MONOGRAPH ON ASCORBIC ACID #116 Vol III
COPIES OF ARTICLES CITED BUT NOT USED
IN MONOGRAPH SUMMARY

2C

VOLUME 3

GRAS MONOGRAPH SERIES

ASCORBIC ACID

(COPIES OF ARTICLES CITED BUT NOT USED IN MONOGRAPH SUMMARY)

prepared for

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

OCTOBER 7, 1974

prepared by

Tracor Jitco, Inc.

VOLUME 3

GRAS MONOGRAPH SERIES

ASCORBIC ACID

(COPIES OF ARTICLES CITED BUT NOT USED IN MONOGRAPH SUMMARY)

prepared for

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

OCTOBER 7, 1974

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

prepared by

Tracor Jitco, Inc.

J Clin Invest 1970, 49, 298-307

Autonomic Reflexes and Vascular Reactivity in Experimental Scurvy in Man

FRANÇOIS M. ABBOUD, JAMES HOOD, ROBERT E. HODGES, and HOWARD E. MAYER

From the Department of Medicine, University of Iowa, College of Medicine, Iowa City, Iowa 52240

ABSTRACT Ascorbic acid is a required cofactor in the conversion of dopamine to norepinephrine in vitro, and the deficiency of this vitamin in guinea pigs is associated with degeneration of autonomic ganglion cells and with cardiac supersensitivity to norepinephrine. Because of these findings, we tested the hypothesis that ascorbic acid deficiency in man alters autonomic cardiovascular reflexes and vasomotor responses to adrenergic stimuli. We studied five normal volunteers who had been deprived of ascorbic acid for a period of 3 months; they had developed symptoms and signs of scurvy and their plasma levels of ascorbic acid averaged 0.178 ±SE 0.07 mg/100 ml. We repeated the studies after giving the subjects vitamin C for a period of 4 months; they had become asymptomatic and their plasma ascorbic acid had increased to an average of 1.68 ±0.151 mg/100 ml.

Blood flow to the left forearm (plethysmograph), arterial and central venous pressures, and heart rate were measured before and after exposure of the lower half of the body to subatmospheric levels of pressure and before and after intravenous and intra-arterial (left brachial artery) infusions of norepinephrine and tyramine.

Average values of blood flow (7.9 ±1.4 ml/min per 100 ml), arterial pressure (91.2 ±4.6 mm Hg), heart rate (68 ±4.4 beats/min), central venous pressure (6.1 ±1.1 mm Hg), and plasma catecholamines (0.68 ±0.20 μg/liter) obtained during ascorbic acid deficiency were not altered significantly after correction of the deficiency. Vasoconstrictor responses to intra-arterial norepinephrine and tyramine were augmented after vitamin repletion. During ascorbic acid deficiency, four subjects had reduced responsiveness of resistance vessels of the forearm to lower body negative pressure as compared to the responsiveness observed after vitamin repletion. Reflex tachycardia during lower body negative pressure and reflex bradycardia during the pressor responses to intravenous tyramine and norepinephrine were similar during the two studies.

The results suggest that the decreased vascular responsiveness to intra-arterial norepinephrine and tyramine and to lower body negative pressure during ascorbic acid deficiency is caused by a defect in the ability of resistance vessels to constrict in response to adrenergic stimuli. Ascorbic acid deficiency in man does not interrupt autonomic reflexes and does not appear to cause significant depletion of endogenous norepinephrine.

INTRODUCTION

Several experimental observations indicate that ascorbic acid deficiency may be associated with abnormal cardiovascular responses. In 1947 Lee and Lee (2) demonstrated in the mesenteric vascular bed of scorbutic guinea pigs decreased reactivity of vessels less than 100 μ to topically applied epinephrine. Thoa and Booker (3) reported in 1963 that increases in arterial pressure and in myocardial contractile force in response to norepinephrine were augmented in scorbutic guinea pigs and more recently Thoa, Wurtman, and Axelrod (4) proposed that this supersensitivity to norepinephrine may be related to decreased binding and inactivation of circulating norepinephrine by the heart. On the other hand, observations on atria isolated from scorbutic pigs failed to show supersensitivity to norepinephrine (5).

Ascorbic acid appears to be an essential cosubstrate for the beta-hydroxylation of dopamine to norepinephrine (6, 7) and the work of Sulkin and Sulkin (8) on the ultrastructure of autonomic ganglion cells indicates

This work was presented at the 41st Annual Meeting of the Central Society for Clinical Research, Chicago, 1 November 1968. A preliminary report has appeared in abstract form (1).

Received for publication 29 July 1969 and in revised form 26 September 1969.

that ascorbic acid is necessary for the integrity of these cells.

The foregoing considerations led us to test the hypothesis that ascborbic acid deficiency in man alters cardiovascular reflexes and vasomotor responses to adrenergic stimuli.

METHODS

After approval of appropriate committees of the College of Medicine and of the Board of Supervisors of the State Prison of Fort Madison, Iowa, five healthy prisoners who volunteered for the study were admitted to the metabolic ward where intense medical and nursing supervision were provided. The subjects' diet consisted of a liquid formula which did not contain vitamin C (Table I) and their caloric intake was adjusted so that each subject maintained constant weight throughout the period of depletion. At the end of approximately 90 days, plasma levels of ascorbic acid had decreased markedly and the men had clinical manifestations of scurvy (9) (Table II). This period of depletion will reduce the total body pool of ascorbic acid to less than 20% of normal (10). Repletion was then begun by allowing the subjects to eat a solid diet containing 2.5 mg of ascorbic acid/day and by supplementing the diet with vitamin C in different amounts for each one of the five subjects as shown in Fig. 1. The first study was carried out during the first week of repletion and a second study was carried out approximately 4 months later (Fig. 1).

The subjects were lying lightly clothed and comfortably supine on a couch. Room temperature was 82°F. A polyethylene cannula (1.2 mm OD) was introduced into the left brachial artery and connected through stopcocks to a pressure transducer (Statham Instruments, Inc., Los Angeles, Calif., P23AA) for measurement of arterial pressure and to syringes for infusion of drugs. A 24 in, 17 gauge intracath (C. R. Bard, Inc., Murray Hill, N. J.) was inserted through the right antecubital vein and advanced a distance of approximately 14–18 in for measurement of central venous pressure. The venous cannula was connected to another transducer (Statham P23BB) placed at the level of right atrium and to syringes for intravenous infusion of drugs.

A strain-gauge plethysmograph compensated for temperature variations (11) was used to measure changes in volume of the forearm. The strands were made of Silastic tubing (0.4 mm ID; 0.8 mm OD) (Dow Corning Corp., Midland, Mich.) filled with mercury. The gauge was applied on the left forearm at a tension of 15 g. The elbow was elevated on foam-padded sand bags and the forearm was slightly flexed and inclined at 45° to the horizontal plane with the wrist and hand supported by a padded sling. In this position, the veins of the forearm were essentially collapsed and could be distended freely when venous congestion was produced. All measurements were recorded on a direct writing oscillograph (Sanborn Co., Waltham, Mass.). A pneumatic cuff was placed around the distal part of the forearm and inflated to suprasystolic pressures during the measurements. This cuff arrested the circulation to the hand and excluded the contribution of the venous return from the hand to changes in volume of the forearm. A second pneumatic cuff

TABLE I

*Vitamin C–Free Synthetic Formula**

Ingredients	Intake
	g/day
Vitamin-free casein (92% protein)	122
Sucrose or Milo oxidized starch	338
Peanut oil	64
Cocoa butter	56
Safflower oil	13
Iodized salt	5
Basic mineral and crystine mixture	
Basic vitamin mixture (Vitamin C–free)	
H_2O (1000–1500 ml)	

* 3000 Calories: 15% Protein, 40% Fat, and 45% Carbohydrate. This liquid formula was given through a nasogastric tube. Additional details concerning the composition of the formula with respect to minerals and vitamins have been published (9).

TABLE II

Manifestations of Scurvy at the Time of the First Study

	Subjects				
	H	Mc	R	P	S
Hyperkeratosis		++	+++	++	+
Follicular congestion		++	+	+	+
Gum swelling				++	++
Petechial and other hemorrhages	+++			+	
Joint effusion	++		+		
Plasma ascorbic acid (mg/100 ml)*	0.12	0.46	0.11	0.07	0.12

The + signs represent a clinical assessment of the severity of the manifestation.

* After repletion of ascorbic acid over a period of 4 months at the time of the second study (session II), the manifestations had disappeared and the plasma levels of ascorbic acid were 1.84, 1.27, 2.06, 1.38, and 1.84 mg/100 ml for subjects H, Mc, R, P, and S respectively. The rise in plasma levels of vitamin C did not appear to correlate with the amount of supplementation. As shown in Fig. 1, subjects H and P received a supplement of ascorbic acid of 4 mg/day, subjects R and S received 64 mg, and subject Mc received 128 mg.

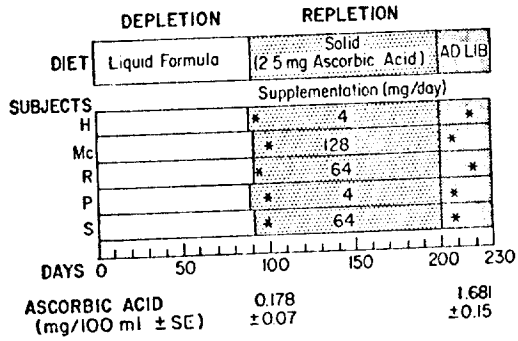


FIGURE 1 The asterisks indicate the times at which the two studies were carried out in each subject. The dietary intake of ascorbic acid was supplemented during the phase of repletion by different daily amounts in each subject as shown in the figure.

TABLE III

Responses to Lower Body Negative Pressure (LBNP) during the Scorbutic

Subject		FBF					BP				
			During LBNP					During LBNP			
		C	−20	−40	−60	After	C	−20	−40	−60	After
		ml/min per 100 ml					*mm Hg*				
H	I	9.3	7.8	7.2	5.3	7.6	102	102	102	100	105
	II	7.2	5.2	6.6	5.4	9.2	107	110	110	100	[illegible]
Mc	I	4.9	3.0	3.1	1.3	9.8	75	73	53	27	[illegible]
	II	4.8	3.3	3.0	2.8	9.3	75	70	70	68	[illegible]
R	I	5.8	3.7	3.1	2.0	9.5	97	95	85	62	68
	II	5.3	3.1	4.3	2.9	8.6	92	88	87	80	80
P	I	12.8	7.4	6.7	7.5	15.5	92	94	95	92	92
	II	9.5	6.6	8.1	8.5	19.8	75	77	79	70	80
S	I	6.6	4.3	4.3	3.9	11.2	90	90	90	77	80
	II	5.2	4.0	4.1	2.8	8.7	97	95	94	78	90
Mean ±SE	I	7.9	5.2*	4.9*	4.0*	10.7*	91	91	85	72*	82
		1.4	1.0	0.9	1.1	1.3	4.6	4.8	8.5	12.9	7.5
	II	6.4	4.4*	5.2*	4.5*	11.0*	89	88	88	79*	83
		0.9	0.6	0.9	1.1	2.2	6.3	7.0	6.8	5.7	5.8

Entries represent blood flow to the forearm (FBF), mean arterial blood pressure (BP), calculated vascular resistance in the forearm (Res.), and heart rate (HR). Resistance was estimated from the quotient (arterial pressure − venous pressure)/(forearm blood flow). The values shown were obtained before LBNP (C), during LBNP at −20, −40, and −60 mm Hg, and at approximately 30 sec after LBNP (After). Each value of FBF represents data from 3 to 6 flow curves. Central venous pressure averaged 6.1 ±1.1, 2.8 ±1.1, 1.8 ±0.8, and 1.3 ±0.7 before and during LBNP at −20, −40, and −60 mm Hg respectively in session I; corresponding values in session II were 6.4 ±0.7, 2.3 ±0.6, 1.4 ±0.5, and 0.7 ±0.6.

* The average values were significantly different from control observations by paired *t* test analyses ($P < 0.05$). Average differences between session I and II were not statistically significant. Immediately after LBNP there was a vasodilator response in the forearm which was transient (Fig. 2); all values returned to control levels within 1–1½ min.

was placed around the arm proximal to the plethysmograph for venous occlusion. Changes in forearm volume produced by sudden inflation of the venous occlusion cuff were used to measure the rate of blood flow to the forearm (12) (Fig. 2).

A suction box was applied around the lower half of the body to the level of the anterior-superior iliac spine and a plastic sheet enveloped it to provide the seal necessary for maintaining subatmospheric levels of pressure within the box. The box was connected to the hose of a commercial vacuum cleaner and the degree of suction was regulated by adjusting the opening of a slot on the side of the connecting hose. The lower half of the body could thus be exposed to subatmospheric levels of pressure at −20, −40, or −60 mm Hg for any desired period of time.

After the subjects had achieved a stable resting state, blood flow to the forearm, arterial pressure, central venous pressure, and heart rate were measured in response to lower body negative pressure (LBNP) and to intra-arterial and intravenous infusions of norepinephrine and tyramine. After obtaining control values, suction was applied to create a negative pressure of 20 mm Hg for 3 min; the level of suction was then increased to achieve a pressure of −40 mm Hg for a period of 3 min and −60 mm Hg for 3 additional min before discontinuing suction. Arterial blood samples for measurements of blood gases, pH, hematocrit, and blood levels of catecholamines were obtained before LBNP and during the last 3 min of LBNP. Responses were also measured during the intra-arterial infusion of two doses of norepinephrine (0.002 and 0.004 μg/kg per min), the intra-arterial infusion of two doses of tyramine (0.3 and 0.6 μg/kg per min), the intravenous infusion of two or three doses of norepinephrine (0.0375, 0.075, and 0.150 μg/kg per min), and finally the intravenous infusion of two doses of tyramine (12.5 and 25 μg/kg per min). The graded doses of each drug were infused consecutively each for 3 min. Resting periods of 15 min were allowed between each set of infusions. The doses of norepinephrine are given in terms of the base of *l*-norepinephrine bitartrate which was diluted in 5% glucose in water and infused at rates of 0.6 and 1.2 ml/min. The dose of tyramine was given in terms of the salt tyramine sulfate. Responses to the interventions were calculated from observations made during the last minute of each intervention.

Phase (Session I) and after Repletion of Ascorbic Acid (Session II)

Res.					HR				
	During LBNP					During LBNP			
C	−20	−40	−60	After	C	−20	−40	−60	After
		U					*beats/min*		
9.9	12.2	13.5	18.1	12.3	65	60	60	90	60
14.9	21.2	16.7	18.5	10.9	65	85	100	105	55
14.0	23.8	16.8	20.4	5.9	80	90	108	100	90
13.8	20.2	22.7	24.1	6.2	75	75	80	90	80
15.8	24.9	26.9	30.6	6.5	65	75	85	102	70
16.4	27.3	19.6	26.6	8.7	70	80	85	90	80
6.8	12.5	14.0	12.1	5.6	55	70	75	90	70
7.3	11.4	9.6	8.3	3.8	75	80	90	110	80
13.1	20.8	20.8	19.7	7.0	75	90	105	155	90
17.5	23.6	22.8	27.6	9.8	90	95	115	155	110
11.9	18.8*	18.4*	20.2*	7.5	68	77	87*	107*	76
1.6	2.7	2.5	3.0	1.2	4.4	5.8	9.1	12.2	6.0
14.0	20.7*	18.3*	21.0*	7.9*	75	83	94*	110*	81
1.8	2.6	2.4	3.6	1.3	4.2	3.4	6.2	11.9	8.7

RESULTS

Each patient had more than one manifestation of ascorbic acid deficiency at the time of the first study and the plasma levels of ascorbic acid were markedly reduced (Table II). At the time of the second study, the clinical manifestations of scurvy had disappeared and plasma levels of ascorbic acid had increased.

Resting values of arterial blood pressure, heart rate, central venous pressure, and blood flow to the forearm were similar in the first and second sessions (Tables III, V and Figs. 3 and 4).

Effect of lower body negative pressure. During session I, LBNP caused a sensation of fainting in two of the five subjects whose mean blood pressures fell by 35 mm Hg (R) and 48 mm Hg (Mc). The other three subjects had little or no reduction in arterial pressure. During the second session, the largest reduction in arterial pressure was 19 mm Hg (S) (Table III). Vascular resistance of the forearm increased during LBNP but the average resistance attained over the 9 min period of suction was lower in three of the five subjects (S, Mc, and H) in the first session as compared to the second session.

The tachycardia and the reductions in central venous pressure were similar in the two sessions.

The values of arterial P_{O_2}, P_{CO_2}, pH, hematocrit, and plasma catecholamine concentrations are in Table IV. There were no consistent differences in these values during the two sessions, both before and during LBNP. A slight reduction in P_{CO_2} took place during LBNP in both sessions.

Responses to intra-arterial infusions of norepinephrine and tyramine. The doses used were insufficient to cause any change in systemic arterial blood pressure or heart

rate but reductions in blood flow to the forearm were apparent. The responses to both norepinephrine and tyramine were less in session I than in session II (Tables V and VI).

Responses to intravenous infusions of norepinephrine and tyramine. Norepinephrine caused a rise in arterial pressure, a decrease in forearm blood flow, an increase in forearm vascular resistance, and bradycardia in each of the five subjects during session I (Fig. 3). After repletion of ascorbic acid, responses tested in only four of the five subjects were not altered consistently.

During session I tyramine caused a rise in arterial pressure with some increase in blood flow to the forearm, a small increase in forearm vascular resistance, and a decrease in heart rate. These responses were not altered consistently in session II (Fig. 4).

DISCUSSION

We are reporting in this paper one of several studies which were carried out on five scorbutic men. One of the studies included an evaluation of the tolerance of these men to a cold environment for several hours. They had been deprived of ascorbic acid for a period of 84–97 days and had just started their repletion regimen when one of us (J. H.) observed that they had an unusual degree of edema of the feet and cyanosis when exposed to the cold. This observation led us to postulate that there may be some alteration in vascular reactivity in human scurvy. After review of the literature, we found several reports which would support the hypothesis that ascorbic acid deficiency might cause an interruption of autonomic cardiovascular reflexes and in particular the adrenergic component of the reflexes. Goldstein, Lauber, Blumberg, and Peisach (6), and Friedman and Kaufman (7) had demonstrated that ascorbic acid was an important cosubstrate involved in the enzymatic synthesis of norepinephrine. The third and last step in the conversion of tyrosine to norepinephrine is the hydroxylation of dopamine which requires copper and the reducing properties of ascorbic acid.

$$\text{Dopamine} + \text{ascorbate} + O_2 \xrightarrow{\text{fumarate}} \text{norepinephrine} + H_2O + \text{dehydroascorbate (7).}$$

On the basis of this finding one may postulate that the rate of synthesis of norepinephrine from dopamine may be reduced in the absence of ascorbic acid. Although defective biosynthesis has not been demonstrated so far in animals deprived of ascorbic acid, Thoa, Wurtman, and Axelrod noted reductions in the catecholamine content of the myocardium of scorbutic guinea pigs (4). Another observation which is probably unrelated to synthesis of the amine is that of Sulkin and Sulkin (8) who demonstrated significant degenerative changes in the ultrastructure of autonomic ganglia of scorbutic guinea pigs; these changes appeared to be specific to the deficiency of this vitamin.

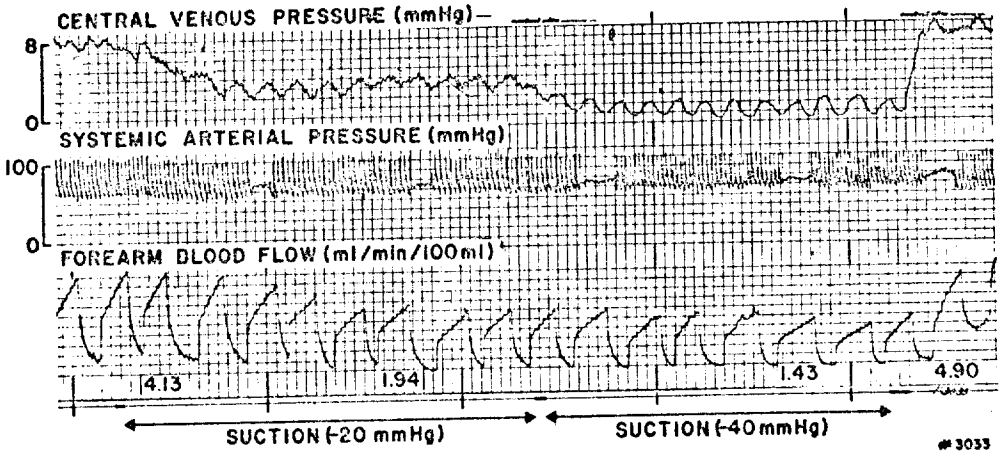


FIGURE 2 Effect of lower body negative pressure (LBNP) on central venous pressure, systemic arterial pressure, and forearm blood flow. For the purpose of this illustration suction was applied at −20 and −40 mm Hg for a total period of approximately 3 min. The fall in central venous pressure and the reduction in blood flow to the forearm were related to the level of negative pressure. The numbers under the flow curves represent the corresponding values of blood flow calculated in ml/min per 100 ml of forearm volume. In this experiment mean arterial pressure was unchanged by LBNP but heart rate increased at −40 mm Hg. Immediately after the end of suction, blood flow to the forearm increased to levels that were higher than control for a period of approximately 30 sec. The distance between two vertical lines represents an interval of 2 sec.

