.57 (2)

*No. of animals used.

also diffuse (Fig 5). The thickness of reticulin fibers increased slightly at 60 days (Fig 6). The laying down of reticulin continued at 90 days, but no collagen fibers were detected at this stage. The collagen fiber formation was delayed; only a few appeared at 115 days, and these were markedly less than in the normal silicotic animals. The lungs presented the appearance of diffuse reticulinosis. In only a few animals the dust appeared in aggregates and reticulin nodulation occurred (Fig 7).

In scorbutic animals ascorbic acid granules could not be observed either in lungs or adrenals. Alcian blue positive ground substance appeared to be the same in amounts as that of normal silicotic animals. While in the latter it had a tendency to decrease at the termination of the experiment, the quantity in scorbutic silicotic animals remained unaltered.

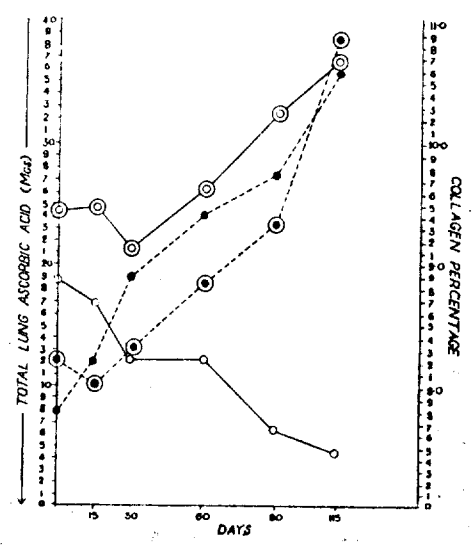
Biochemical Studies.—The mean values of total ascorbic acid and percentage collagen in the lungs of normal silicotic animals are shown in Table 3 and Fig 8.

It was observed that there was a steady increase of ascorbic acid concentration from 30 days in normal silicotic animals ($P < 0.01$); it showed no significant change during early periods and remained significantly higher than in normal controls ($P < 0.05$ at 90 days and $P < 0.01$ at 115 days). Ascorbic acid concentration of normal control animals did not show any change.

Collagen percentage showed an appreciable increase from 15 to 115 days in normal silicotic animals. Comparing 15 days and 30 days with 115 days, the difference is significant ($P < 0.05$). The values were significantly higher than in normal controls ($P < 0.05$ at 15, 30, and 60 days and $P < 0.01$ at 115 days).

The experiment showed a direct relationship between total ascorbic acid concentration and collagen percentage in the lungs of normal silicotic animal, with a steady increase of ascorbic acid concentration, and a

Fig 8.—Relation between ascorbic acid and collagen in lungs of normal silicotic and scorbutic silicotic guinea pigs. **Encircled solid circle** represents scorbutic silicotic collagen. **Solid circle** represents normal slicotic collagen. **Open circle** represents scorbutic silicotic ascorbic acid. **Encircled open circle** represents normal silicotic ascorbic acid.



corresponding increase in percentage of collagen (Fig 8).

Table 4 shows the mean values of total ascorbic acid and collagen percentage in the lungs of scorbutic silicotic animals.

A highly significant decrease of ascorbic acid concentration, both in the scorbutic silicotic and scorbutic control animals, was observed from 15 days ($P < 0.01$) until the termination of the experiment at 115 days. Scorbutic control animals, however, showed a still lower ascorbic acid than the scorbutic silicotic ($P < 0.01$ at 90 days and $P < 0.05$ at 115 days).

Percentage of collagen showed a steady increase in scorbutic silicotic animals, as was seen in experiment 1 with normal silicotic animals.

There was no relationship between ascorbic acid concentration and collagen percentage in the lungs of scorbutic silicotic animals. Increase in collagen percentage was associated with a decrease in the ascorbic acid content (Fig 8).

Total ascorbic acid concentration in adrenals and blood of normal silicotic and scorbutic silicotic animals is depicted in Table 5 and 6.

It was observed that there was a steady increase of adrenal ascorbic acid from 60 days in normal silicotic animals, and its concentration remained significantly higher than in normal controls ($P < 0.01$). Blood ascorbic acid did not reveal any change in normal silicotic and normal control animals.

A highly significant decrease of adrenal ascorbic acid was observed from 15 days onwards, both in scorbutic silicotic and scorbutic control animals ($P < 0.01$). The scorbutic control animals, however, showed sharper decline than the scorbutic silicotic animals ($P < 0.05$ at 90 days). Blood ascorbic acid of scorbutic animals showed a trend similar to that of adrenal ascorbic acid.

Table 5.—*Total Ascorbic Acid Concentration in Adrenals* of Nonscorbutic and Scorbutic Animals During Development of Pulmonary Silicosis*

Days of Exposure	Nonscorbutic		Scorbutic	
	Silicotic	Nonsilicotic	Silicotic	Nonsilicotic
0	81.26 ± 0.51 (6)†	81.26 ± 0.51 (6)†	58.26 ± 0.72 (6)†	58.26 ± 0.72 (6)†
15	82.55 ± 1.45 (3)	78.75 ± 2.52 (4)	28.15 ± 1.31 (3)	22.46 ± 3.22 (2)
30	85.14 ± 1.48 (3)	77.05 ± 4.43 (3)	22.53 ± 2.51 (3)	29.21 ± 1.83 (2)
60	83.54 ± 2.14 (3)	86.00 ± 1.50 (4)	20.11 ± 1.09 (3)	20.13 ± 2.60 (2)
90	91.98 ± 1.72 (6)	77.56 ± 2.84 (4)	12.22 ± 1.32 (6)	7.87 ± 0.66 (6)
115	100.22 ± 4.03 (3)	81.28 ± 4.83 (4)	7.92 ± 1.14 (3)	6.70 ± 0.98 (3)

*Milligrams per 100 gm wet weight.
†No. of animals used.

Comment

The present experiments demonstrate a direct relationship between total ascorbic acid concentration and collagen content in the lungs of silicotic animals kept on normal diet. With an increase of collagen a corresponding increase in ascorbic acid content of lungs and adrenals was noted. An increase in ascorbic acid at sites of collagen formation has been reported in tissues undergoing wound healing.[11] Our experiments show that similar deposits of ascorbic acid also occur in silicotic collagen formation. In silicosis of rats it has been observed also that there is a decrease of ascorbic acid excretion and, therefore, possibly increased retention, which may explain a possible mode of increase of ascorbic acid in the silicotic process.[12] Further, an accelerated synthesis of ascorbic acid has been observed by the action of a number of compounds like chloretone, paraldehyde, and barbiturates[13]; whether it is true of silica requires further investigation. It has been shown also that ascorbic acid is metabolized to respiratory carbon dioxide in guinea pigs.[11] In recent years Gross[15,16] has conclusively shown damage to the respiratory unit of the lung by silica dust. In our experiments this damage may have decreased the metabolism of ascorbic acid and, therefore, increased the concentration in the lung tissue. Wolbach[17] not-

Table 6.—Total Ascorbic Acid Concentration in Blood* (mg/100ml) of Nonscorbutic and Scorbutic Animals During Development of Pulmonary Silicosis

Days of Exposure	Nonscorbutic		Scorbutic	
	Silicotic	Nonsilicotic	Silicotic	Nonsilicotic
0	0.70 ± 0.04 (6)†	0.70 ± 0.04 (6)†	0.58 ± 0.03 (6)†	0.58 ± 0.03 (6)†
15	0.75 ± 0.09 (3)	0.82 ± 0.06 (4)	0.46 ± 0.06 (3)	0.43 ± 0.05 (2)
30	0.59 ± 0.13 (3)	0.69 ± 0.07 (4)	0.33 ± 0.03 (3)	0.26 ± 0.04 (2)
60	0.65 ± 0.07 (3)	0.69 ± 0.08 (4)	0.28 ± 0.05 (3)	0 (2)
90	0.74 ± 0.07 (6)	0.88 ± 0.09 (4)	0 (6)	0 (6)
115	0.78 ± 0.10 (2)	0.69 ± 0.08 (4)	0 (3)	0 (3)

*Milligrams per 100 ml.
†No. of animals used.

ed that the chief function of ascorbic acid in the body is concerned with the formation of intercellular substance. This intercellular substance is produced by fibroblasts as matrix for the formation of reticulin and collagen fibers.[18] It is possible that the increase of ascorbic acid during early periods in our experiment may be for the laying down of the intercellular matrix and, during later periods, for the maintenance of formed collagen. The exact mechanism related to this increase and buildup of ascorbic acid, whether due to increased metabolism, decreased catabolism, or both, is not known and requires further investigation.