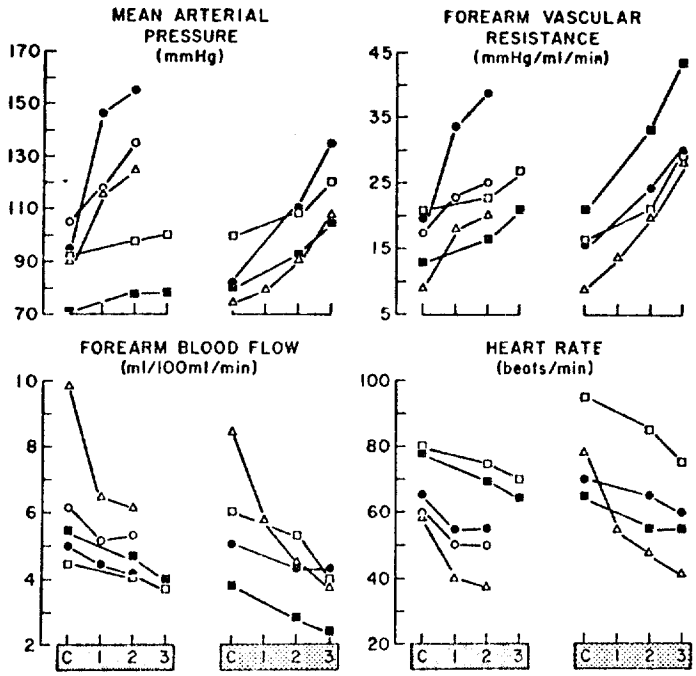


FIGURE 3 Responses to intravenous administration of norepinephrine. Each symbol represents a different subject. C refers to control observations before the infusion and 1, 2, and 3 refer to the three doses used (0.0375, 0.075, 0.15 μg/kg per min). The values to the left of each graph were obtained during the scorbutic phase (session I) and those to the right were obtained during session II after repletion of ascorbic acid.

We reasoned that if the biochemical and pathophysiological defects which were observed in vitro and in vivo in the guinea pigs occurred also in man we should be able to identify a defect in cardiovascular reflexes in the scorbutic subjects. Such a defect would not necessarily explain the edema and cyanosis that were observed in the cold; but because of its importance and the scarcity of studies on vascular responsiveness in human scurvy, we decided to evaluate the integrity of adrenergic responses in these five subjects who had already become scorbutic. This was done by testing the effects of pooling of blood in the lower extremities and of intra-arterial and intravenous norepinephrine and tyramine. Exposure of the lower half of the body to subatmospheric pressures

TABLE IV

Blood Gases, pH, Hematocrit, and Plasma Catecholamines in Arterial Blood before (C) and during LBNP (−60 mm Hg) in the Scorbutic Phase (I) and after Repletion of Ascorbic Acid (II)

		P_{O_2}		P_{CO_2}		pH		Hematocrit		Plasma catecholamines	
		C	LBNP	C	LBNP	C	LBNP	C	LBNP	C	LBNP
		mm Hg		*mm Hg*		*U*		*per cent*			
Mean ±SE	I	72.6	62.2	37.4	33.3*	7.46	7.47	43.4	43.9	0.68	0.54
		4.8	10.8	0.8	1.4	0.011	0.017	2.6	2.2	0.20	0.17
	II	77.8	80.2	35.1	32.4*	7.45	7.45	44.9	44.9	0.43	0.43
		4.8	4.7	0.9	0.6	0.001	0.006	2.6	2.3	0.05	0.03

* The average values were significantly different from control (paired *t* test). Values in sessions I and II were not statistically different. The reduction in P_{CO_2} may reflect some degree of hyperventilation during LBNP.

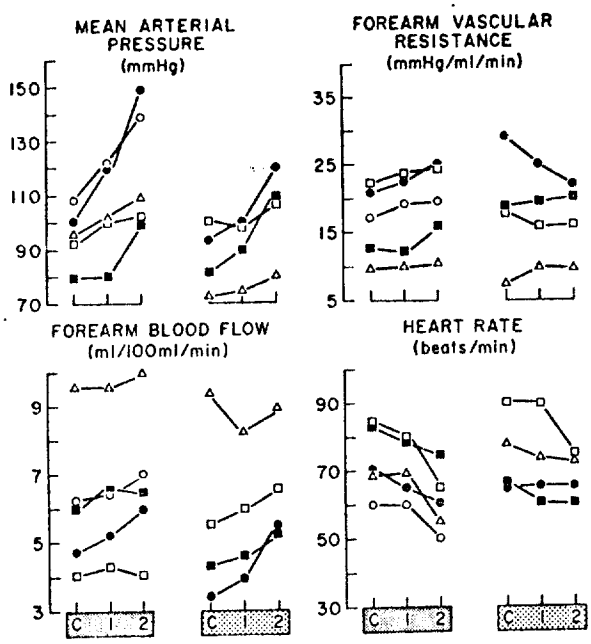


FIGURE 4 Responses to intravenous administration of tyramine (12.5 and 25 μg/kg per min). See legend to Fig. 3.

causes pooling of blood in the lower parts, decreases central venous pressure, and triggers reflex tachycardia and reflex vasoconstriction in the forearm. The responses are similar to those seen during upright tilt (13); they reflect to a major degree the integrity of the autonomic nervous system and they are abolished in autonomic neuropathy (14, 15). When endogenous catecholamines are depleted, the cardiovascular effects of norepinephrine may be augmented (16) but those of tyramine are reduced since tyramine acts through the release of endogenous catecholamines (17–19). Thus, the relative responses to norepinephrine and tyramine during ascorbic acid deficiency and after its correction provide an indirect index of endogenous catecholamines in these two states.

The results indicate that during the first session when plasma levels of ascorbic acid were negligible and the clinical manifestations of scurvy were evident tachycardia resulted from pooling of blood in the lower extremities and bradycardia was triggered by elevation of arterial pressure with intravenous norepinephrine and tyramine. Similar responses were seen 4 months later during the second session after full repletion of the vitamin and disappearance of all signs and symptoms of the deficiency suggesting that autonomic innervation of the heart was not affected by the vitamin deficiency.

The findings with intra-arterial norepinephrine and tyramine would not support the concept that there is significant depletion of endogenous catecholamines during experimental scurvy in man. Had there been depletion, the vasoconstrictor response to tyramine would have been reduced and the response to norepinephrine would have been augmented, and this differential effect of the two sympathomimetic amines would have been reversed after repletion of ascorbic acid. The results indicate, however, that the responses to both norepinephrine and tyramine were reduced during ascorbic acid deficiency. Thus, although the vasoconstrictor effect of intra-arterial tyramine was reduced in session I as compared to session II, this reduction does not indicate depletion of catecholamines since the vasoconstrictor effect of intra-arterial norepinephrine, which acts directly on adrenergic alpha receptors, was also reduced in session I. The decreased responsiveness should therefore be attributed to either a nonspecific reduction in contractility of vascular smooth muscle in scurvy or a diminution in responsiveness to adrenergic stimuli. In their studies on mesenteric vessels of scorbutic guinea pigs, Lee and Lee reported decreased responsiveness of venules and arterioles less than 100 μ in topical applications of epinephrine; the responsiveness of larger vessels was not altered (2).

The levels of arterial pressure and of forearm vascular resistance which were attained during LBNP also suggest that the responsiveness of resistance vessels of the forearm to LBNP was less in session I than in session II in four of the five subjects. Two subjects (R and Mc) had marked reductions in arterial blood pressure accompanied by a sensation of dizziness and fainting during LBNP in session I. These effects were not seen after repletion of ascorbic acid; and although the levels of vascular resistance of the forearm were not consistently lower during LBNP in session I in these two subjects, the more pronounced hypotension would indicate that vascular tone was less during LBNP in session I than in session II. Two other subjects (S and H had lower levels of vascular resistance associated with slightly lower levels of pressure during LBNP in session I than in session II suggesting also that vascular tone was less during LBNP in the first session.

The constrictor effects of intravenous norepinephrine and tyramine on resistance vessels of the forearm were not augmented consistently after repletion of ascorbic acid as were the responses to the intra-arterial infusions. It should be mentioned in this connection that responses to intravenous infusions include in addition to the direct action of the drugs on blood vessels a possible central action or myogenic and neurogenic reflex effects which may either mask, modify, or override any direct effects.

Deprivation from ascorbic acid was maintained for a period of 3 months. It might be argued that this period was insufficient to induce scurvy in view of previous

studies in which the deficiency had to be maintained for more than 4 months before the appearance of symptoms and signs of scurvy (20–23). In this as in previous studies by our group (9, 10, 24), the 3 month period was sufficient to decrease the total body pool of ascorbic acid to less than 20%, to decrease the plasma levels to less than 0.1 mg/100 ml in most subjects and to cause definite clinical manifestations of scurvy. Rigid dietary restriction may have been a factor in our ability to induce scurvy in a shorter period of time than was possible in earlier studies.

In the present experiments, session I was held 3–10 days after the beginning of repletion of ascorbic acid rather than at the end of the depletion phase. The reason was that the clinical observation which led to the studies on vascular responsiveness was made at the end of the period of depletion and during the first few days of repletion as was mentioned in the foregoing text. The short period of repletion did not affect the scorbutic state of these men; and at the time of the first study, the clinical manifestations were as pronounced as on the last day of depletion and the plasma levels of ascorbic acid were still very low. Subject Mc who had had the largest amount of supplemental ascorbic acid at the time of the first study also had the greatest reduction in vasoconstrictor responses to LBNP as compared to responses obtained 4 months later.

The degree to which the vasoconstrictor responses were augmented after the 4 month repletion period did not appear to correlate with the dose of supplemental ascorbic acid that had been administered; a dose of 10 mg/day or less may well exceed the requirements for maintaining or restoring "normal" vasomotor responsiveness.

Perhaps the most important conclusion which can be drawn from these findings is that experimental scurvy in man is not accompanied by the physiologic or pharmacologic responses which would be associated with degeneration of autonomic ganglia or with defective synthesis of norepinephrine. The in vitro biochemical findings and the observations made on autonomic ganglia of scorbutic guinea pigs may not be transferable to experimental human scurvy. In this regard, however, one should mention that enzymatic beta-hydroxylation of dopamine to norepinephrine is not a rate-limiting step in the synthesis of the neuromediator (25), and there-

TABLE V

Effect of Intra-Arterial Infusions of Norepinephrine and Tyramine on Blood Flow to the Forearm (ml/min per 100 ml) during the Scorbutic Phase (I) and after Repletion of Ascorbic Acid (II)

Subject		C	Norepinephrine		C	Tyramine	
			μg/kg per min			*μg/kg per min*	
			0.002	*0.004*		*0.3*	*0.6*
H	I	7.5	3.3	2.8	7.8	5.5	4.0
	II	9.8	2.5	1.4	8.1	5.3	4.9
Mc	I	4.3	2.6	2.1	5.3	4.2	2.9
	II	4.3	2.0	1.4	4.2	2.3	1.2
R	I	5.2	2.9	2.4	5.7	3.6	2.3
	II	4.0	1.0	0.8	5.9	2.5	1.8
P	I	11.3	5.1	2.0	11.3	7.6	5.2
	II	7.0	1.6	1.5	6.2	4.0	3.5
S	I	4.7	4.3	3.5	4.3	3.8	3.3
	II	4.0	1.7	1.8	4.1	2.2	1.8
Mean ±SE	I	6.60	3.64*	2.56*	6.88	4.94*	3.54*
		1.29	0.46	0.27	1.24	0.74	0.50
	II	5.82	1.76*	1.38*	5.70	3.26*	2.64*
		1.14	0.24	0.16	0.74	0.60	0.68
P values‡ Ivs/II		>0.05	<0.005		>0.05	<0.05	

* Indicates that the values during infusions were significantly different from control ($P < 0.05$ by paired t test).

‡ Indicates the probability values for the statistical significance of the differences between sessions I and II which were calculated by paired t test for control values (C) and by analysis of variance (Table VI) for values obtained during the infusions.

TABLE VI
Analysis of Variance of Responses to Intra-Arterial Norepinephrine and Tyramine in the Two Sessions

Source of variation	Degrees of freedom	Norepinephrine Mean square	Norepinephrine F	Tyramine Mean square	Tyramine F
Subjects	4	0.68	1.67	6.62	12.97*
Sessions	1	11.76	29.18*	8.32	16.31*
Doses	1	2.56	6.35‡	5.10	10.00‡
Sessions × Doses	1	0.66	1.64	0.76	1.49
Error	12	0.403		0.510	

* Indicates $P < 0.005$ and the absence of a symbol indicates that the F value was not statistically significant (Snedecor, G. W. 1956. Statistical Methods Applied to Experiments in Agriculture and Biology, Iowa State University Press, Ames, edition 5). The significance of the variation ascribed to "Sessions" indicates that responses to the sympathomimetic amines were different in the two sessions and the significance of the variation of "Doses" indicates that there was a dose-related effect. The lack of significance of the variation ascribed to Sessions × Doses interactions indicates that the dose-response curves in the two sessions were parallel.
‡ Indicates $P < 0.05$.

fore, a deficiency of ascorbic acid which is important for that step might not be expected to result in significant reductions in the synthesis of endogenous norepinephrine. In guinea pigs, scurvy may be induced within 2–3 wk and the metabolic pathways of ascorbic acid are different in pigs and in man so that the pathophysiology of scurvy may be different in the two species.

ACKNOWLEDGMENTS

This study was supported by research grants from the American and Iowa Heart Associations, the U. S. Public Health Service (HE-09835, HE-02644, and TI-AM5306), by a U. S. Army Medical Research and Development Command Contract (DADA17-67-C-7111), and by a Research Career Program Award HE-K3-17013 from the U. S. Public Health Service.

REFERENCES

1. Abboud, F. M., J. Hood, and R. E. Hodges. 1968. Vascular reactivity in experimental scurvy in man. *J. Lab. Clin. Med.* **72**: 849. (Abstr.)
2. Lee, R. E., and N. Z. Lee. 1947. The peripheral vascular system and its reactions in scurvy: an experimental study. *Amer. J. Physiol.* **149**: 465.
3. Thoa, N. B., and W. M. Booker. 1963. Cardiovascular dynamics in normal and scorbutic guinea pigs infused with dopamine and norepinephrine. *Fed. Proc.* **22**: 448. (Abstr.)
4. Thoa, N. B., R. J. Wurtman, and J. Axelrod. 1966. A deficient binding mechanism for norepinephrine in hearts of scorbutic guinea pigs. *Proc. Soc. Exp. Biol. Med.* **121**: 267.
5. Bhagat, B., W. L. West, and I. M. Robinson. 1966. Sensitivity to norepinephrine of isolated atria from scorbutic guinea pigs. *Biochem. Pharmacol.* **15**: 1637.
6. Goldstein, M., E. Lauber, W. E. Blumberg, and J. Peisach. 1965. Dopamine-β-hydroxylase—a copper enzyme. *Fed. Proc.* **24**: 604. (Abstr.)
7. Friedman, S., and S. Kaufman. 1965. 3,4-Dihydroxyphenylethylamine β-hydroxylase: a copper protein. *J. Biol. Chem.* **240**: PC552.
8. Sulkin, D. F., and N. M. Sulkin. 1967. An electron microscopic study of autonomic ganglion cells of guinea pigs during ascorbic acid deficiency and partial inanition. *Lab. Invest.* **16**: 142.
9. Hodges, R. E., E. M. Baker, J. Hood, H. E Sauberlich, and S. C. March. 1969. Experimental scurvy in man. *Amer. J. Clin. Nutr.* **22**: 535.
10. Baker, E. M., R. E. Hodges, J. Hood, H. E. Sauberlich, and S. C. March. 1969. Metabolism of ascorbic-1 ^{14}C acid in experimental human scurvy. *Amer. J. Clin. Nutr.* **22**: 549.
11. Whitney, R. J. 1953. The measurement of volume changes in human limbs. *J. Physiol. (London).* **121**: 1.
12. Wood, J. E. 1965. The Veins; Normal and Abnormal Function. Little, Brown and Company, Boston. 62–65.
13. Brigden, W., S. Howarth, and E. N. Sharpey-Schafer. 1950. Postural changes in the peripheral blood flow of normal subjects with observations on vasovagal fainting reactions as a result of tilting, lordotic posture, pregnancy and spinal anesthesia. *Clin. Sci.* **9**: 78.
14. Mason, D. T., I. J. Kopin, and E. Braunwald. 1966. Abnormalities in reflex arterial and venous constriction to changes in posture in familial dysautonomia. *Circulation.* **34** (Suppl. 3): 164.
15. Abboud, F. M., and J. W. Eckstein. 1966. Autonomic defect in orthostatic hypotension. *J. Lab. Clin. Med.* **68**: 851. (Abstr.)
16. Burn, J. H., and M. J. Rand. 1958. The action of sympathomimetic amines in animals treated with reserpine. *J. Physiol. (London).* **144**: 314.
17. Trendelenburg, U. 1961. Modification of the effect

of tyramine by various agents and procedures. *J. Pharmacol. Exp. Ther.* **134**: 8–17.

18. Abboud, F. M., and J. W. Eckstein. 1964. Effects of small oral doses of reserpine on vascular responses to tyramine and norepinephrine in man. *Circulation.* **29**: 219.
19. Abboud, F. M., and J. W. Eckstein. 1964. Venous and arterial responses to norepinephrine in dogs treated with reserpine. *Amer. J. Physiol.* **206**: 299.
20. Medical Research Council. Vitamin-C subcommittee of the accessory food factors committee. R. A. Peters, Chairman. 1948. Vitamin-C requirement of human adults. Experimental study of vitamin-C deprivation in man. Preliminary report. *Lancet.* **1**: 853.
21. Crandon, J. H., C. C. Lund, and D. B. Dill. 1940. Experimental human scurvy. *N. Engl. J. Med.* **223**: 353.
22. Farmer, C. J. 1944. Some aspects of vitamin C metabolism. *Fed. Proc.* **3**: 179.
23. Pijoan, M., and E. L. Lozner. 1944. Vitamin C economy in the human subject. *Bull. Johns Hopkins Hosp.* **75**: 303.
24. Hood, J., and R. E. Hodges. 1969. Ocular lesions in scurvy. *Amer. J. Clin. Nutr.* **22**: 559.
25. Nikodijevic, B., C. R. Creveling, and S. Udenfriend. 1963. Inhibition of dopamine beta-oxidase *in vivo* by benzyloxyamine and benzylhydrazine analogs. *J. Pharmacol. Exp. Ther.* **140**: 224.

Physiological Studies of Gamma-Irradiated Tomato Fruits. III. Effects on Ascorbic Acid Content, Acidity and Texture[1,2]

By Adel S. Abdel-Kader,[3] Leonard L. Morris and E. C. Maxie,
University of California, Davis

Abstract. Gamma irradiation of tomato fruits reduces their ascorbic acid content, in greater proportion in pink and table-ripe than in mature-green fruits. Final total ascorbic acid, however, is higher in irradiated pink and table-ripe fruits than in subsequently ripened irradiated mature-green fruit, because of a higher initial concentration of the vitamin.

Irradiated tomatoes had a lower total acidity and higher pH than unirradiated fruit. Soluble solids content was not affected by irradiation, whereas firmness was reduced markedly.

Introduction

Possible changes in nutritive value must be considered in evaluating the commercial feasibility of irradiation of fruits. Such changes have been summarized by Maxie and Abdel-Kader (17).

Sampling procedures must receive careful attention since the chemical composition of tomatoes is affected by many preharvest and postharvest phenomena, and may vary in morphologically comparable fruits. Scott and Kramer (29) observed that the ascorbic acid content of tomatoes increases with advancing ripeness, and that vine-ripened fruits have a higher content than artificially ripened ones. They reported a loss of ascorbic acid during storage of green tomatoes at 70°F and of riper fruit at 35 and 50°. Craft and Heinze (5) found at least 8% higher ascorbic acid concentration in red-ripe tomatoes in the greenhouse than in yellow-red fruits on the same clusters. There was no difference between yellow-red and mature-green tomatoes.

Proctor et al. (24, 25) reported that ascorbic acid is one of the more radiosensitive vitamins. There is a wide variation in the reported ascorbic acid content of gamma-irradiated fruits. Doses in the range of 200-500 Krad reduced the ascorbic acid content of tomatoes (31). The reported destruction of ascorbic acid in strawberries by gamma irradiation varies from 0% at 500 Krad (6) to 3–16% at 200 Krad (19) and 81% at 400 Krad (2). Mathur and Lewis (16) found a 40% loss in mangos subjected to 12 Krad. Maxie et al. (19) found no differences in ascorbic acid content between orange fruits subjected to 0 and 200 Krad even after 95 days at 0°C. In contrast, they found that lemons irradiated with 100, 200, 300, and 400 Krad respectively contained 94, 29, 10, and 5% as much ascorbic acid

[1]Received for publication June 18, 1968.

[2]Based on a portion of a dissertation submitted by the senior author to the Graduate Division, University of California, Davis, in partial satisfaction of the requirements for the Ph.D. degree (June, 1966). Research supported by the United States Atomic Energy Commission, Contract UCD-34P80.

[3]Present address: Department of Plant Production, College of Agriculture, Ein Shams University, Cairo, Egypt.

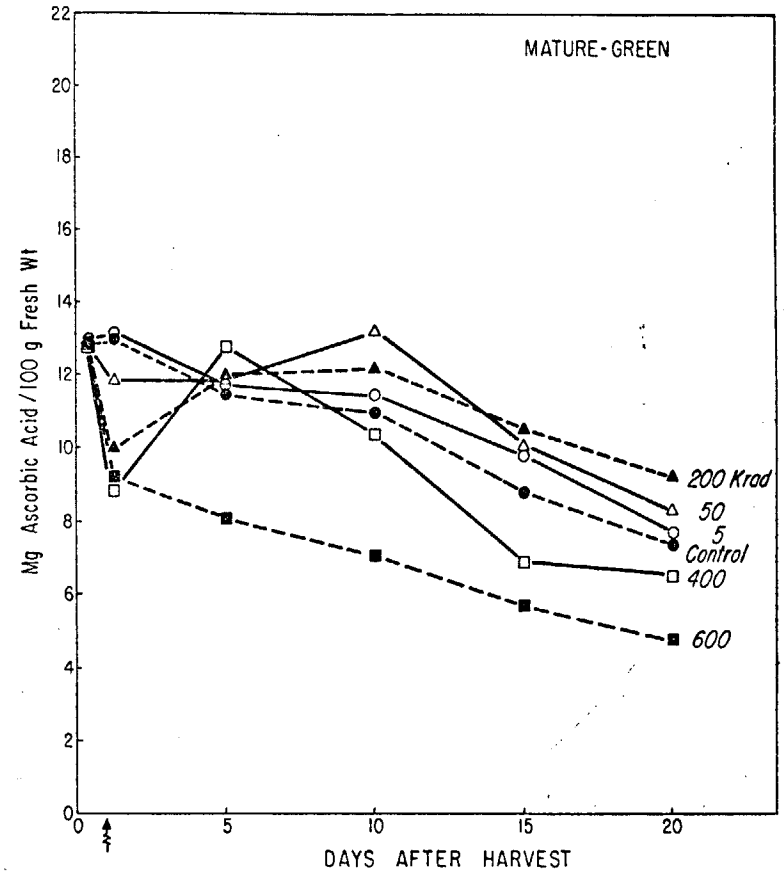


Fig. 1. Effect of gamma irradiation on ascorbic acid content of mature-green 'Early Pak No. 7' tomatoes held at 20°C.

as control fruits after 40 days at 15°C. Romani et al. (28) reported an immediate decrease of 15% in ascorbic acid in oranges irradiated at 400 Krad. Maxie et al. (21) observed losses of ascorbic acid of 23 and 35% at doses of 150 and 300 Krad in 'Krummel' peaches. They also found that 'Bing' cherries subjected to 200 and 400 Krad lost 3 and 10.5% more ascorbic acid during 24 hours at 5°C than did control fruits.

Important factors determining the flavor of tomatoes are acidity and total soluble solids. Craft and Heinze (5) concluded that per cent soluble solids increased slightly in mature-green tomatoes stored for short periods, but decreased with longer storage, whereas total acidity and pH did not change appreciably. Lutz (14) reported that the hydrogen ion concentration of tomatoes does not change greatly

Proc. Amer. Soc. Hort. Sci. 93: 843-853

0009

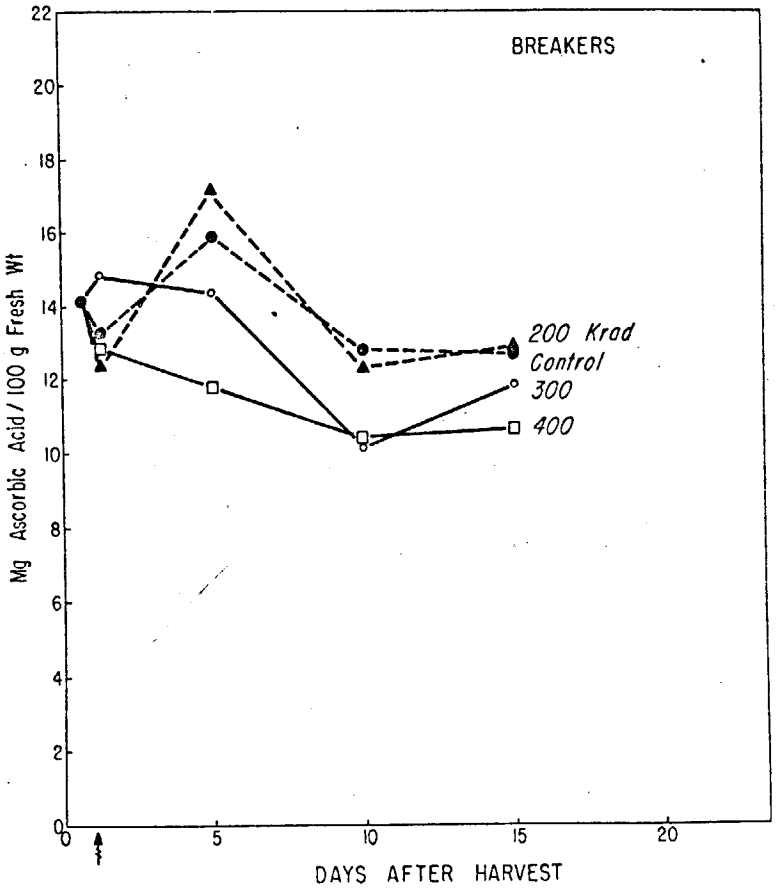


Fig. 2. Effect of gamma irradiation on ascorbic acid content of breaker-stage 'Early Pak No. 7' tomatoes held at 20°C.

during ripening. Fernandes and Clarke (7) reported an accelerated loss of titratable acidity in irradiated apples during storage at 37°F. Maxie et al. (22) showed a marked decrease in total acidity in irradiated lemon fruits during 40 days at 59°F.

Softening induced by radiation has been observed in apples (1, 3, 8, 23), pears, peaches, and nectarines (19), plums and raspberries (32), grapes (21), lemons and oranges (19), strawberries (4, 9), and sweet cherries (15), and is a major factor in loss of quality and susceptibility of irradiated fruits to mechanical injury. The relation of radiation-induced softening to changes in pectic substances has been studied extensively. Kertesz et al. (11) reported a degradation of pectin solution at 8,300 rep. Glegg et al. (8) showed that the threshold dose

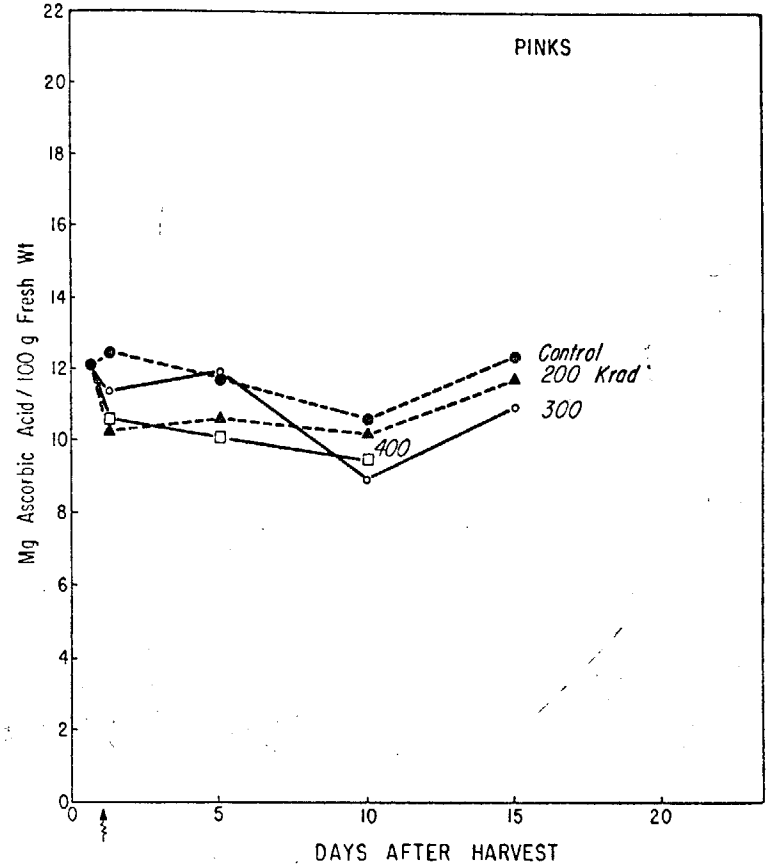


Fig. 3. Effect of gamma irradiation on ascorbic acid content of pink-stage 'Early Pak No. 7' tomatoes held at 20°C.

for softening was 34.7 Krad in 'Chantenay' carrots, and 316 Krad in 'Detroit Dark Red' beets. According to Boyle et al. (1), the threshold values that caused softening of 7 varieties of apples ranged from 4.2 to 107 Krad.

This study was designed to investigate the effects of gamma irradiation on ascorbic acid content, total soluble solids, pH, titratable acidity, and textural changes in tomato fruits.

Materials and Methods

Fruits of 'Early Pak No. 7' and/or 'Ace' that were harvested at different stages of ripeness were obtained near Merced, California. The fruits were sorted for uniformity, and matched subsamples of 100 fruits per treatment were treated within 36 hr of harvest. Irradi-

0009

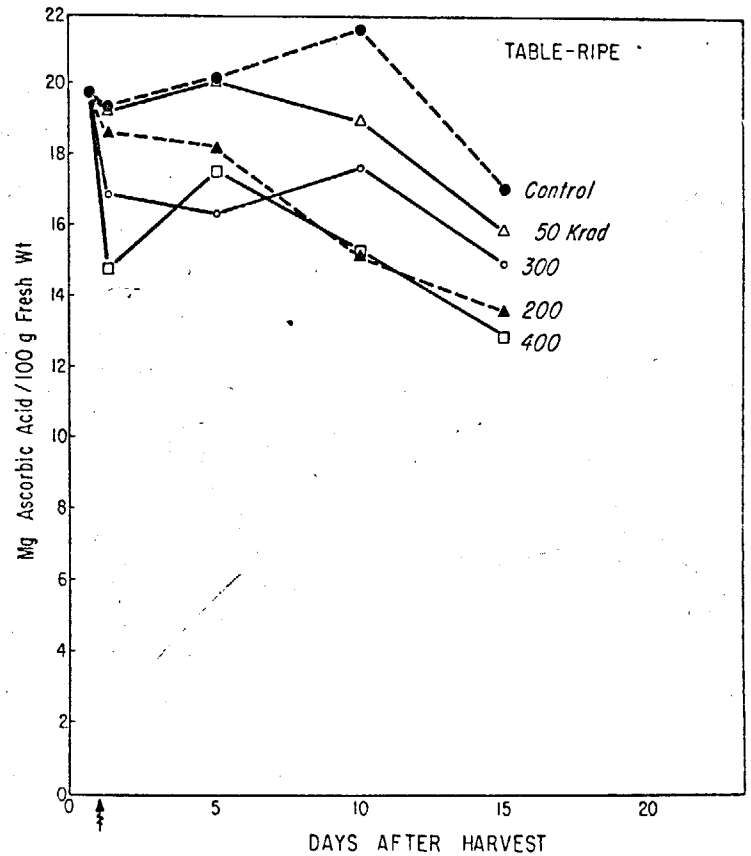


Fig. 4. Effect of gamma irradiation on the ascorbic acid content of table-ripe 'Early Pak No. 7' tomatoes held at 20°C.

ation was in the Mark II Experimental Food Irradiator (27). Fruits were held at approximately 20°C and 90% relative humidity. Representative subsamples of 10 fruits from each treatment were selected for analyses immediately before and after irradiation, and then at succeeding 5-day intervals.

Ascorbic acid was determined by the method of Loeffler and Ponting (13). Twenty-five small segments were taken at random from each of the 10 fruits in the subsample, and the juice was extracted with a hand press. The pH of the juice was determined with a Beckman pH meter. Acidity, expressed as % citric acid, was determined by titration of a 10-ml portion of juice with 0.1 N NaOH to an end point of pH 8.2. Per cent soluble solids was measured with a Bausch and Lomb hand refractometer. An Allo-Kramer shear press (12) fitted with a standard shearing cell was used to measure textural

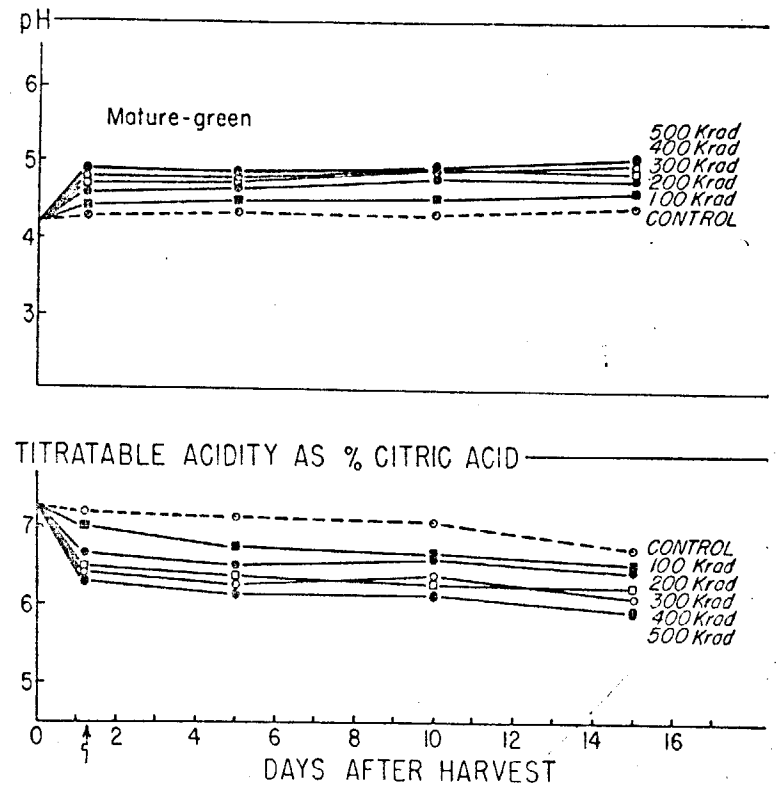


Fig. 5. The effect of gamma irradiation on pH and titratable acidity of juice from mature-green 'Ace' tomato fruits held at 20°C.

changes on 100 g of tissue from the stem end of individual fruits. Since the area under the curve and the peak height showed good correlation, only data on the former are presented. Each determination represents the average of 10 fruits, and all experiments were repeated at least once.

Results and Discussion

Fig. 1 shows the effect of gamma irradiation on the ascorbic acid content of mature-green 'Early Pak No. 7' tomatoes held 20 days at 20°C. Ascorbic acid in control fruits declined progressively with time. Doses of 5 and 50 Krad had no effect, while doses of 200 and 400 Krad caused an immediate decline, followed on the 5th day by recovery to about the levels in the control fruit. Fruits subjected to 400 Krad underwent a second decline at about the 10th day, with the amount thereafter remaining somewhat less than in the control fruit. A dose of 600 Krad caused an immediate loss of about 25%, with a

6000

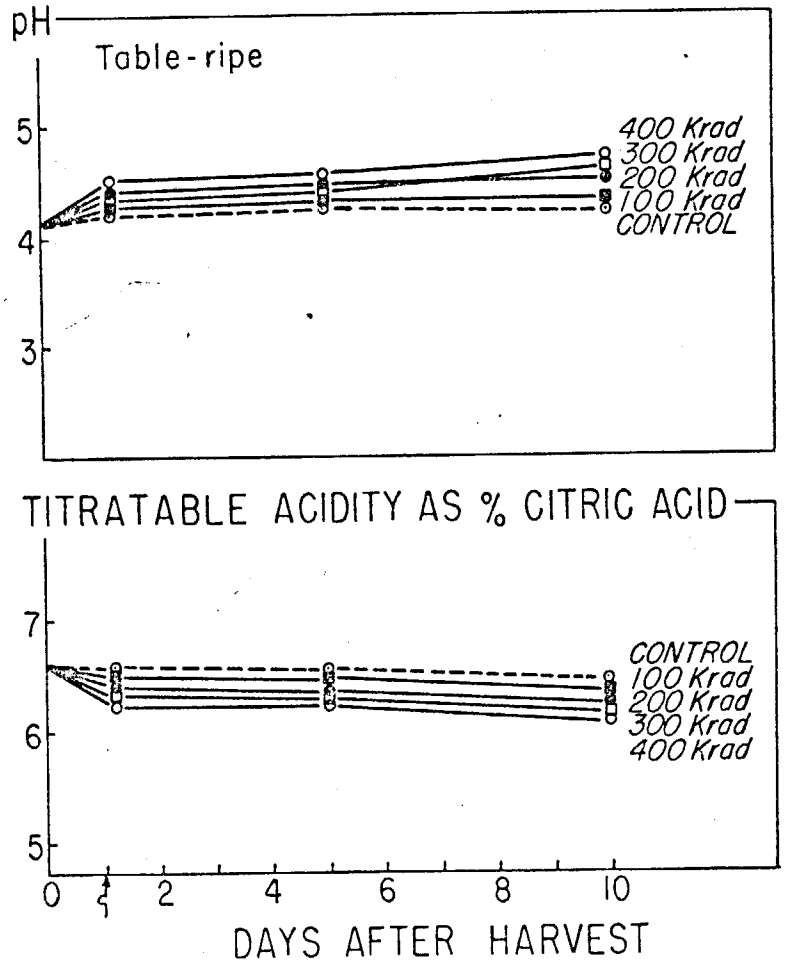


Fig. 6. The effect of gamma irradiation on pH and titratable acidity of juice from table-ripe 'Ace' tomato fruits held at 20°C.

continued progressive decline thereafter. In fruits irradiated at the breaker stage, 200 Krad had no effect on ascorbic acid content, whereas 300 and 400 Krad caused respective reductions of 8% and 15% after 15 days (Fig. 2). Fruits irradiated at the pink stage suffered small losses in ascorbic acid content at all doses (Fig. 3).

Fig. 4 compares ascorbic acid analyses of controls and fruits irradiated at the table-ripe stage. Doses of 50, 200, 300, and 400 Krad caused respective reductions of 6, 21, 12, and 24%. A large part of the losses occurred immediately after irradiation, with some recovery on the 5th day in fruits treated with 400 Krad. It should be noted

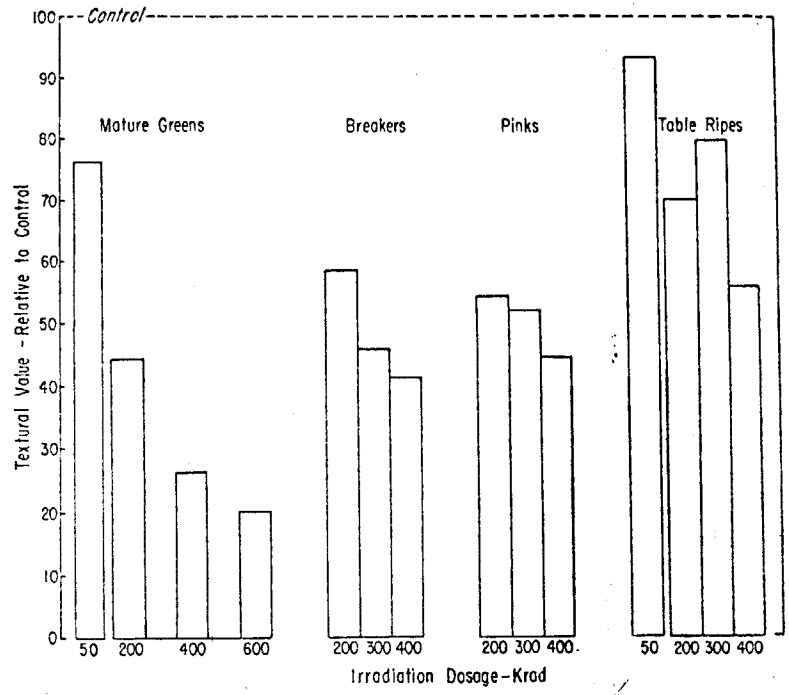


Fig. 7. Textural values immediately after treatment of 'Early Pak No. 7' tomatoes irradiated at various stages of ripeness. The textural values are expressed as the percentage of untreated fruits of the same maturity.

that ascorbic acid was always higher than 15 mg/100 g during the first 10 days in storage (a normal marketing period), although fruits harvested at mature-green and ripened in storage had only 10 mg/100 g when they reached the table-ripe stage (Fig. 1). Thus, irradiated table-ripe tomatoes contained more ascorbic acid than did unirradiated mature-green fruits, because of the higher initial content. This observation agrees with findings of Scott and Kramer (29) and Craft and Heinze (5), who reported that tomato fruits ripened on the plant had a higher ascorbic acid content than tomatoes harvested as mature-green and subsequently ripened in storage.