It is well recognized that in scurvy there is a general decline in collagen formation.[19-23] In the present study, however, collagen synthesis in scorbutic silicotic animals was more than anticipated. An explanation for this anomaly is to be sought in the following two points: (1) Histologically less collagen was noticed in the lungs of scorbutic animals than in the controls, whereas the reverse was true of reticulin. The increased reticulin formation in scorbutic silicotic animals may be due to the fact that only very small amounts of ascorbic acid are needed for its formation. Danielli et al[24] studied the effects of small doses of ascorbic acid on wound healing and came to the conclusion that on low doses of ascorbic acid large amounts of reticulin could be formed. (2) In the chemical assay based on estimation of hydroxyproline, no distinction is made between reticulin and collagen and, as such, the rise in concentration of the former constituent in scurvy is presumably balanced by a fall in the concentration of collagen per se, giving a picture almost similar to the normal. This is in line with the findings of Gersh,[25] who suggested that in acute scurvy morphological collagen shows a decrease, whereas chemically no difference exists from that found in nonscurvy animals. Further, in vitro studies have also revealed that hydroxyproline synthesis takes place normally in the absence of ascorbic acid.[26]

In scorbutic silicotic animals morphological collagen formation was inhibited but not completely stopped. This is evidenced by the presence of a small number of collagen fibers at 115 days. This confirms the earlier work on collagen formation in sponge implants in which histological examination revealed a fine collagenous capsule around sponge implants in deficient animals and a dense capsule in normal animals.[27] The higher concentration of ascorbic acid in the lungs of silicotic scorbutic animals in comparison to nonsilicotic scorbutic animals suggests that even in ascorbic acid depletion there is an accumulation of ascorbic acid in lungs and adrenals.

Kofoed and Robertson[28] investigated the requirement for ascorbic acid in the synthesis of sulphated glycosaminoglycans and came to the conclusion that ascorbic acid is not essential for chondriotin sulphate synthesis in cartilage. In our experiments the amount of acid mucopolysaccharide ground substance in silicotic scorbutic animals did not differ from that seen in normal silicotic animals; whether the composition of this alcian blue positive ground substance in the two cases was similar remains to be explored. Thus it is concluded that while in normal silicotic animals hydroxyproline synthesis, collagen formation, and ascorbic acid deposition go together, in scorbutic silicotic

animals only hydroxyproline synthesis proceeded at a rate comparable to that of the normal silicotic animals.

After our paper was completed, we came across the excellent research of Chvapil et al, published in *Environmental Research* (1:89 [June] 1967). Dr. Chvapil has worked simultaneously on a different model on the interrelation between ascorbic acid levels and collagen synthesis in lung tissue. We confirm each other's findings.

Quartz powder used in this investigation was supplied by Dr. Atma Ram, Director, Central Glass and Ceramic Research Institute, Calcutta, and its analysis was performed by Dr. Ravi Prakash, Director, Geology and Mining, Lucknow, India.

Mr. Mohammad Waseem helped in biochemical investigations; Mr. Mulkraj provided technical assistance; Mr. Panangat A. George performed the statistical analysis; and Mr. Musleh Ahmad and Mr. Sunil K. Nath were responsible for photomicrography.

References

1. Cartwright, J.: *The Preparation and Assessment of Size Graded Mineral Samples*, Research Report No. 128, UK: Ministry of Fuel and Power, Safety in Mines Research Establishment, Aug, 1956, pp 1-59.

2. Kettle, E.H., and Hilton, R.: The Technique of Experimental Pneumoconiosis, *Lancet* 1:1190-1192 (June 4) 1932.

3. Belt, T.H., and King, E.J.: *Tissue Reactions Produced Experimentally by Selected Dusts From South Wales Coalmines, in Chronic Pulmonary Disease in South Wales Coalminers: III. Experimental Studies*, Special Report Series, No. 250, Medical Research Council, London: His Majesty's Stationery Office, 1945, pp 29-68.

4. Gordon, H., and Sweets, H.H., Jr.: A Simple Method for the Silver Impregnation of Reticulin, *Amer J Path* 12:545-552 (July) 1936.

5. Mcmanus, J.F.A., and Mowry, R.W.: *Staining Methods, Histologic and Histochemical*, New York: Paul B. Hoeber, Inc., Medical Bk. Dept. of Harper & Row, 1965, pp 61-62.

6. Pearse, A.G.E.: *Histochemistry, Theoretical and Applied*, ed 2, Boston: Little, Brown & Co., 1961, p 838.

7. Deane, H.W., and Morse, A.: The Cytological Distribution of Ascorbic Acid in the Adrenal Cortex of the Rat Under Normal and Experimental Conditions, *Anat Rec* 100:127-141 (Jan) 1948.