The results reported here on the effect of gamma irradiation on the ascorbic acid content of tomatoes agree with results reported for peaches and 'Bing' cherries (21), oranges (28), strawberries (19), and mangos (16).

In 'Ace' fruits, pH increased and titratable acidity decreased with increasing dose (Fig. 5 and 6). The effect was much less in table-ripe than in mature-green fruits. These results agree with results for apples (7) and lemons (22). Although Romani (26) concluded that the loss in acid in irradiated fruit is not a direct decarboxylation, Maxie

6000

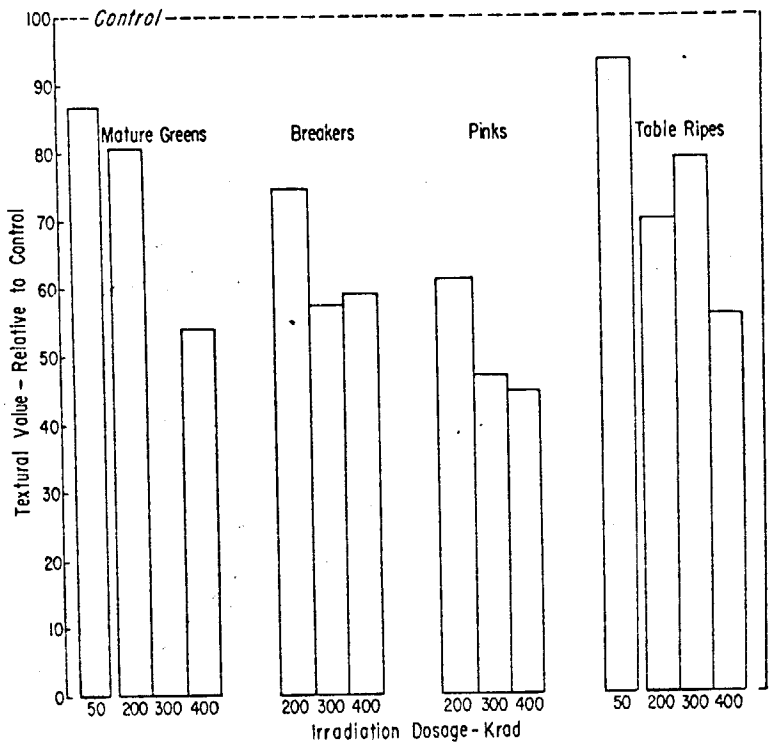


Fig. 8. Textural values at the table-ripe stage of 'Early Pak No. 7' tomatoes irradiated at various stages of ripeness. The textural values are expressed as the percentage of untreated fruits of the same maturity.

et al. (18) clearly showed radiolytic decarboxylation of citric, fumaric, succinic, and malic acids irradiated in solution.

Since per cent soluble solids changed very little following irradiation, regardless of stage of ripeness, these data are not presented.

Fig. 7 and 8 show the effects of gamma irradiation on the textural values of fruits irradiated at the mature-green, breaker, pink, and table-ripe stages. Firmness is lost proportionally to dose immediately after irradiation (Fig. 7), but the effect decreases after the fruit ripens (Fig. 8).

The mechanism by which radiation induces softening is not clear. Kertesz et al. (10) and Somogyi and Romani (30) showed good correlations between tissue softening, decrease in protopectin, and increases in pectin and pectate. In another paper Kertesz et al. (11) suggested that other factors, such as cell turgidity, were involved in the radiation-induced softening.

The effect of irradiation on susceptibility to mechanical injury

and to decay needs to be evaluated in commercial shipments. Under comparable conditions of temperature and relative humidity, irradiated fruits will probably behave differently after a long-distance shipment than when held stationary in storage. Maxie and Sommer (20) concluded that textural changes will be the most limiting factor in the application of irradiation for most fruits.

Literature Cited

1. Boyle, F. P., Z. I. Kertesz, R. E. Glegg, and M. A. Connor. 1957. Effect of ionizing radiation on plant tissues. II. Softening of different varieties of apples and carrots by gamma rays. *J. Food Res.* 22:89–95.
2. Clarke, I. D. 1959. Possible applications of ionizing radiations in the fruit, vegetable, and related industries. *Intern. J. Appl. Rad. & Isotopes* 6:175–181.
3. ———. 1962. Some effects of gamma radiations on the texture and pectic substances in apples. 1st. Intern. Cong. Food Sci. Tech., 1962, London.
4. Cooper, G. M., and D. K. Salunkhe. 1963. Effect of gamma radiation, chemical and packaging treatments on refrigerated life of strawberries and sweet cherries. *Food Technol.* 17:123–126.
5. Craft, C. C., and P. H. Heinze. 1954. Physiological studies of mature-green tomatoes in storage. *Proc. Amer. Soc. Hort. Sci.* 64:343–350.
6. De Zeeuw, D. 1961. Expériences sur la preservation des fruits frais par irradiation. *Irrad. Alim.* 1(3):6–8.
7. Fernandes, S. J. G., and I. D. Clarke. 1962. Effect of ionizing radiation the acid metabolism of apples. *J. Sci. Food & Agr.* 13:23–28.
8. Glegg, R. E., F. P. Boyle, L. W. Tuttle, D. F. Wilson, and Z. I. Kertesz. 1956. Effect of ionizing radiations on plant tissues. I. Quantitative measurements of the softening of apples, beets and carrots. *Radiation Res.* 5:127–133.
9. Johnson, Carol F., E. C. Maxie, and E. M. Elbert. 1965. Physical and sensory tests on fresh strawberries subjected to gamma irradiation. *Food Technol.* 19:119–123.
10. Kertesz, Z. I., R. E. Glegg, F. P. Boyle, G. F. Parsons, and L. M. Massey, Jr. 1964. Effect of ionizing radiations on plant tissues. III. Softening and changes in pectins and cellulose of apples, carrots and beets. *J. Food Sci.* 29:40–48.
11. ———, B. H. Morgan, L. W. Tuttle, and M. Lavin. 1956. Effect of ionizing radiations on pectin. *Rad. Res.* 6:372–381.
12. Kramer, Amihud. 1961. The shear-press, a basic tool for the food technologist. *The Food Scientist* 5(10):7–16.
13. Loeffler, H. J., and J. D. Ponting. 1942. Photometric method for the determination of ascorbic acid. *Ind. Eng. Chem. Anal. Ed.* 14:846–849.
14. Lutz, J. M. 1944. Maturity and handling of greenwrap tomatoes in Mississippi. *U. S. Dept. Agr. Circ.* 695:1–12.
15. Massey, L. M., Jr., W. B. Robinson, J. F. Spaid, D. F. Splittstoesser, J. P. Van Buren, and Z. I. Kertesz. 1965. Effect of gamma radiation upon cherries. *J. Food Sci.* 30:759–765.
16. Mathur, P. B., and N. F. Lewis. 1961. Storage behaviour of gamma irradiated mangos. *Intern. J. Appl. Rad. & Isotopes* 11:43–45.
17. Maxie, E. C., and A. S. Abdel-Kader. 1967. Food irradiation—physiology of fruits as related to feasibility of the technology. *In:* Advances in Food Research. Academic Press, New York. 15:105–145.
18. ———, H. L. Rae, I. L. Eaks, and N. F. Sommer. 1966. Studies on radiation-induced ethylene production by lemon fruits. *Rad. Bot.* 6:445–455.
19. ———, and N. F. Sommer. 1963. Radiation technology in conjunction with postharvest procedures as a means of extending the shelf-life of fruits and vegetables. U. S. Atomic Energy Comm. Research & Dev. Rpt. UCD-34P80-1. 181 p.

6000

20. ————, and ————. 1964. Status of gamma irradiation as a technology for perishable commodities. Proc. Fruit and Veg. Handling Conf., Univ. of California, Davis (March, 1964), pp. 96–100.
21. ————, ————, and D. S. BROWN. 1964. Radiation technology in conjunction with postharvest procedures as a means of extending the shelf-life of fruits and vegetables. U. S. Atomic Energy Comm. Research & Dev. Rpt. No. UCD-34P80-2. 166 p.
22. ————, ————, and ————. 1965. Radiation technology in conjunction with postharvest procedures as a means of extending the shelf-life of fruits and vegetables. U. S. Atomic Energy Comm. Research & Dev. Rpt. No. UCD-34P80-3. 190 p.
23. PHILLIPS, W. R., P. A. POAPST, and K. W. MACQUEEN. 1960. Irradiation of apples for fruit storage. Rpt. Canad. Comm. Fruit Veg. Pres. (1959–1960).
24. PROCTOR, B. E., and S. A. GOLDBLITH. 1949. Effect of soft X-rays on vitamins (niacin, riboflavin and ascorbic acid). *Nucleonics* 5(3):56.
25. ————, and J. P. O'MEARA. 1951. Effect of high-voltage cathode rays on ascorbic acid. *Ind. Eng. Chem.* 43:718–721.
26. ROMANI, R. J. 1964. Radiation physiology of fruit respiration during and immediately following kilorad doses of ionizing radiation. *Rad. Bot.* 4:299–307.
27. ————, B. J. ROBINSON, H. L. RAE, E. C. MAXIE, and N. F. SOMMER. 1963. Fruit irradiation—physical methods. *Rad. Bot.* 3:345–350.
28. ————, JAN VAN-KOOY, L. LIM, and J. B. BOWERS. 1963. Radiation physiology of fruit. Ascorbic acid, sulfhydryl and soluble nitrogen content of irradiated citrus. *Rad. Bot.* 3:363–369.
29. SCOTT, L. E., and A. KRAMER. 1949. The effect of storage upon the ascorbic acid content of tomatoes harvested at different stages of maturity. *Proc. Amer. Soc. Hort. Sci.* 54:277–280.
30. SOMOGYI, L. P., and R. J. ROMANI. 1964. Irradiation-induced textural change in fruits and its relation to pectin metabolism. *J. Food Sci.* 29:366–371.
31. Stanford Research Institute. 1961. Radiation preservation of selected fruits and vegetables. An analysis of R & D programming and market factors. U. S. Atomic Energy Comm., Office of Tech. Information, Washington, D. C. 227 p.
32. TRUELSEN, T. A. 1963. Radiation pasteurization of fresh fruits and vegetables. *Food Technol.* 17:100–103.

6000

J. Am. Med. Assoc. 111: 1555-1565, 1938

Special Article

VITAMIN C

PHARMACOLOGY AND THERAPEUTICS

ARTHUR F. ABT, M.D.
AND
CHESTER J. FARMER, A.M.
CHICAGO

This article and others recently published or to be published comprise a new series on the present status of our knowledge of the vitamins. They have been prepared under the general auspices of the Council on Pharmacy and Chemistry and the Council on Foods. The opinions expressed are those of the authors and not necessarily the opinions of either council. Reprints are not available but the articles will be published later in book form.—Ed.

Vitamin C is specifically involved in the prevention or treatment of scurvy. Since this factor was isolated, its structure determined and the pure substance synthesized, considerable advances in our knowledge of the physiology of vitamin C have been made and some evidence of the value of this substance in conditions other than scurvy has been developed. Exact knowledge of the pharmacologic action of vitamin C is still rather limited, but it is growing rapidly because of the development of chemical methods for estimating vitamin C in foods and body tissues and fluids. The present review is an attempt to summarize this knowledge as well as the present status of our knowledge of the therapeutic usefulness of vitamin C.

AVAILABLE PREPARATIONS OF VITAMIN C

It must not be forgotten that vitamin C is an accessory food substance and that ordinarily foods are relied on for a supply of this essential factor. As described in the paper on sources of vitamin C,[1] there are some foods which are especially potent. Chief among these are the citrus fruit juices, which long have been used in the prevention and treatment of scurvy. Fresh orange juice contains on the average from about 40 to 60 mg. of vitamin C per hundred cubic centimeters. The daily requirements of an adult are considered to be about 25 mg. for the prevention of scurvy and more than that for normal requirements. The exact requirements for normal health are not known but nutritionists have estimated that a standard allowance for an adult would be from 600 to 1,200 international units, equivalent to from 30 to 60 mg. of vitamin C. Canned tomato juice is also a rich source of vitamin C, as are the canned citrus fruit juices.[2]

It is customary to give vitamin C to all babies early in life. This is usually administered in the form of fresh orange juice, beginning even as early as the third week of life with a teaspoonful and, if well tolerated, increasing gradually until the baby is taking about 2 ounces daily by the third month. On this basis the infant will be receiving up to about 25 mg. or more of vitamin C a day. Other sources of vitamin C may be substituted for the fresh orange juice and this substitution should be on the basis of the vitamin C content.

Synthetic preparations of crystalline vitamin C are now available. Ascorbic acid is freely soluble in water and may be administered orally in tablet form or dissolved in water. For parenteral use, either intramuscularly or intravenously, Fisher and Leake[3] have recommended that ascorbic acid be dissolved in sterile water and neutralized with one-half its weight of sodium bicarbonate immediately before injection. If this procedure is followed, the vitamin will not lose in potency and local reaction will be avoided.

Before the isolation of the crystalline vitamin, doses larger than 500 mg. daily (approximate content of 1 quart of orange juice) were impracticable. We have noted in some of our experimental work that certain persons will not tolerate even 1 pint of orange juice daily. Since the crystalline vitamin has become available, it may be used to supplement the dietary intake or to be given in large doses.

The crystalline vitamin has an indicated therapeutic value in infant feeding under certain conditions. There are some infants who are allergic to or intolerant to fruit juices and other foods rich in vitamin C. This intolerance or sensitivity is presumably due to the proteins of the juices or foods containing the natural vitamin, and in such cases the crystalline vitamin may be advantageously substituted. The substitution of the crystalline vitamin for the natural food sources may also have a therapeutic indication in certain acute and chronic gastrointestinal diseases in which adequate absorption of vitamin C from natural food sources is altered or interfered with.

In certain of the conditions to be discussed, relatively large doses of crystalline vitamin C are required to obtain therapeutic effects. Although the normal daily human requirements have been estimated to be from 15 to 40 mg. or even up to 60 mg.,[1] it is difficult to explain why certain therapeutic effects can be obtained only by administering doses of from ten to twenty-five times this amount. Our present state of knowledge is so limited that a precise statement of what constitutes proper dosage is not practicable. In New and Nonofficial Remedies it has been suggested that crystalline vitamin C be administered in quantities of 10 mg. for the prevention of infantile scurvy. "The therapeutic dose is about 30 mg. daily, although many physicians prefer to administer larger amounts to adults."[5]

One question raised with regard to the higher dosages that are possible with crystalline vitamin C is the possibility of toxicity. It is rather generally accepted that vitamin C is nontoxic when administered in moderate doses to the human being and excess amounts are excreted. Repeated doses of as much as from 1 to 6 Gm. have been administered orally and intravenously to adults, with no evidence of toxic action.[6] Friedman, McGoey and Ralli[7] in a study of vitamin C clearance have repeatedly obtained plasma concentrations ranging from 5 to 22 mg. per hundred cubic centimeters (normal values are from 0.7 to 1.5 mg.) with no evidence of toxicity. However, Schade[8] observed vagotonic

From the Northwestern University Medical School.

1. Bessey, Otto A.: Vitamin C: Methods of Assay and Dietary Sources, J. A. M. A. **111:** 1290 (Oct. 1) 1938.
2. The Vitamin C Content of Commercially Canned Tomato Juice and Other Fruit Juices as Determined by Chemical Titration: A Report of the Council on Foods, J. A. M. A. **110:** 650 (Feb. 26) 1938.
3. Fisher, B. H., and Leake, C. D.: The Parenteral Administration of Cevitamic Acid (Ascorbic Acid) Solution, J. A. M. A. **103:** 1556 (Nov. 17) 1934.
4. Smith, Schef L.: Vitamin C Nutrition and Requirements, J. A. M. A., to be published.
5. New and Nonofficial Remedies, Chicago, American Medical Association, 1938, p. 1[illegible].
6. Personal clinical experience.
7. Friedman, G. J.; McGoey, C., and Ralli, E. P.: Personal communication to the authors; abstract, Proc. Am. Physiol. Soc. Bull., 1938.
8. Schade, H. A.: Beitrag zur Frage der Indication von Vitamin C (L-Ascorbinsäure) auf Pylorusfunktionsvorgänge, Klin. Wochschr. **14:** 5[illegible] (Jan. 15) 1935.

symptoms in growing children at the height of the vitamin C action (fatigue, exhaustion, slow pulse, increased intestinal peristalsis). Widenbauer[9] noted vagotonic symptoms such as dermographia, hyperemia, erythema, increased peristalsis and bradycardia in infants on maximum doses of vitamin C. These symptoms were not attributed to a hypervitaminosis but to drug sensitivity or idiosyncrasy to ascorbic acid. In the guinea pig Randoin[10] has noted that, if a scorbutic animal is given relatively large quantities of vitamin C, the animal may lose weight and the symptoms of scurvy reappear.

FUNCTIONS AND PHARMACOLOGIC ACTIONS

The chief function assigned to vitamin C in the body is concerned with the formation of colloidal intercellular substances.[11] The intercellular substances which appear to be regulated by vitamin C are of mesenchymal origin — the collagen of all fibrous tissue structures, all nonepithelial cement substances, including the intercellular

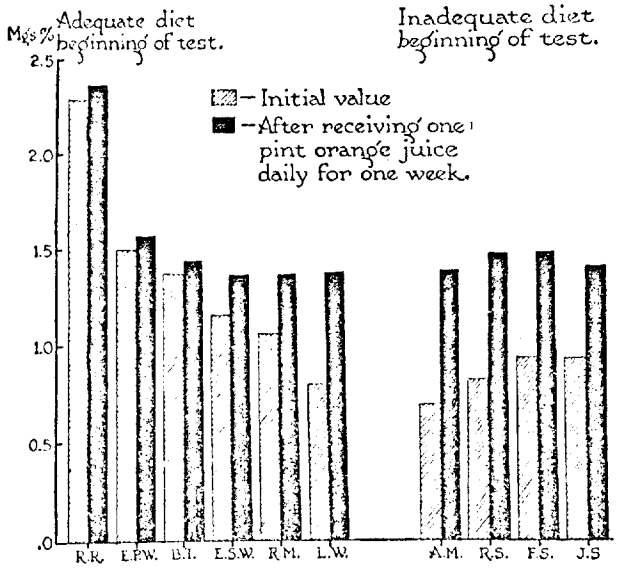


Chart 1. Vitamin C content of the blood as influenced by diet: ascorbic acid values of young adults.

substance of the capillary wall, dentin, cartilage, and the matrices of bone. It has also been suggested that vitamin C may function as a respiratory catalyst, aiding cellular respiration by acting as a hydrogen transport. The theory suggesting this function has received support in relation to plant and vegetable life[12] but has not been proved for animal tissue.

It has been shown that man, the guinea pig and the primates are incapable of synthesizing vitamin C. In tissue slice experiments, Widenbauer and Korschorreckt[13] reported an increase in vitamin C, as measured by iodine titration, when slices of intestine from the mouse or rat, animals which are capable of synthesizing vitamin C, are incubated in a sterile dextrose-salt medium. When slices of liver, spleen, stomach or brain were used, no evidence of vitamin C formation could be found. These authors concluded that animals which do not require a dietary supply of vitamin C synthesize ascorbic acid from dextrose in the intestine.