8. Stegemann, H.: Microdetermination of Hydroxyproline With Chloramine-T and p-Dimethylaminobenzaldehyde, *Z Physiol Chem* 311:41-45, 1958.

9. Roe, J.H., and Kuether, C.A.: 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 (Feb) 1943.

10. Schalfert, R.R., and Kingsley, G.R.: A Rapid, Simple Method for the Determination of Reduced, Dehydro-, and Total Ascorbic Acid in Biological Material, *J Biol Chem* 212:59-68 (Jan) 1955.

11. Abt, A.F.; von Schuching, S.V.; and Roe, J.H.: Connective Tissue Studies: III. Ascorbic Acid, Collagen and Hexosamine Distribution and Histology of Connective Tissue in Scars Produced in Guinea Pigs on Various Vitamin C Dietary Levels Following Wounding by Abdominal Incision, *J Nutr* 70:427-437 (April) 1960.

12. Grubina, A., Jr., et al: Effect of Riboflavin on the Course of Experimental Silicosis in the White Rat, *Vop Pitan* 20:40-45, 1961.

13. Longenecker, H.E.; Fricke, H.H.; and King, C.G.: The Effect of Organic Compounds Upon Vitamin C Synthesis in the Rat, *J Biol Chem* 135:497-510 (Sept) 1940.

14. Burns, J.J.; Burch, H.B.; and King, C.G.: The Metabolism of 1-C^{14}-L-Ascorbic Acid in Guinea Pigs, *J Biol Chem* 191:501-514 (Aug) 1951.

15. Gross, P.: The Alveolar Membrane as a Target Tissue: Morphologic Aspects, *Dis Chest* 41:1[illegible]-172 (Feb) 1962.

16. Gross, P., and Brieger, H.: Silicotic Bronchiolitis Obliterans, *Arch Environ Health* 12:[illegible] (Jan) 1966.

17. Wolbach, S.B.: The Pathologic Changes Resulting From Vitamin Deficiency, *JAMA* 108:7-13 (Jan 2) 1937.

18. Curran, R.C.: Observations on the Formation of Collagen in Quartz Lesions, *J Path Bact* 66:271-282 (July) 1953.

19. Wolbach, S.B., and Howe, P.R.: Intercellular Substances in Experimental Scorbutus, *Arch Path Lab Med* 1:1-24 (Jan) 1926.

20. Penney, J.R., and Balfour, B.M.: The Effect of Vitamin C on Mucopolysaccharide Production in Wound Healing, *J Path Bact* 61:171-178 (April) 1949.

21. Robertson, W., van B., and Schwartz, B.: Ascorbic Acid and the Formation of Collagen, *J Biol Chem* 201:689-696 (Sept) 1953.

22. Gross, J.: Studies on the Formation of Collagen: I. Properties and Fractionation of Neutral Salt Extracts of Normal Guinea Pig Connective Tissue, *J Exp Med* 107:247-263 (Feb) 1958.

23. Gore, I., et al: Aortic Acid Mucopolysaccharides and Collagen in Scorbutic Guinea Pigs, *J Nutr* 87:311-316 (Nov) 1965.

24. Danielli, J.F.; Fell, H.B.; and Kodicek, E.: The Enzymes of Healing Wounds: II. The Effect of Different Degrees of Vitamin C Deficiency on the Phosphatase Activity in Experimental Wounds in the Guinea-Pig, *Brit J Exp Path* 26:367-376 (Dec) 1945.

25. Gersh, J.: *Transactions of the Second Conference on Connective Tissues*, New York: Josiah Macy, Jr., Foundation Publications, 1952, p 15.

26. Woessner, J.F., and Gould, B.S.: Collagen Biosynthesis: Tissue Culture Experiments to Ascertain the Role of Ascorbic Acid in Collagen Formation, *J Biophys Biochem Cytol* 3:685-695 (Sept) 1957.

27. Gould, B.S.: Biosynthesis of Collagen: III. The Direct Action of Ascorbic Acid on Hydroxyproline and Collagen Formation in Subcutaneous Polyvinyl Sponge Implants in Guinea Pigs, *J Biol Chem* 232:637-649 (June) 1958.

28. Kofoed, J.A., and Robertson, W., van B.: Ascorbic Acid and the Synthesis of Chondroitin Sulfate, *Biochim Biophys Acta* 124:86-94 (Oct) 1966.