Occurrence in Body Fluids.—Vitamin C has been found in the body fluids. It is excreted in the urine, the amounts varying with the dietary intake.[14] The level of vitamin C in the blood plasma also varies directly with the dietary intake[15] (chart 1). The methods for chemical assay of the blood plasma have been given in another article of the series.[15a] In our opinion the recommendation that potassium cyanide be used as a stabilizer of ascorbic acid is unfortunate. We and others[15b] have recently shown that potassium cyanide not only does not prevent the loss of ascorbic acid from the blood plasma, but its use may also give erroneous results of considerable magnitude. The use of potassium cyanide in carrying out the method for determining ascorbic acid in blood should be discontinued. Low blood plasma values found in man have been associated with active scurvy.[16] In the scorbutic guinea pig the lowest plasma values have occurred just before death.[17] According to Borsook,[18] practically all the ascorbic acid in plasma and serum is in the reduced state.

Plaut and Bülow[19] have observed a direct relationship between the vitamin C content of the cerebrospinal fluid and the dietary intake, which corresponds to the observations on blood plasma. Stuteville[20] noted the presence of vitamin C in saliva. Abt, Farmer and Epstein[21] measured the ascorbic acid content of saliva obtained from students, before and after diets supplemented by vitamin C. No definite relationship could be established between the dietary intake and the salivary content. The salivary level was also found to be independent of the level in the blood. According to Zimmet and Dubois-Ferrière,[22] the concentration of vitamin C in the saliva of children increased progressively from a level of 0.04 mg. per hundred cubic centimeters at 4 years of age to 0.11 mg. per hundred cubic centimeters at 16 years.

9. Widenbauer, F.: Toxisch. Nebenwirkungen von Ascorbinsäure—C Hypervitaminose, Klin. Wchnschr. **15**: 1158 (Aug. 15) 1936.

10. Randoin, G. L.: On the Necessity for a Biological Supervision of Food with Special Reference to Its Vitamin Content, Quart. Bull. Health Organ. League of Nations **5**: 693 (Sept.) 1936.

11. Wolbach, S. B.: Pathologic Changes Resulting from Vitamin Deficiency, J. A. M. A. **108**: 7 (Jan. 2) 1937.

12. Szent-Györgyi, Albert: On the Function of Hexuronic Acid in the Respiration of the Cabbage Leaf, J. Biol. Chem. **90**: 385 (Jan.) 1931.

14. Harris, L. J.; Ray, S. N., and Ward, Alfred: The Excretion of Vitamin C in Human Urine and Its Dependence on the Dietary Intake, Biochem. J. **27**: 2011 (Nov.) 1933. Harris, L. J., and Ray, S. N.: Diagnosis of Vitamin C Submutrition by Urine Analysis, with Note on Antiscorbutic Value of Human Milk, Lancet **1**: 71 (Jan. 12) 1935. von Euler, Hans, and Klussmann, E.: Physiologische Versuch über Vitamin C (Ascorbinsäure) und Redukton, Ztschr. f. physiol. Chem. **217**: 167 (April) 1933. Johnsen and Zilva.[24]

15. Farmer, C. J., and Abt, A. F.: Ascorbic Acid Content of Blood, Proc. Soc. Exper. Biol. & Med. **32**: 1625 (June) 1935; Determination of Reduced Ascorbic Acid in Small Amounts of Blood, ibid. **34**: 146 (March) 1936. Greenberg, L. D.; Ruchart, J. F., and Phatak, N. M.: Studies on Reduced Ascorbic Acid Content of the Blood Plasma, ibid. **35**: 135 (Oct.) 1936.

15a. Bessey, Otto A.: Vitamin C: Methods of Assay and Dietary Sources, J. A. M. A. **111**: 1290 (Oct. 1) 1938.

15b. Farmer, C. J., and Abt, A. F.: The Invalidation of Plasma Ascorbic Acid Values by Use of Potassium Cyanide, Proc. Soc. Exper. Biol. & Med. **38**: 369 (April) 1938. Friedman, Gerald J.; Rubin, Saul H., and Kees, Walter: Effect of Addition of KCN to Whole Blood on Indophenol-Reducing Power of Plasma, ibid. **38**: 358 (April) 1938. Minellan, R. L.: The Relation Between Plasma Ascorbic Acid Concentration and Diet in the Newborn Infant, J. Pediat. **13**: 309-313 (Sept.) 1938.

16. Abt, A. F., and Epstein, I. M.: Cevitamic Acid in the Treatment of Infantile Scurvy, J. A. M. A. **104**: 634 (Feb. 23) 1935.

17. Abt, A. F.: Cevitamic Acid of the Blood Plasma, Am. J. Dis. Child. **51**: 682 (Sept.) 1937.

18. Borsook, Henry; Davenport, H. W.; Jeffreys, C. E. P., and Warner, R. C. P.: Oxidation of Ascorbic Acid, J. Biol. Chem. **117**: 237 (Jan.) 1937.

19. Plaut, F., and Bülow, M.: Weitere untersuchungen über das C-Vitamin im Gehirn und im Liquor cerebrospinalis, Ztschr. f. d. ges. Neurol. u. Psychiat. **152**: 84, 1935.

20. Stuteville, O. H.: Presence of Vitamin C in Saliva, Proc. Soc. Exper. Biol. & Med. **32**: 1454 (June) 1935.

21. Abt, A. F.; Farmer, C. J., and Epstein, I. M.: Normal Cevitamic (Ascorbic) Acid Determinations in Blood Plasma and Their Relationship to Capillary Resistance, J. Pediat. **8**: 1 (Jan.) 1936.

The vitamin C content of sweat,[23] aqueous humor [24] and gastric juice [25] has been determined. A study of the occurrence of vitamin C in the feces has been made in our laboratory.[26] In the normal individual not over 6 to 10 mg. daily are excreted, even with oral feeding of large amounts. Under abnormal conditions (diarrhea, colitis and the like) the amount depends largely on the number and type of stools. Ishibashi [27] has reported studies on the vitamin C content of the feces of infants. According to him, peroral administration of the vitamin produced no fecal rise.

Absorption and Excretion.—Vitamin C appears to be selectively absorbed by the tissues of the intestinal tract,[28] principally the small intestine. Klodt [29] believes that bile may aid absorption. In achlorhydria, blood plasma levels are usually lower than normal. This may be caused by decreased absorption of active vitamin C from the intestine, which in turn is caused by a lessened acidity of the intestinal contents or by a a changed bacterial flora. In support of the latter view Kendall and Chinn [30] have shown that ascorbic acid is destroyed by certain bacteria isolated from stomach and intestinal contents of achlorhydric patients. Further evidence of the loss of vitamin C activity prior to absorption has been afforded by the observation of Wilder and Wilbur [31] that variable amounts of vitamin C are lost in the stool or destroyed in the gastrointestinal tract even under normal conditions. Hou [32] found that ascorbic acid administered by subcutaneous injection was twice as effective as that given by mouth in the protection of guinea pigs against scurvy. He has suggested as the probable explanation that part of the ascorbic acid administered orally is lost in the gastrointestinal tract. Until definite information is available to the contrary, it must be assumed that not only are there individual variations in absorption of vitamin C from the intestinal tract but also under certain conditions the absorption may be definitely abnormal.

After absorption vitamin C may be detected in increased quantities in the blood plasma, by which it is transported to the various tissues of the body. Whether a storage of vitamin C occurs in the animal body is still a debatable question.[33] This is indicated by the necessity for a continuous food supply in species incapable of synthesizing vitamin C. Because of the ease with which vitamin C can be determined chemically, many studies have been made concerning its metabolism. Large doses of the vitamin have been administered and measurements made of the amounts excreted in the urine. Johnson and Zilva [34] consider that "saturation" of the tissues with vitamin C has been reached when the ingestion of a certain amount of vitamin C is roughly equaled by the daily excretion of the vitamin in the urine. Succeeding investigators have attempted to determine the saturation of the organism by oral or intravenous administration of test doses of ascorbic acid and subsequent measurement of its excretion in the urine. The majority of investigators employing urinary excretion tests have failed to recognize the possibility that absorption from the intestinal tract may not be complete.[35] Further, they have arbitrarily stated that various amounts ranging from 30 to 75 per cent of the test dose are excreted on reaching "a state of saturation." If subjects on a previous minimal vitamin C diet require a total of from 2,000 to 3,000 mg. of ascorbic acid before appreciable amounts are excreted in the urine, must it be assumed that the total amount of vitamin administered found its way to the tissues? It should be obvious that, before quantitative statements are derived, consideration must be given to questions of destruction in and absorption from the intestinal tract, and excretion by way of the feces.

We have repeatedly observed that, on the oral administration of large quantities of vitamin C, only small amounts are excreted in the urine, while a steady rise in plasma level occurs. If the oral ingestion is continued for a sufficient period, a point of plasma elevation will be attained, following which a rapid urinary excretion of a large part of the ingested ascorbic acid occurs. We believe that the plasma level at which this quantitative urinary excretion occurs shows considerable individual variation.

Everson and Daniels [36] state that ascorbic acid metabolism may be compared with nitrogen metabolism in that the urinary excretion of vitamin C is of both endogenous and exogenous origin. Hawley and others [37] report that the amount of vitamin C excreted in the urine may vary according to the acid-alkali content of the diet, a highly alkaline urine having lower amounts than a highly acid urine. Daniels [38] reports that therapeutic doses of acetylsalicylic acid increase the excretion of vitamin C in the urine of children, while Youmans [39] states that it does not increase or decrease excretion of vitamin C in the urine of adults. It has been reported by Ahmad [40] that diets high in protein and fat cause an increased urinary excretion of vitamin C. On the other hand, Heinemann [41] noted that the total reducing power of the urine varies directly with the protein intake, while the vitamin C excretion remains uninfluenced.

Diuretic Effect.—Abbasy [42] has noted that, following the administration of a test dose (700 mg. of ascorbic acid to 10 stone, 140 pounds, 63.5 Kg. of body weight), the volume of urine, as well as the excretion of vitamin

23. Cornbleet, Theodore; Klein, R. L., and Pace, E. R.: Vitamin C Content of Sweat, Arch. Dermat. & Syph. **34:** 253 (Aug.) 1936.
24. Rosner, Lawrence, and Bellows, John: Ascorbic Acid Oxidase in Determining Vitamin C in Lens and Aqueous Humor, Proc. Soc. Exper. Biol. & Med. **34:** 193 (May) 1936. Buschke, W.: Blutzirkulation im Auge und Vitamin C, Arch. f. Augenh. **109:** 691, 1936.
25. Peters, G. A., and Martin, H. E.: Ascorbic Acid in Gastric Juice, Proc. Soc. Exper. Biol. & Med. **36:** 76 (Feb.) 1937.
26. Farmer, C. J., and Chinn, Herman: Unpublished data.
27. Ishibashi, T.: Vitamin C of the Urine and Feces of Newly Born Infants, Acta paediat., Japan. **43:** 187 (Feb.) 1937.
28. Zilva, S. S.: Ascorbic Acid Content of Intestine of Guinea Pig, Biochem. J. **29:** 100 (Nov.) 1935.
29. Klodt, W.: Ueber den Einfluss der Galle auf die Vitamin C Resorption, Med. Welt. **10:** 477 (April) 1936.
30. Kendall, A. I., and Chinn, Herman: A Method for Isolation of Ascorbic Acid Fermenting Bacteria, Proc. Soc. Exper. Biol. & Med. **38:** 8 (Feb.) 1938; Decomposition of Ascorbic Acid by Certain Bacteria, J. Infect. Dis. **62:** 330 (May-June) 1938.
31. Wilder, R. M., and Wilbur, D. L.: Diseases of Metabolism and Nutrition, Arch. Int. Med. **59:** 512 (March) 1937.
32. Hou, H. C.: Comparison of Oral and Subcutaneous Administration of Protective Doses of Ascorbic Acid (Vitamin C), Proc. Soc. Exper. Biol. & Med. **32:** 1391 (June) 1935.
33. Zilva, S. S.: Vitamin C Requirements of the Guinea Pig, Biochem. J. **30:** 1419 (Aug.) 1936.
34. Johnson, S. W., and Zilva, S. S.: The Urinary Excretion of Ascorbic Acid and Dehydroascorbic Acids in Man, Biochem. J. **28:** 1393 (No. 4) 1934.
35. Storage of Vitamin C, editorial, J. A. M. A. **108:** 395 (Jan. 30) 1937.
36. Everson, G. J., and Daniels, A. L.: Vitamin C Studies with Children of Preschool Age, Nutrition **12:** 15 (July) 1936.
37. Hawley, E. E.; Frazer, J. P.; Button, L. L., and Stephens, D. J.: Effect of the Administration of Sodium Bicarbonate and of Ammonium Chloride on the Amount of Ascorbic Acid Found in the Urine, Nutrition **12:** 215 (Aug.) 1936.
38. Daniels, Amy L., and Everson, Gladys J.: Influence of Acetylsalicylic Acid (Aspirin) on Urinary Excretion of Ascorbic Acid, Proc. Soc. Exper. Biol. & Med. **35:** 70 (Oct.) 1936.
39. Youmans, J. B.; Corlette, M. B.; Frank, Helen, and Corlette, Mildred: Failure of Acetylsalicylic Acid to Affect Excretion of Ascorbic Acid (Vitamin C) in Urine, Proc. Soc. Exper. Biol. & Med. **36:** 73 (Feb.) 1937.
40. Ahmad, Bashir: Observations on Excretion of Vitamin C in Human Urine, Biochem. J. **30:** 11 (Jan.) 1936.
41. Heinemann, Martin: On Relation Between Diet and Urinary Output of Thiosulfate and Ascorbic Acid; Human Requirements for Vitamin C, Biochem. J. **30:** 2399 (Dec.) 1936.
42. Abbasy, M. A.: The Diuretic Action of Vitamin C, Biochem. J. **31:** 339 (Feb.) 1937.

C in the urine, showed a definite rise. He therefore attributed a diuretic action to the vitamin. As a result of an extended study of the urinary output of nine patients with cardiac disease, Evans[43] states that vitamin C was found to induce a greater diuresis than digitalis but less than theobromine, theobromine with sodium salicylate, and ammonium chloride.

Blood Pressure. It was noted by Kasahara and Kawamura[44] that guinea pigs showed a rise in blood pressure when injected with 100 mg. per kilogram or greater doses of ascorbic acid and that monkeys showed a rise in blood pressure starting with injections of 40 mg. per kilogram and a greater rise with doses of from 100 to 200 mg. per kilogram. The authors conclude that the blood pressure rises in monkeys and guinea pigs following injections only of large amounts of ascorbic acid.

Capillary Action. While it has been shown histologically that vitamin C regulates the intercellular substance of the capillary wall, a clear relationship between capillary resistance of the skin and dietary intake of vitamin C has not been definitely established.[45] Göthlin[46] has recently attempted to correlate capillary resistance measured by his positive pressure method with the ascorbic acid content of blood. He found increased capillary fragility in individuals with blood levels of 1 mg. per liter or less. In more than twenty cases of both infantile and adult scurvy observed by us, we have never obtained such low blood values.

Effect of Anesthesia.—According to Bowman and Muntwyler,[47] urinary excretion of vitamin C is increased following ether anesthesia in the dog, rat and guinea pig. The ascorbic acid content of the kidneys, liver, adrenals and spleen was found to be lessened after anesthesia. Bersin and his co-workers[48] found that excretion of ascorbic acid was diminished in the urine following ether and chloroform anesthesia in guinea pigs, on administration of a test dose of vitamin C intravenously.

RELATION OF VITAMIN C TO SCURVY

Scurvy is a disease for which vitamin C is the sole therapeutic agent. The specificity of vitamin C in scurvy was demonstrated long before the isolation of the vitamin and, as soon as the vitamin was available as a pure chemical substance, it was successfully used both clinically and experimentally in the treatment of scurvy. According to Finkle,[49] a low body level of vitamin C is etiologic for no other disease than scurvy, with the possible exception of lupus erythematosus. As has been previously stated, the blood plasma levels are extremely low in human scurvy. Abt,[50] in reporting his early studies on human scurvy, showed that the dietary history and clinical signs and symptoms parallel the low blood plasma values and that the plasma content shows a rapid rise in a healing scurvy. With the aid of the micromethod described by us, tolerance tests were made on patients recovering from scurvy, the ascorbic acid content of the plasma being determined hourly and curves plotted. The initial level rises slightly but usually returns to a low level.[17] As the patient continues on a high vitamin C intake, fasting blood levels on successive days attain higher values. Tolerance tests performed at these times by the oral administration of 10 mg. of ascorbic acid per kilogram of body weight show, with fair uniformity, a higher value at the peak of each succeeding curve. The peak is usually reached by the second hour, and the rise (difference between the initial fasting level and the value attained at the peak) increases until a state is attained in which the body promptly excretes the major part of the ingested ascorbic acid.

Ascorbic acid may be given intramuscularly or intravenously for the cure of scurvy when nausea, emesis or extreme prostration prevents its oral administration. It is interesting to note that Hess and Unger[51] in 1918 administered neutralized orange juice intravenously to three patients for whom oral administration was impossible.

Jackson and Park[52] have reported a case of congenital scurvy. By determining the vitamin C content of the blood obtained simultaneously from the cord of the infant and the vein of the mother immediately after delivery, Abt, Farmer and Epstein[21] found practically identical values from the two sources. From these observations it may be concluded that the fetus is dependent for its source of vitamin C on the maternal dietary. If the vitamin intake of the mother is extremely low during pregnancy, congenital scurvy may consequently result.

Goettsch[53] has noted in treating infantile scurvy that a single massive dose of vitamin C permits the healing of the scurvy as effectively as the same amount of vitamin in divided doses. Observing calcification of subperiosteal hematomas, she noted that healing commenced as early as the forty-eighth hour after the intravenous injection of the large doses of vitamin. Aub[54] reports that vitamin C is a factor in calcium deposition in the bones. Its addition to a scorbutic diet causes a rapid deposition of calcium at the epiphysial ends of bones and in trabeculae. The effect is probably due to the necessity of adequate amounts of vitamin C for normal production of the organic matrix in which the lime salts are deposited.

Hess[55] has noted the development of scurvy in infants on a diet which contained sufficient vitamin to be ordinarily antiscorbutic. These infants recovered when given larger amounts of vitamin C orally. Hess felt that the development of scurvy in these cases was due to a failure of absorption of the ingested vitamin. Hagmann[56] reported that scurvy developed in a 2½ months old infant receiving 4 drachms (15 cc.) of orange juice daily. Large doses of vitamin C taken

43. Evans, William: Vitamin C in Heart Failure. Lancet **1:** 308 (Feb. 5) 1938.

44. Kasahara, M., and Kawamura, R.: Ueber den Einfluss der L-Ascorbinsäure auf den Blutdruck. Klin. Wchnschr. **16:** 1543 (Oct.) 1937.

45. Abt, Farmer and Epstein,[21] Wilder and Wilbur,[39] Ahlasy.[42]

46. Göthlin, G. F.: When Is Capillary Fragility a Sign of Vitamin C Substitution in Man? Lancet. **2:** 703 (Sept. 18) 1937; Acta paediat. **20:** 71, 1937.

47. Bowman, D. E., and Muntwyler, E.: Further Experiments upon the Excretion of Ascorbic Acid in the Urine Following Ether Anesthesia, J. Biol. Chem. **11:** 113, 1936; Ascorbic Acid Content of Tissue Following Ether Anesthesia. Proc. Soc. Exper. Biol. & Med. **35:** 557 (Jan.) 1937.

48. Bersin, T.; Lauber, H. J., and Nafziger, H.: Der Einfluss der Narkose und Operation auf den Vitamin C Haushalt, Klin. Wchnschr. **16:** 1272 (Sept. 11) 1937.

49. Finkle, Philip: Vitamin C Saturation Levels in Body in Normal Subjects and in Various Pathological Conditions, J. Clin. Investigation **16:** 587 (July) 1937.

50. Abt, A. F.: Newer Knowledge of Vitamin C. Radiol. Rev. & Mississippi Valley M. J. **59:** 146 (May) 1937.

51. Hess, A. F., and Unger, L. J.: Experiments on Antiscorbutics; Report of an Antiscorbutic for Intravenous Use, Proc. Soc. Exper. Biol. & Med. **15:** 141 (May) 1918.

52. Jackson, Deborah, and Park, E. A.: Congenital Scurvy: Case Report, J. Pediat. **7:** 741 (Dec.) 1935.

53. Goettsch, Elvira: Treatment of Infantile Scurvy with Cevitamic Acid, Am. J. Dis. Child. **49:** 1441 (June) 1935.

54. Aub, J. C.: The Use of Calcium and the Choice of a Calcium Salt, J. A. M. A. **109:** 1276 (Oct. 16) 1937.

55. Hess, A. F.: Infantile Scurvy, in System of Pediatrics (I. A. Abt, Editor), Philadelphia, W. B. Saunders Company, 1923, vol. II, chapter 50, p. 849.

56. Hagmann, E. A.: Active Scurvy in an Infant Receiving Orange Juice, J. Pediat. **11:** 489 (Oct.) 1937.

orally failed to cure the scurvy, while vitamin C given intravenously caused an effective therapeutic response. Hagmann attributes the failure of the orally administered vitamin to effect a cure to a temporary faulty absorption from the gastrointestinal tract.

It has been noted that the urinary excretion of vitamin C in scurvy is low. Schultzer[57] has recently reported an average daily excretion of 11 mg. in the urine of a scorbutic patient before treatment. After daily intravenous injections of 40 mg. of the crystalline vitamin, the twenty-four hour urinary excretion rose to 16 mg. in the second week and to 26 mg. in the fourth week. Schultzer points out that on the basis of earlier experience the oral administration of 15 mg. of vitamin C daily was considered an adequate protective dose. Since a daily intravenous injection of 40 mg. of the vitamin was found to be an adequate curative dose, he suggests that the daily human requirement may be less than 40 mg. It has been[58] pointed out that, in the guinea pig, parenterally administered vitamin C is approximately twice as effective as a similar dose given orally.

Zilva,[59] employing the urinary excretion tests in experimental animals, casts doubt on the nature of this latent condition.

In our work we have repeatedly pointed out that fasting blood plasma levels parallel the vitamin C intake. In healthy individuals on what is considered an adequate vitamin C intake the blood plasma value will be 0.7 mg. per hundred cubic centimeters or above. Blood plasma values below 0.7 mg. per hundred cubic centimeters are subnormal or at least suboptimal. Active scurvy may occur with values ranging up to 0.4 to 0.5 mg. per hundred cubic centimeters (chart 2). Most authors reporting on blood plasma determinations are in close agreement with these figures with the exception of Ingalls,[60] whose figures are very low: from 0.15 to 0.30 mg. per hundred cubic centimeters of blood plasma in asymptomatic scurvy and from 0 to 0.15 mg. in active scurvy. The low values obtained by this author may be due to the fact that potassium cyanide[4,6] was added to the blood, which was in some cases permitted to stand instead of being immediately deproteinized with metaphosphoric acid and titrated with 2:6 dichlorophenolindophenol. It remains for future investigation to prove or disprove the existence of a latent or prescorbutic state. With respect to the development of human scurvy, it should be emphasized that scurvy may surreptitiously develop in patients under treatment for other conditions, if adequate protective vitamin C content of the diet has been overlooked.

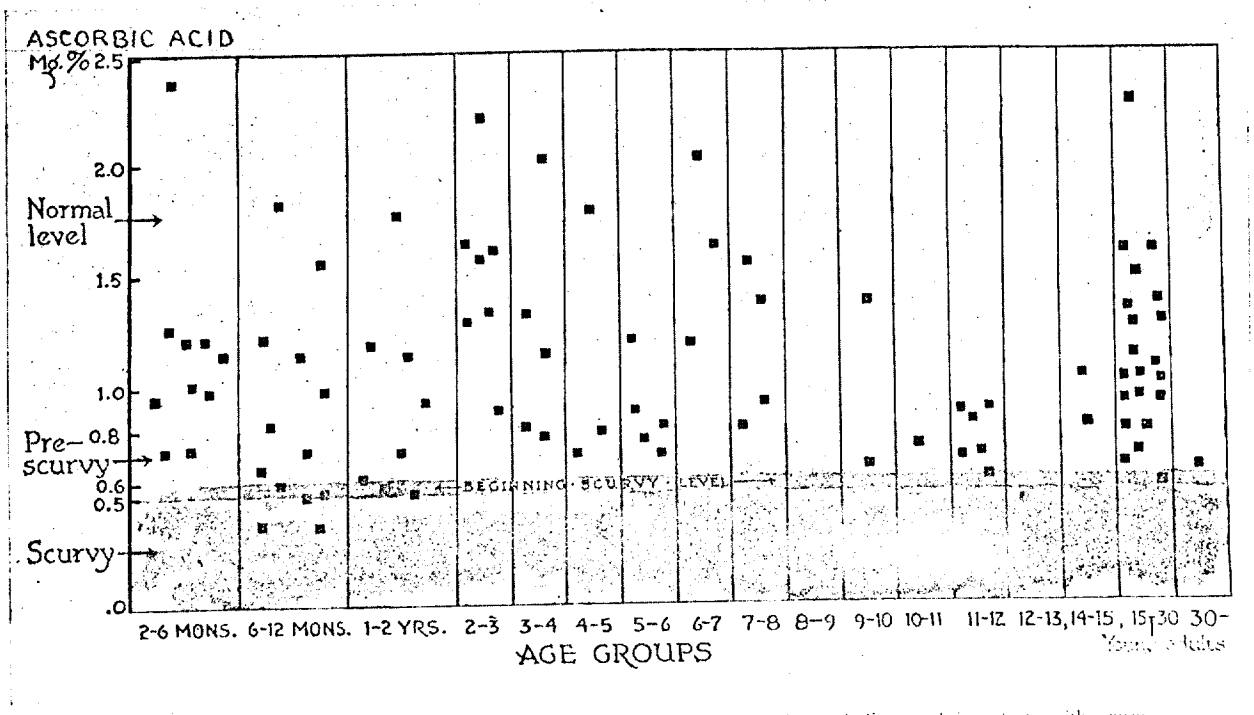


Chart 2.—Range of blood values for different age groups on adequate and subnormal diets, and in patients with scurvy.

LATENT SCURVY

The question of a prescorbutic state or latent scurvy was first emphasized by Hess[59] in 1917. It was his contention that this state might be analogous to latent tetany. Hess and succeeding observers attempted to demonstrate this condition by the use of various tests for capillary fragility in the skin. Since the chemical isolation of the vitamin, various observers have attempted, through urinary excretion tests, to demonstrate this condition. As we have previously stated,

ANEMIA AND VITAMIN C

Mettier, Minot and Townsend[61] reported that anemia is commonly found in adults with a vitamin C deficiency. They believe that vitamin C can have a

57. Schultzer, P.: Saturation of a Scurvy Patient with Small Doses of Ascorbic Acid, Biochem. J. **31**: 1951 (Nov.) 1937. Abt, A. F., and Farmer, C. J.: Exhibit on Vitamin C, Kansas City Session A. M. A., May 1936.

58. Hou,[32] Wright, I. S.; Lilienfeld, Alfred, and MacLenathen, Elizabeth: Determination of Vitamin C Saturation, Arch. Int. Med. **60**: 264 (Aug.) 1937.

59. Hess, A. F.: Subacute and Latent Infantile Scurvy: The Cardiorespiratory Syndrome (A New Sign), J. A. M. A. **68**: 235 (Jan. 27) 1917.

60. Ingalls, T. H.: Studies on Urinary Excretion and Blood Concentration of Ascorbic Acid in Infantile Scurvy, J. Pediat. **10**: 577 (May) 1937.

61. Mettier, S. R.; Minot, G. R., and Townsend, W. C.: Scurvy in Adults, Especially Effect of Food Rich in Vitamin C on Blood Formation, J. A. M. A. **95**: 1089 (Oct. 11) 1930.

specific effect on erythropoiesis when there has been a chronic lack of this vitamin. Anemia is more frequently associated with scurvy in the adult than in the infant, judging from clinical reports. Alfred Hess[62] noted that anemia was not constantly associated with infantile scurvy; in fact, in some of his scorbutic infants he reported a polycythemia. Rohmer and Bindschedler[63] reviewed the literature, noted the disagreement of the various authors on the association of anemia with infantile scurvy, and concluded that anemia is not an essential symptom of vitamin C deficiency. Hans Aron[64] also reported that anemia is not always associated with scurvy and that when it occurs it is not due to loss of blood, as was formerly believed. He stated also that in many instances it is difficult to determine whether one is dealing with a scurvy accompanied by anemia or an alimentary anemia complicated by scurvy. Parsons and Hawksley[65] suggested that hematopoiesis does not occur in the absence of vitamin C. They believed that in some cases of deficiency anemia more than one factor may be lacking and stated that "a child suffering from nutritional anemia may also develop scurvy, and the anemia of scurvy, in which case for its complete cure vitamin C as well as iron will be required." It is the opinion of Cooley[66] that there is no direct relationship between any of the avitaminoses and a definite type of anemia. Gingold[67] concluded that ascorbic acid has no effect on the normal or pathologic hemopoietic organs and is not, therefore, indicated in the treatment of pernicious anemia, secondary anemias or leukemias.

We have recently noted a 10 months old infant suffering with scurvy who was being fed on a diet containing a mixture of numerous proprietary products, so that the diet was high in calcium, iron and vitamins A, B_1 and D; the diet lacked only vitamin C. The infant was of a ruddy complexion, the red blood count was 4,800,000 and the hemoglobin 75 per cent (Sahli). The conditions in the case corresponded to our experimental experience with young guinea pigs, namely that anemia does not develop either clinically or experimentally when an adequate supply of iron is present in the scorbutic diet. When anemia accompanies lack of vitamin C it is probably due to a generally deficient diet in which substances other than vitamin C, especially iron, have been lacking. An accompanying infection may be a causative factor in the production of such anemia. While it is felt by some that the anemia of vitamin C deficiency may be one of commission, the result of a dysfunctioning of the red blood cell-forming elements, it is probably more often a condition produced by omission, namely of iron, in the generally deficient diet.

Heilmeyer[68] injected iron ascorbate intravenously in patients suffering from secondary anemia and noted good results, especially in those patients in whom oral administration of iron was interfered with by metabolic disturbances. He further administered iron ascorbate orally and noted a better absorption with this preparation than with other iron products. He was able to obtain a full iron effect with doses of from 300 to 600 mg. daily. Glanzmann[69] has recommended iron ascorbate in the treatment of infantile anemia of the alimentary type. Pijoan[70] later made a similar report. It is evident that the need of a preparation such as iron ascorbate is limited.

LEUKEMIA AND VITAMIN C

Stephens and Hawley[71] have noted that the vitamin C content of leukocytes is considerably greater than that of the plasma or erythrocytes. They noted an extremely high value for the ascorbic acid of the whole blood in leukemias. Eufinger and Gachtgens[72] have reported the treatment of a case of myeloid leukemia in which the white count was brought back to normal by the injection of 2,000 mg. of ascorbic acid.

RELATION OF VITAMIN C TO HEMORRHAGIC DISEASES

Following the isolation of vitamin C in crystalline form there was a great impetus for investigations of its therapeutic value in hemorrhagic diseases and hemorrhage in other diseases. At first, numerous reports appeared claiming good results from the use of vitamin C in symptomatic purpura, thrombopenic purpura, Schönlein's and Henoch's purpuras, and hemophilia. Favorable claims also were made for such conditions as leukemia, acute hemorrhagic nephritis, metrorrhagia, pulmonary and gastric hemorrhages, and intestinal hemorrhage in ulcerative colitis and following perforations in typhoid. These were usually accounts of success in isolated cases.[73] It soon became evident that these early enthusiastic claims could not be substantiated.

In examining several patients with thrombopenic purpura whose blood components had been studied for a considerable time prior to the therapeutic attempts with vitamin C, we were able, with Dr. Mila Pierce,[74] to demonstrate no appreciable improvement in the thrombopenia. The capillary resistance of the skin remained abnormally low after the administration of large doses of vitamin C both orally and intravenously. Several investigators[75] in this country have similarly been unable to report beneficial effects after treatment with vitamin C in cases of true thrombopenic purpura and hemophilia.

62. Hess, A. F.: Scurvy, Past and Present, Philadelphia, J. B. Lippincott Company, 1920.

63. Rohmer, P., and Bindschedler, J. J.: L'anémie préscorbutique du nourrison, Acta. pediat. **13:** 399 (June) 1932.

64. Aron, Hans: Die Nährschäden des Kindes, Berlin and Vienna, Urban and Schwarzenberg, 1928, p. 105.

65. Parsons, L. G., and Hawksley, J. C.: Studies in Anemias of Infancy and Early Childhood; Anhematopoietic Anemias (Deficiency Diseases of Erythron); Nutritional Anemia and Anemias of Prematurity, Scurvy and Celiac Disease, Arch. Dis. Childhood **8:** 117 (April) 1933.

66. Cooley, Thomas B.: Practice of Pediatrics, Joseph Brennemann, Editor, Hagerstown, Md., W. F. Prior Company, 1937, vol. III, chapter 16, p. 9.

67. Gingold, N.: The Action of Ascorbic Acid (Vitamin C) on the Hemopoietic Apparatus Under Normal and Pathologic Conditions (Exclusive of Hemorrhagic States), Sang **11:** 392, 1937.

68. Heilmeyer, H.: Die Behandlung eisen empfindlicher Anämien mit

69. Glanzmann, E.: Zur Behandlung der Kinderänamien mit ascorbinsauren Eisen, Schweiz. Med. Wchnschr. **67:** 436 (May 15) 1937.

70. Pijoan, M.: Antiscorbutic Properties of a Salt of Iron and Ascorbic Acid, Science **86:** 80 (July 23) 1937.

71. Stephens, D. J., and Hawley, E. E.: Partition of Reduced Ascorbic Acid in Blood, J. Biol. Chem. **115:** 653 (Oct.) 1936.

72. Eufinger, H., and Gachtgens, G.: Ueber die Einwirkung des Vitamin C auf das Pathologisch veränderte weisse Blutbild, Klin. Wchnschr. **15:** 150 (Feb. 1) 1936.

73. Boger, A., and Schröder, H.: Ueber die Stillung schwerster Blutungen bei allen Formen der hemorrhagischen Diathese und der Hemophilie durch parenterale Zufuhr von C-Vitamin, München. med. Wchnschr. **81:** 1335 (Aug. 24) 1934. Vogt, E.: Ueber die Behandlung gynäkologischer Blutungen mit Vitamin C, ibid. **82:** 263 (Feb. 14) 1935. Engelkes, N.: Treatment of Hemorrhagic Disorders with Vitamin C, Lancet **2:** 1285 (Dec. 7) 1935.

74. Abt, A. F.: Physiology of Ascorbic Acid in Normal and Abnormal States, J. Physiol. U. S. S. R. **22:** 807 (July) 1937.

75. Wright, I. S., and Lilienfeld, Alfred: Pharmacologic and Therapeutic Properties of Crystalline Vitamin C (Cevitamic Acid) with Especial Reference to Its Effects on Capillary Fragility, Arch. Int. Med. **57:** 241 (Feb.) 1936. Stephens, D. J., and Hawley, E. E.: Relationship of Vitamin C to the Hemorrhagic Diatheses, J. Lab. & Clin. Med. **22:** 173 (Nov.) 1936. Finkle, Philip: Vitamin C Saturation Levels in Body in

In a recent review on the hemorrhagic states, Witts[76] corroborated the negative therapeutic effect of vitamin C in nonscorbutic hemorrhagic states. This author mentioned the possibility of scurvy being associated with a hemorrhagic disease, the disappearance of some of the symptoms following treatment with the vitamin being due to its effect on the associated scurvy. An interesting report by Neuweiler[77] concerns the vitamin C content of the blood serum of women during normal menstruation. In this study the author found that the vitamin C content of the blood serum did not vary during the menstrual periods of three normal women. Szent-Györgyi and others,[78] in their early work with impure preparations of vitamin C, believed that successful therapeutic effects were attained in some of the nonscorbutic hemorrhagic diseases. He and his co-workers subsequently isolated a flavin which they term "vitamin P" and which they believed was responsible for the effect. In a recent report they have stated that this substance requires for its activity the presence of traces of ascorbic acid and, in the entire absence of ascorbic acid, vitamin P is inactive. According to these results it may be concluded that pure vitamin C has a negative therapeutic action on hemorrhagic diseases of a nonscorbutic etiology. The evidence for the existence of vitamin P is doubtful, however, and it is clear that further work will have to be done in this field before conclusions can be reached.

RELATION OF VITAMIN C TO OTHER CONDITIONS

Teeth.—Because gingivitis and hemorrhage into the gums constitute such a striking symptom of scurvy, the claim that vitamin C has a direct relationship with the well-being of the teeth has been made for a long period. Hanke,[79] in a study on children, concluded that the addition of 1 pint of orange juice and the juice of one lemon to a diet lacking in vitamin C, but otherwise normal, led in many cases to disappearance of gingivitis and an arrest or a reduction of the intensity of dental caries. Keeton[80] has administered 400 mg. of vitamin C daily for four or five days prior to the extraction of a tooth and reported that the effect on healing is striking.

Most of the evidence that vitamin C is related to normal development of the teeth has been deduced largely from experimental work on the guinea pig. The incisor teeth of the guinea pig differ from those of man in that they are constantly growing. One is led to question, therefore, the permissibility of carrying over to man, without reservation, all experimental evidence on this point.

Fish and Harris[81] in their recent work on guinea pigs hold untenable the view of the earlier writers, who noted the formation of "pulp bones" in partial vitamin C deficiency and regarded this occurrence as a type of neoplastic change. By use of a technic in which a series of deficient and normal diets were fed, they noted that vitamin C is necessary for promoting the functional activity of formative cells such as the odontoblasts, ameloblasts and osteoblasts. They noted that in mild chronic vitamin C deficiency the "pulp bone" is the result of a partial degeneration of the odontoblasts, forming a scar tissue.

Wolbach[11] has pointed out that in the growing teeth of guinea pigs a lack of vitamin C causes the formation of dentin to cease and the pulp to become separated from the dentin by a liquid presumably produced by the odontoblasts. In the healing process in guinea pigs the formation of dentin is resumed. Wolbach concluded that, in infants, teeth in the process of formation may possibly be affected by scurvy in a manner similar to that noted in the guinea pig, but that specific evidence of this in the human being is lacking. Hou[82] has reported similar observations in the scorbutic guinea pig. As evidence of a change in mineral content, Toverud[83] has shown that the incisor teeth of scorbutic guinea pigs contain less ash than those of normal animals. Furthermore, the ash is lower in calcium and higher in magnesium that that from teeth of normal controls. The older writers recognize the loosening of teeth in the scurvy of both man and animals, and Boyle, Bessey and Wolbach[84] more recently have pointed out that, besides striking alterations in tooth pulp and dentin, there may be changes in the peridental soft and calcified tissues. They concluded that there may be two types of pyorrhea, a local inflammatory disease and a systemic process causing diffuse atrophy of the alveolar bone. This systemic type of pyorrhea, as it occurs in infantile scurvy, is similar to that found in guinea pigs on a vitamin C-deficient diet. In a limited number of patients with this type of pyorrhea, they have noted a correlation between low blood ascorbic acid values and rarefaction of the alveolar bone. They believe that a low vitamin C intake is an important factor in the production of this type of systemic pyorrhea. The authors made no statement as to the therapeutic effect which vitamin C might have on this type of pyorrhea. It is probable that the beneficial effects noted by Hanke after the addition of large amounts of orange juice were due to amelioration of this systemic type of pyorrhea known to be caused by deficiency of the vitamin. Although there is still a dearth of exact knowledge of vitamin C in its relation to dental and gingival disease in man, there is a general unanimity of opinion that an adequate intake of vitamin C is necessary for normal tooth growth and tooth structure, and the maintenance of healthy gums in man.

Eye.—Whether deficiency of vitamin C has any significance in the etiology of cataract has been a matter of dispute. Various workers have found that, as in other tissues of the body, the concentration of vitamin C in the crystalline lens of scorbutic guinea pigs is lower than normal.[85] Low values have been reported in the cataractous lens.[86] Some investigators have been able to produce cataract in some guinea pigs by feeding them

76. Witts, L. J.: The Hemorrhagic States, Brit. M. J. **2**: 689 (Oct. 9) 1937.
77. Neuweiler, W.: Vitamin C-Spiegel in Blute und Menstruation, Klin. Wchnschr. **16**: 926 (Sept. 4) 1937.
78. Szent-Györgyi, A.; Armento, S.; Bentsath, A.; Beres, T., and Rusznyak, S.: Ueber den Einfluss von Substanzen der Flavongruppe auf die Permeabilität der Kapillaren, Vitamin P, Deutsche med. Wchnschr. **62**: 1325 (Aug. 14) 1936.
79. Hanke, Milton H.: Diet and Dental Health, University of Chicago Press, 1933, p. 143.
80. Keeton, R. W.: Relation of Metabolic Diseases to Dentistry, J. Am. Dent. A. **24**: 1336 (Aug.) 1937.
81. Fish, E. W., and Harris, L. J.: The Effects of Vitamin C Deficiency on Tooth Structure in Guinea Pigs, Phil. Tr. Roy. Soc. London, 223B, 1933, p. 489.
82. Hou, H. C.: A Comparative Study of the Vitamin C Content of Several Varieties of Chinese Oranges, Chinese J. Physiol. **9**: 223 (Aug. 15) 1935.
83. Toverud, G.: The Influence of Diet on Teeth and Bone, J. Biol. Chem. **58**: 358 (Dec.) 1923.
84. Boyle, P. E.; Bessey, O. A., and Wolbach, S. B.: Experimental Alveolar Bone Atrophy Produced by Ascorbic Acid Deficiency and Its Relation to Pyorrhea Alveolaris, Proc. Soc. Exper. Biol. & Med. **36**: 733 (June) 1937.
85. Von Euler, H., and Malmberg, M.: Ueber die Antiskorbutische Wirkung der Augenlinsen und über ihren Gehalt an Reduktonen und Sulfhydrylen, Ztschr. f. physiol. Chem. **230**: 225 (Oct.) 1934. Johnson.
86. Von Euler, H., and Martius C.: Ueber den Gehalt der Augenlinsen und Sulfhydrylverbindungen und an Ascorbinsäure, Ztschr. f. physiol. Chem. **222**: 65 (Oct.) 1933.

a scurvy-producing diet,[87] especially if the aqueous humor was drained by paracentesis, but others have not been able to repeat the experiment.[88] Vitamin C is present in the normal lens and vitamin C depletion might conceivably predispose the lens to a greater degree of susceptibility to agents causing cataract which ordinarily do not affect the normal eye. Bellows[89] has studied the level of vitamin C in the blood of cataractous patients and found it to be definitely lower on the average than that of noncataractous persons of similar social status and age group (from 60 to 80 years).

Skin. Vitamin C apparently has a definite relation to pigmentation of the skin. A lessened pigmentation in patients suffering with Addison's disease has been observed[90] after vitamin C had been administered for some time. Schroeder and Einhauser[91] administered 300 mg. of vitamin C intravenously for fourteen days and noticed a decreased pigmentation of the face in a case of pernicious anemia. Cornbleet[92] noted that the excess pigment in the skin in Addison's disease and in scurvy is absorbed when vitamin C is administered. Volpe[93] treated several patients suffering with psoriasis with large doses of ascorbic acid and noted striking improvement. We ourselves have noted depigmentation of the skin following massive vitamin C therapy in Addison's disease. In the case of a Negro, a daily oral dose of 450 mg. over a period of several months produced a noticeable mottling depigmentation below the eyes. It should be noted that large doses, far beyond the normal requirement, have been used to produce these therapeutic results.

The suggestion has been made[19] that vitamin C may be specific in lupus erythematosus as well as in scurvy.

ENDOCRINE DISTURBANCES

Several observers have pointed out the possibility of a relationship between ascorbic acid and thyroid gland function. Demole and Ippen[94] have demonstrated that loss of weight in guinea pigs on a scorbutic diet could be checked by the administration of 0.5 mg. of ascorbic acid daily. If, however, the animals received 0.1 mg. of thyroxin subcutaneously, the weight loss continued until the amount of daily oral ascorbic acid administration had been raised to from 10 to 20 mg. Demole and Ippen interpreted this as evidence of the antithyrotoxic action of ascorbic acid. Heyl[95] has reported a morphologic change in the thyroid gland induced by ascorbic acid similar in appearance to that obtained by the injection of the thyrotropic hormone of the anterior pituitary lobe. If the extract of the anterior pituitary is subjected to oxidative processes, shown by control experiment to be severe enough to destroy ascorbic acid, the pituitary extract still remained active. The author concluded that, in the case of the thyrotropic action of the anterior pituitary, a substance other than vitamin C is concerned. Spence and Scowen[96] have shown that the daily administration of large doses of ascorbic acid does not prevent the occurrence of thyroid hyperplasia in guinea pigs receiving injections of extract of anterior pituitary containing thyrotropic hormone. Marine and his associates[97] have noted the protective action of ascorbic acid against hypertrophy of the thyroid gland in guinea pigs injected with the thyrotropic factor. Sure and Theis[98] indicate that in experimental hyperthyroidism produced in rats by feeding toxic doses of thyroxin a vitamin C disturbance occurred as evidenced by the reduction of the vitamin C content of the adrenal, the thymus and the kidney, similar to that encountered in scurvy in the guinea pig. According to Paal and Brecht,[99] the administration of thyroxin and thyrotropic hormone to guinea pigs and rats leads to a rise in the ascorbic acid content of the adrenal gland and liver. When cortical extract free from ascorbic acid was given together with these substances, no such rise occurred. These controversial reports indicate the need for further experimental work on the relationship between vitamin C and the thyroid gland.

The relationship of vitamin C to dextrose tolerance in the guinea pig was studied by Sigel and King;[100] successive stages of vitamin C depletion induced a corresponding rise in the fasting blood sugar level and a distinctly lowered dextrose tolerance. Pfleger and Scholl[101] have noted that in diabetic patients saturation with vitamin C intensifies the action of insulin, so that the carbohydrate metabolism of the patient could be regulated with smaller doses of insulin. They also noted that increased vitamin C ingestion lessened fatigue in diabetic patients.

The relationship between vitamin C and Addison's disease has been studied. As shown by Harris and Ray,[102] both the cortex and the medulla of the normal adrenal gland have a high ascorbic acid content. In three cases of Addison's disease studied by Wilkinson and Ashford,[90] using the urinary test of Harris and Ray, there appeared to be a vitamin C subnutrition. Wilkinson's patients were being simultaneously treated with small doses of adrenal cortex extract and vitamin C. On this combined therapy a decrease in pigmentation of the skin was noted in two cases three months after discharge. Hoff[103] has also noted a decrease in pathologic pigmentation in Addison's disease.

In two patients with Addison's disease brought to our laboratory by Dr. Carl Johnson, daily oral administration of 450 mg. of ascorbic acid alone caused considerable depigmentation after from six to ten weeks but failed to improve other addisonian symptoms. This

87. Manjukowa, N. K., and Frandkin, M. J.: Neue experimentelle Beiträge über die Pathogenese der Katarakt, Arch. f. Ophth. **133:** 378, 1935.

88. Johnson, S. W.: Cataract and Ascorbic Acid in Guinea Pig Eye, Biochem. J. **30:** 1450 (Aug.) 1936.

89. Bellows, J. G.: Biochemistry of Lens: Cevitamic Acid Content of Blood and Urine of Subjects with Senile Cataract, Arch. Ophth. **15:** 78 (Jan.) 1936.

90. Wilkinson, J. F., and Ashford, C. A.: Vitamin C Deficiency in Addison's Disease, Lancet **2:** 967 (Oct. 24) 1936. Hoff.[103]

91. Schroeder, H., and Einhauser, M.: Ueber einen Zusammenhang zwischen gestörter Vitamin-C-Resorption und pathologischer Pigmentierung bei Gastroenteritis und Achylia gastrica, München. med. Wchnschr. **83:** 925 (June 5) 1936.

92. Cornbleet, Theodore: Vitamin C and Pigment, Arch. Dermat. & Syph. **35:** 471 (March) 1937.

93. Volpe, L.: Ueber weitere Erfolge in der Psoriasis-Behandlung mit Vitamin C, Schweiz. med. Wchnschr. **67:** 498 (May 29) 1937.

94. Demole, V., and Ippen, E.: Die Antithyreotoxische Wirkung von Ascorbinsäure, Ztschr. f. physiol. Chem. **235:** 226, 1935.

95. Heyl, J. G.: Thyreotroper Effekt von Vitamin C (Ascorbinsäure) und Unabhängigkeit der thyreotropen Wirkung des Hypophysen vorder-

96. Spence, A. W., and Scowen, E. F.: The Effect of Ascorbic Acid on Experimental Goiter, Biochem. J. **29:** 562 (Jan. 15) 1935.

97. Marine, David; Baumann, E. J., and Rosen, S. H.: Effect of Ascorbic Acid on Thyroid and Suprarenals of Guinea Pigs, Proc. Soc. Exper. Biol. & Med. **31:** 870 (April) 1934.

98. Sure, Barnett, and Theis, R. M.: Hyperthyroidism and Vitamin C, Proc. Soc. Exper. Biol. & Med. **37:** 646 (Jan.) 1938.

99. Paal, H., and Brecht, K.: Ascorbinsäure und Schilddrüsenfunktion, Klin. Wchnschr. **16:** 261 (Feb. 20) 1937.

100. Sigel, A., and King, C. G.: Relationship of Vitamin C to Glucose Tolerance in Guinea Pig, J. Biol. Chem. **116:** 489 (Dec.) 1936.

101. Pfleger, R., and Scholl, F.: Diabetes and Vitamin C, Wien. Arch. f. inn. Med. **31:** 109 (Oct. 31) 1937.

102. Harris, L. J., and Ray, S. N.: Vitamin C in the Suprarenal Medulla, Biochem. J. **27:** 2006 (No. 6) 1933.

103. Hoff, F.: Beiträge zum Problem der krankhaften Hauptpig-

is in agreement with the observation of Siwe,[104] who also noted a decreased pigmentation without relief from the characteristic symptoms of Addison's disease.

GASTROINTESTINAL DISEASES

We have already referred to the subject of absorption, destruction and elimination of vitamin C in the gastrointestinal tract. Alt, Chinn and Farmer[105] found low blood values for vitamin C in patients suffering with achlorhydria. Einhauser[106] has reported an altered absorption of vitamin C in patients suffering with catarrhal gastro-enteritis. Although Stepp[107] points out that gastrointestinal disturbances can be the cause as well as the result of lack of vitamins, it is debatable whether a lack of vitamin C can be looked on as a direct contributing factor to gastric ulcer. In Schultzer's[108] studies the improbability of C-avitaminosis as an etiologic factor in peptic ulcer is pointed out. Harris, Abbasy, Yudkin and Kelly[109] and Archer and Graham[110] reported low urinary excretion values for patients suffering with gastric ulcer. However, these low excretion values may have been related to the low dietary content of the diets prescribed for ulcer. Several investigators[111] have recently pointed out that the usual diets given patients suffering with peptic ulcer are likely to be deficient in vitamin C. The exact relationship of this dietary deficiency to hemorrhage from the ulcers is as yet unproved, although it would certainly seem advisable to supplement peptic ulcer diets with vitamin C. Portnoy and Wilkinson,[112] using urinary excretion, blood plasma level and intradermal tests, have shown a marked vitamin C deficiency in patients suffering from peptic ulcer. The severest degree of deficiency was found in patients with hematemesis. We ourselves have noted a mild scurvy develop in several patients on strict ulcer diets, because of a lack of vitamin C in the dietary.

Gachtgens[113] has reported on hypovitaminoses of intestinal origin in pregnancy. He points out that there is an increased vitamin C requirement in pregnancy as the fetus gains its supply of ascorbic acid directly from the food intake of the mother. Gachtgens reports the case of a pregnant woman who, because of a severe ulcerative colitis and a restricted diet, acquired a severe deficiency of vitamin C, and he points out that, whenever gastrointestinal disturbances develop during pregnancy, it may be necessary to supply the maternal organisms with adequate amounts of vitamin C administered parenterally.

RELATION OF VITAMIN C TO IMMUNITY

A considerable amount of experimental work has been published with regard to the relationship of vitamin C to immunity, and this material has recently been comprehensively reviewed.[114] It is not within the scope of this article to take up in detail all the reports covering this subject. Among the first observers to note an altered immunologic response when ascorbic acid was administered in sufficient doses were Sulzberger and Oser.[115] These authors in a controlled experiment noted that large doses of ascorbic acid reduced and inhibited the susceptibility of the skin of the guinea pig to experimental sensitization with neoarsphenamine. Cormia[116] noted that a high vitamin C diet should be of value to all patients receiving arsphenamine and that intensive therapy with vitamin C may be helpful in treating patients with post-arsphenamine dermatitis. Landfisch[117] similarly reported that he was able to treat twenty-five patients who had previously shown definite sensitization to neoarsphenamine and marked symptoms of intolerance when the arsenical was administered alone by combining ascorbic acid injections with neoarsphenamine or by administering the ascorbic acid one-half hour before the neoarsphenamine. Further evidence of the increased tolerance of the organism for arsphenamine after ascorbic acid therapy has been reported recently by Dainow.[118] Vitamin C is apparently of value in controlling the intolerance to arsenicals which must be intravenously administered.

Meyer[119] believes that the toxic symptoms produced by chronic benzene poisoning may be due to an avitaminosis C. Experiments with guinea pigs showed that the toxic effect of an injection of 0.25 cc. of benzene four times a week was counteracted when a daily dose of 20 mg. of ascorbic acid was added to a scorbutic basal diet. As a possible mechanism for this effect, Vauthey[120] has recently suggested that ascorbic acid exerts a stimulatory effect on the liver, resulting in reduced toxicity of the chemical medicament.

Epstein[121] noted that large doses of vitamin C aided in the desensitization of patients suffering with bronchial asthma. Jusatz,[122] using rabbits on a deficiency diet, was able to augment the specific precipitant titer from ten to a hundred fold by adding ascorbic acid to each dose of horse protein intravenously injected. The oral administration of vitamin C failed to increase the specific precipitant titer. Madison and Manwaring[123] have confirmed Jusatz's experiments in rabbits using horse serum plus 100 mg. of crystalline vitamin C. These authors also noted

---

104. Siwe, S.: Das Verhalten der C-Vitamin bei Morbus Addisoni, Klin. Wchnschr. **14:** 1311 (Sept. 11) 1935.

105. Alt, H. L.; Chinn, Herman, and Farmer, C. J.: The Blood Cevitamic Acid in Patients with Achlorhydria, read before the Central Society for Clinical Research, Chicago, Nov. 5, 1937.

106. Einhauser, M.: C-Vitamin und Gastroenteritis, Ztschr. f. d. ges. exper. Med. **98:** 461, 1936.

107. Stepp, W.: Neue Gesichtspunkte in der klinischen Anwendung der rein dargestellten Vitamine, Angewandte Chemie **50:** 30 (Jan. 2) 1937.

108. Schultzer, P.: Studies on Capillary Resistance: Improbability of C-Vitaminosis as Etiologic Factor in Peptic Ulcer, Acta med. Scandinav. **83:** 555, 1934.

109. Harris, L. J.; Abbasy, M. A.; Yudkin, John, and Kelly, Simon: Vitamins in Human Nutrition: Vitamin C Reserves of Subjects of Voluntary Hospital Class, Lancet **1:** 1488 (June 27) 1936.

110. Archer, H. E., and Graham, George: The Subscurvy State in Relation to Gastric and Duodenal Ulcer, Lancet **2:** 364 (Aug. 15) 1936.

111. Rivers, A. B., and Carlson, L. A.: Vitamin C as Supplement in Therapy of Peptic Ulcer: Preliminary Report, Proc. Staff Meet., Mayo Clin. **12:** 383 (June 16) 1937. Easterman, G. B.: Peptic Ulcer, Medical Management, Minnesota Med. **20:** 766 (Dec.) 1937. Ingalls, T. H., and Warren, H. A.: A Symptomatic Scurvy, New England J. Med. **217:** 443 (Sept. 9) 1937.

112. Portnoy, Benjamin, and Wilkinson, J. F.: Intradermal Test for Vitamin C Deficiency, Brit. M. J. **1:** 328 (Feb. 12) 1938.

113. Gachtgens, G.: Die Beziehungen intestinal bedingter Hypovitaminosen zur Schwangerschaft, Klin. Wchnschr. **16:** 411 (March 27) 1937.

114. Robinson, E. C.: The Vitamins and Resistance to Infection, Medicine **13:** 123 (May) 1934. Perla, David, and Marmorston, Jessie: Role of Vitamin C in Resistance, Arch. Path. **23:** 543 (April) 1937; Role of Vitamin C in Resistance, ibid. **23:** 683 (May) 1937.

115. Sulzberger, M. B., and Oser, B. L.: Influence of Ascorbic Acid of Diet on Sensitization of Guinea Pigs to Arsphenamine, Proc. Soc. Exper. Biol. & Med. **32:** 716 (Feb.) 1935.

116. Cormia, F. E.: Experimental Arsphenamine Dermatitis; Influence of Vitamin C in Production of Arsphenamine Sensitiveness, Canad. M. A. J. **36:** 397 (April) 1937.

117. Landfisch, S.: Synthetic Ascorbic Acid New Auxiliary Remedy in Arsphenamine Therapy, Polska gaz. lek. **16:** 575 (July 18) 1937.

118. Dainow, L.: Intolerance aux arsenobenzenes et vitamin C, Presse méd. **45:** 1670 (Nov. 24) 1937.

119. Meyer, A.: Chronische Benzolvergiftung und Vitamin C, Ztschr. f. Vitaminforsch. **6:** 83 (Jan.) 1937.

120. Vauthey, M.: Vitamin C (L-Ascorbic Acid) and Tolerance for Chemical Medicaments, Chem. Abstr. **32:** 995 (Feb. 10) 1938.

121. Epstein, Alexander: Vitamin C in Desensitization; Advances in Vitamin Treatment, Schweiz. med. Wchnschr. **66:** 1087 (Nov. 7) 1936.

122. Jusatz, H. J.: Der Einfluss der Vitamine auf den Immunitätszustand des tierischen Organismus; wasserlösliche Vitamine, Ztschr. f. Immunitätsforsch. u. exper. Therap. **88:** 483 (Aug. 4) 1936.

123. Madison, R. R., and Manwaring, M. H.: Ascorbic Acid Stimulation of Specific Antibody Production, Proc. Soc. Exper. Biol. & Med. **37:** 402 (Nov.) 1937.

that, when the horse serum and ascorbic acid were injected separately, either at different times into the same vein or into different veins, the antibody stimulation was less pronounced than the effect obtained by mixing the horse serum and vitamin C before injection. Lemke[124] has reported that vitamin C, when injected intravenously into hypersensitive guinea pigs, desensitizes them. However, van Niekerk[125] states that vitamin C has no protective action for guinea pigs against anaphylactic shock to horse serum. He noted that no protective action resulted when the second dose of horse serum was combined with from 25 to 100 mg. of ascorbic acid. Hochwald[126] states that, if 100 mg. of vitamin C is injected into sensitized guinea pigs a few minutes before the shocking dose, no anaphylaxis develops. Solomonica[127] observed that, if his guinea pigs were injected with vitamin C prior to or during the sensitizing period, many of them survived. The protective effects of ascorbic acid were much lessened if the vitamin was given later, e. g. shortly before the shocking dose.

Vitamin C has recently been related to the complement content of the blood.[128] Ecker[129] and his co-workers have shown a definite correlation between the complement titer of guinea pig serum and the vitamin C content of the blood serum. When the animals were placed on a scorbutic diet the complement titer dropped, paralleling the decrease in ascorbic acid blood values. The values rose when vitamin C was fed to the scorbutic animals.

Jungeblut[130] noted that vitamin C, when added to tetanus toxin in vitro, inactivates the toxin though it fails to confer complete protection against tetanus intoxication in vivo. This author furthermore states that, as far as its in vitro action is concerned, vitamin C cannot be regarded as a specific detoxicant for any given virus or toxin.

It therefore seems probable that vitamin C, particularly when administered intravenously, is capable of protecting the body against certain drug intolerances, as noted for the arsenicals, of stimulating experimental antibody production, and perhaps of protecting against experimental anaphylaxis. The failure of oral administration is interesting and requires further experimental work for its explanation. The whole field of the relationship of vitamin C to natural resistance and immunity is but in its infancy, and much more experimental and clinical evidence must be accumulated before any definite therapeutic claims seem justifiable.

INFECTIONS AND INFECTIOUS DISEASES

Recent reviews suggest that a low vitamin C intake may be one of the causes of lowering natural resistance. There is evidence also that in the course of certain infections there is a greater demand for vitamin C.[131] There have been numerous suggestive reports of the possible role of vitamin C in infectious diseases but the evidence is not yet clearcut. The effect of vitamin C on the integrity of the tissues is probably concerned with its role in the production of normal intercellular cement substances. This in turn may be prerequisite to the maintenance of natural resistance of the organism to infection.

Evidence showing the desirability of trying the effect of administering vitamin C, along with other treatment of infections, has been obtained chiefly by studying the vitamin C content of the blood plasma and the amount of vitamin C excreted in the urine. Low values for either or both have been observed in mild coryzal fever, acute pharyngitis, persistent chronic nasal sinusitis, chronic otitis media, acute respiratory infection, active osteomyelitis, rheumatoid arthritis, and rheumatic fever in children.

The administration of vitamin C has been tested in a number of disease conditions, and favorable reports have appeared in pneumonia, pertussis and rheumatic fever. Negative results have been reported in poliomyelitis, and the results are inconclusive in diphtheria.

It is evident that vitamin C is not a specific therapeutic agent in the treatment of any of these diseases. It should not be forgotten, however, that the importance of an ample diet has long been recommended in the treatment of febrile conditions, and such patients have perhaps an increased requirement for all of the vitamins.

WOUND HEALING AND SURGICAL CONDITIONS

Hans Aron[61] noted that in scorbutic children wounds of the skin often remained open without a tendency to heal. Following therapy with vitamin C, he observed that these wounds healed quickly. In a 6 weeks old infant whose laparotomy wound opened postoperatively, Lanman and Ingalls[132] noted a definite early scurvy at autopsy. The authors then performed laparotomy on guinea pigs which had been on previous scorbutic diets. At intervals after operation the animals were killed and the authors report that the wounds broke down more easily in the scorbutic pigs than in the nonscorbutic controls. On histologic examination they noted defective repair of the corium and a poor production of collagen in the scars of the scorbutic animals. This is in agreement with the role which vitamin C plays in the formation of collagen. Taffel and Harvey[133] have recently confirmed the work of Lanman and Ingalls and expressed the opinion that vitamin C deficiency in man may indeed be one of the major causative factors in these cases of wound disruption where there is no evidence of infection of the wound. From the evidence presented one may conclude that vitamin C has an important role in the healing of surgical wounds. When a history of low vitamin C intake or a low plasma value is obtained preoperatively, it would seem logical to administer vitamin C preoperatively and postoperatively. Hanke[134] noted apparent acceleration of bone healing in fractures, following administration of vitamin C. Lauber, Bersin

124. Lemke, H.: Beeinflussung des anaphylaktischen Shocks der Meerschweinchen durch C-Vitamin, Monatschr. f. Kinderh. **67:** 244 (Nov.) 1936.

125. van Niekerk, J.: Anaphylaxis and Vitamin C, J. Allergy **8:** 446 (July) 1937.

126. Hochwald, A.: Allergiefragen und vitamin C, Zentralbl. f. inn. Med. **56:** 769 (Sept. 21) 1935.

127. Solomonica, Bruno: Vitamin C and Anaphylactic Shock in Guinea Pigs, J. Immunol. **31:** 209 (Sept.) 1936.

128. Vitamin C and Complement, editorial, J. A. M. A. **110:** 2157 (June 25) 1938.

129. Ecker, E. E.; Pillemer, L.; Wertheimer, D., and Gradis, H.: Ascorbic Acid and Complement Function, J. Immunol. **34:** 19 (Jan.) 1938.

130. Jungeblut, C. W.: Inactivation of Tetanus Toxin by Crystalline Vitamin C (l-Ascorbic Acid), J. Immunol. **33:** 203 (Sept.) 1937.

131. The clinical reports have been most numerous. The reader is

132. Lanman, T. H., and Ingalls, T. H.: Vitamin C Deficiency and Wound Healing: Experimental and Clinical Study, Ann. Surg. **105:** 616 (April) 1937.

133. Taffel, M., and Harvey, S. C.: Effect of Absolute and Partial Vitamin C Deficiency on Healing of Wounds, Proc. Soc. Exper. Biol. & Med. **38:** 418 (May) 1938.

134. Hanke, H.: Experimentelle Untersuchungen über Beeinflussung

and Nafziger[135] report an increased demand for vitamin C in infection of a surgical nature. Myerson[136] suggests that vitamin C deficiency is one of the possible factors in hemorrhage following tonsillectomy.

CONCLUSIONS

Vitamin C is definitely indicated as a therapeutic agent in the prevention and cure of scurvy. Whether vitamin C has a definite causal relationship to any other pathologic process is as yet uncertain. The vast amount of investigative and clinical research reported to date seems to be indicative of a further therapeutic role for vitamin C, though not enough progress has been attained to allow definite therapeutic conclusions to be formulated. Certain suggestive conditions may be briefly outlined when, aside from the prevention and cure of scurvy, vitamin C may be therapeutically indicated.

An ample supply is indicated in the diet of the pregnant woman and nursing mother. If the possibility of a prescorbutic state is accepted or if the existence of latent scurvy is recognized, it is imperative that adequate diagnostic methods and criteria be formulated in order to prevent the development of such states, which would be conditions of suboptimal nutrition and possibly deleterious to general health. We believe that fasting blood plasma values are to date the best method for determining the possibility of such a state. In order to prevent the occurrence of such a possibility, the minimal human requirement for vitamin C must be definitely known. Whether this requirement varies for different ages and different individuals is unknown.

The question of absorption following oral ingestion is of the utmost importance. Absorption has been entirely overlooked by most investigators and, when mentioned in the literature, has been discussed chiefly as a theoretical possibility. Little or nothing is known concerning the assimilation of vitamin C and its metabolism in abnormal and normal states. The implication that there is an increased need and an augmented metabolism for vitamin C in many infections is generally noted in all the numerous reports available. The exact proof for this important fact is lacking. How fever, toxemia and specific bacteria act on vitamin C in individual infections is still unknown. How vitamin C is absorbed, assimilated and utilized in specific infections is still unknown. The relation of vitamin C to the endocrines has been noted, and there are implications that it may be specifically related to hyperthyroidism, diabetes and Addison's disease. The exact facts are still lacking; this is more or less true of the other specific conditions here reviewed.

The failure of the oral administration of vitamin C in certain conditions in which its parenteral administration gives positive experimental or clinical results, and the positive results following its use in extremely large doses when its use in small doses was unavailing, are paradoxical questions which need elucidation.

Perhaps the value of a view on the therapeutic indications and implications for vitamin C at this time lies in its urge to spur us on to fill the gaps in our present knowledge which, it will be hoped, are as evident to the reader as they have been to the reviewers.

135. Lauber, H. J.; Bersin, T., and Nafziger, H.: Der Ascorbinsäurebedarf bei chirurgischen Infectionen, Klin. Wchnschr. **16**: 1274 (Sept.) 1937.

136. Myerson, M. C.: Hemorrhage Following Tonsillectomy, Am. J. Surg. **36**: 151 (April) 1937.

J. NUTR. 70(4): 427-37, 1960.

0056

Jour Nutrition 70(4) 427-437 Illus 1960

Connective Tissue Studies[1,2]

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

ARTHUR F. ABT, SUSANNE VON SCHUCHING AND JOSEPH H. ROE
Radioisotope Service, Veterans Administration Center, Martinsburg, West Virginia

Aschoff and Koch, in 1919, were the first to note that the basic factor in human scurvy was an alteration of the intercellular connective tissue. In the following year, Hess ('20) described the tissue changes in scurvy in his classical monograph, Scurvy, Past and Present.

In 1924, Höjer in a most comprehensive report, augmented the description of the tissue changes in scurvy noted by Hess. Probably the most important finding reported by Höjer was that in scurvy the rate of collagen formation is diminished and that a widespread deficiency of collagen exists in the connective tissue. Bourne ('42) has also noted a diminished rate of formation of collagen in scurvy. In 1926, Wolbach and Howe first observed that newly formed fibrous tissue in wound repair is free from collagen, whereas they found no striking abnormality of the collagen fibers of connective tissue already existing in scorbutic guinea pigs.

In previous studies we have reported a direct relationship between the amount of vitamin C in the diet and the strength of the healing wound, as well as between the dietary level of ascorbic acid and its concentration in scar tissues, in the tissues of other organs and in the blood.

For the past several years we have likewise studied the role of vitamin C in the formation and maintenance of connective tissue. These studies have been made in the guinea pig both on *recently* healed abdominal wounds, reported by Abt, et al. ('59a), as well as on similar *long healed* wounds, reported by Abt and coworkers ('59b).

We have previously demonstrated that ascorbic acid is essential for (a) *the production* of connective tissue to ensure immediate postoperative healing, as well as for (b) *the maintenance* of connective tissue in previously formed scar tissue. We have also shown that ascorbic acid accumulates in scar tissue immediately following wounding and persists for long periods of time.

A part of our study has been directed to measuring in scar tissue the principal constituents of connective tissue: collagen, and ground substance. We have used determinations of hydroxyproline as a measure of collagen and determinations of hexosamine as a measure of ground substance at varying levels of dietary ascorbic acid intake.

MATERIALS AND METHODS

Young male guinea pigs of the Hartley strain, weighing 220 to 250 gm, were used. Similar dietary management was instituted and the abdominal operative technique was performed as previously reported by Abt, von Schuching and Roe ('59a). Tissues

Received for publication October 7, 1959.

[1] Presented at the 23rd Annual Meeting of the American Institute of Nutrition, Atlantic City, New Jersey, April 15, 1959.

[2] This investigation was supported in part by Research Grant USPHS A-1949(C1) from the NIH, Public Health Service.

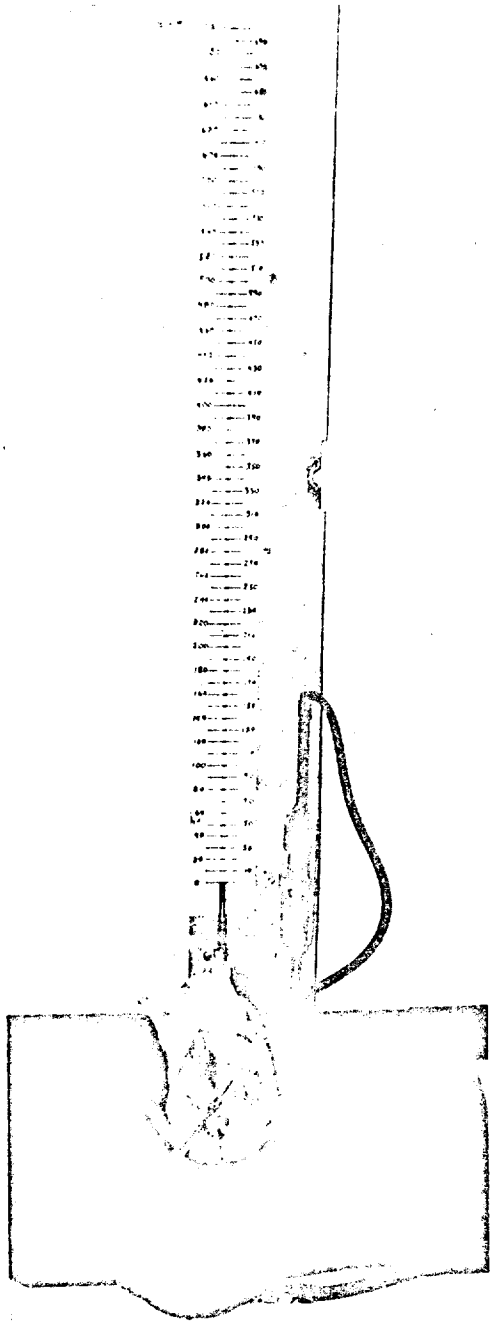


Fig. 1 Illustrates the Mercury Manometer used to test the pressure of rupture of the wound in the anesthetized guinea pig.

for analysis were taken immediately after the wound rupture determinations. Figure 1 illustrates the mercury manometer used to test the pressure of rupture of the wound in the anesthetized guinea pig. Mucopolysaccharides were determined as hexosamine by the method of Elson and Morgan ('33), as modified by Roseman and Dafiner ('56). Collagen was estimated as hydroxyproline by the modification by Martin and Axelrod ('53) of the method of Neuman and Logan ('50). The ascorbic acid content of blood and tissues was determined by the method of Roe and Keuther ('43). Histologic sections were fixed in formalin or Bouin's fluid and stained for connective tissue by Foot's modification of Masson's Trichrome stain.

RESULTS

Ascorbic acid distribution. We have previously noted that the concentration of ascorbic acid in the wound tissue rose above the concentration in distant tissue in recently healed wounds. We have further noted that this higher concentration of ascorbic acid persists in scar tissue after long intervals of healing. Following these original observations we have further fractionated the scar tissue of abdominal wounds for ascorbic acid concentration, to determine in exactly which tissue the highest level of ascorbic acid existed.

We have separated the central core of the scar which consists nearly exclusively of connective tissue and termed this the "scar core connective tissue." The tissue adjacent to the wound and not more than 1 to 2 mm from it, we have designated the "paravulneral tissue" (Stein and Wolman, '58). From the same animal, samples of abdominal muscle were also taken 0.7, 1.5 and 3 cm from the edge of the wound, as well as of muscle from the leg and back. Connective tissue was obtained from the achilles and patellar tendon and from the ligamentum nuchae.

The highest values for ascorbic acid from these enumerated tissues, were obtained from the scar core connective tissue and from the connective tissue of the tendons and ligaments. Thus, the increase of ascorbic acid in scar tissue after wounding is definitely linked to the connective tissue. Our results are noted in figure 2.

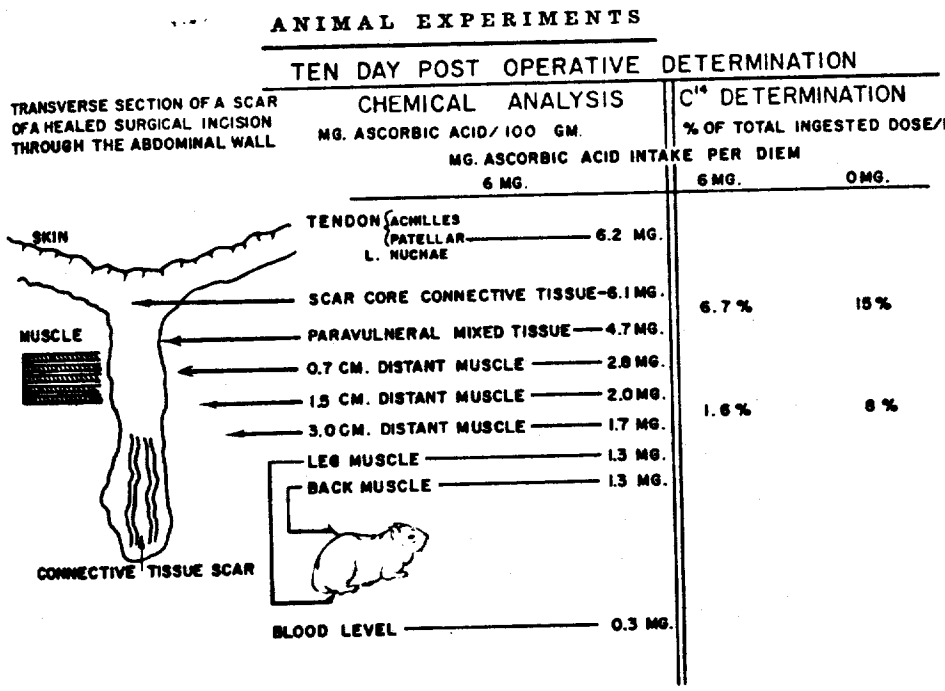


Fig. 2 Fractionation of ascorbic acid in scar and distant muscle, chemical analysis, and C^{14} determination. The most important finding is the highest concentration of ascorbic acid in the scar connective tissue. Marked differences are also noted in the retention of C^{14} in the tissues of normal and scorbutic animals.

Collagen and hexosamine distribution. In table 1 the collagen content, calculated as hydroxyproline, in gm/100 gm of dry tissue is shown. The following tissues were analyzed: abdominal skin, abdominal muscle, scar skin, scar muscle, teeth, leg bone and cartilage. Patellar cartilage was analyzed in the experiment using a commercial chow[3] as the dietary source, whereas in the animals receiving the semi-synthetic diet of Reid and Briggs ('53), costal cartilage was analyzed. Statistical analysis showed no significant differences in collagen content between the control and scorbutic groups except in the costal cartilage of those fed the scorbutic diet for 19 days. In view of the failure to observe significant differences in all of the other analyses, the latter finding is probably the result of technical error.

Table 2 shows the hexosamine content in milligrams per 100 gm of dry tissue, or milligrams per 100 ml for serum. Values for abdominal skin, abdominal muscle, scar skin, scar muscle, leg bone, cartilage and serum were determined. No significant differences in values between control and scorbutic groups were found.

When a comparison is made between the hexosamine values of the normal tissue at the time of operation, and the values for scar tissue in the operated area at increasing intervals after the operation, a rise is noted in the amount of hexosamine in the scar tissue in both control and scorbutic animals in all instances except for the data for three days after operation. The increases were statistically significant in most cases.

Summarizing the results obtained (tables 1 and 2), neither collagen nor hexosamine levels showed a significant difference between the control and scorbutic groups. The collagen values were compared at intervals after the operation. Except for a decrease in the skin value three days after operation, no other changes were observed. At 6 days after operation, the

[3] Purina Chow.

TABLE 1

Collagen content, converted from hydroxyproline analyses, of wound and other tissues in guinea pigs fed commercial chow and semi-synthetic diets, with and without ascorbic acid. Data represent averages of 5 animals per series. Animals were on identical diets, control guinea pigs receiving 90 mg of ascorbic acid per animal daily. Values are gm per 100 gm of dry tissue

Daily oral intake of vitamin C	Total days on diet	At operation		At sacrifice					
		Abdominal skin	Abdominal muscle	Days post-operative	Scar skin	Scar muscle	Teeth	Leg bone	Cartilage
mg	*no.*			*no.*					
				Commercial chow diet[1]					
100 by dropper	20	—	—	10	36.7 ± 4.98[2]	11.3 ± 1.36	10.18 ± 2.80	9.66 ± 0.38	25.6 ± 5.18[3]
0	20	—	—	10	30.0 ± 2.35	11.0 ± 1.95	9.80 ± 4.05	9.18 ± 1.63	31.2 ± 3.05
P of difference		—	—		0.02	> 0.5	> 0.5	> 0.5	0.1
				Reid-Briggs semi-synthetic diet					
90 mixed in diet	13	51.8 ± 2.67	10.2 ± 3.62	3	38.4 ± 4.58	7.9 ± 2.79	13.50 ± 1.30	13.30 ± 1.20	22.5 ± 2.08[4]
0	13	48.3 ± 4.92	8.7 ± 1.10	3	36.7 ± 0.70	8.5 ± 1.58	13.80 ± 0.70	12.10 ± 1.66	24.0 ± 1.36
P of difference		0.2	0.4		> 0.5	> 0.5	> 0.5	0.2	0.3
90 mixed in diet	16	38.3 ± 7.75	10.5 ± 1.68	6	38.4 ± 5.04	8.0 ± 0.81	—	—	—
0	16	43.1 ± 3.10	11.2 ± 1.94	6	38.1 ± 4.18	8.3 ± 0.89	—	—	—
P of difference		0.24	> 0.5		> 0.5	> 0.5	—	—	—
90 mixed in diet	19	40.2 ± 8.02	9.0 ± 2.01	9	32.2 ± 4.14	13.0 ± 2.38	11.40 ± 0.70	11.70 ± 1.28	22.0 ± 1.22
0	19	47.0 ± 8.86	6.1 ± 2.28	9	30.9 ± 2.98	9.6 ± 1.81	12.40 ± 0.85	11.80 ± 0.70	25.5 ± 1.37
P of difference		0.24	0.08		> 0.5	0.04	0.08	> 0.5	< 0.010
90 mixed in diet	22	41.9 ± 4.51	11.6 ± 2.16	12	35.5 ± 5.52	10.5 ± 0.26	12.60 ± 0.86	12.20 ± 1.38	22.9 ± 3.31
0	22	40.5 ± 3.00	11.2 ± 2.55	12	34.2 ± 5.51	11.1 ± 4.20	11.60 ± 0.47	12.30 ± 0.59	24.0 ± 2.73
P of difference		> 0.5	> 0.5		> 0.5	> 0.5	0.05	> 0.5	> 0.5

[1] Purina chow. [2] Standard deviation. [3] Patellar. [4] Costal.

0056

TABLE 2

Hexosamine content of wound and other tissues in guinea pigs fed commercial chow and semi-synthetic diets, with and without ascorbic acid. Data represent averages of 5 animals per series. Animals were fed identical diets, control guinea pigs receiving 90 mg of ascorbic acid per animal daily. Values are mg per 100 gm of dry tissue or mg per 100 ml of serum

Daily oral intake of vitamin C	Total days on diet	At operation		At sacrifice					
		Abdominal skin	Abdominal muscle	Days post-operative	Scar skin	Scar muscle	Leg bone	Cartilage	Serum
mg	*no.*			*no.*					*mg/100 ml*
				Commercial chow diet[1]					
100 By dropper	20	—	—	10	352.0 ± 86.0[2]	—	345.0 ± 310.0	511.0 ± 249.0[3]	75 ± 47.0
0	20	—	—	10	327.0 ± 66.0	—	219.0 ± 166.0	608.0 ± 107.0	116 ± 75.0
P of difference					> 0.5		0.3	> 0.5	0.2
				Reid-Briggs semi-synthetic diet					
90 Mixed in diet	13	372.4 ± 75.0	247.6 ± 76.0	3	357.8 ± 78.0	442.8 ± 129.0	287.8 ± 20.8	270.4 ± 60.0[4]	129 ± 33.0
0	13	362.0 ± 42.5	181.3 ± 129.0	3	378.8 ± 49.4	430.2 ± 140.0	263.6 ± 23.2	245.0 ± 24.0	103 ± 43.4
P of difference		> 0.5	> 0.5		0.5	> 0.5	0.5	> 0.5	0.5
90 Mixed in diet	16	235.4 ± 42.5	207.8 ± 54.6	6	448.0 ± 38.4	378.0 ± 114.0	—	—	85 ± 21.5
0	16	319.8 ± 84.0	190.8 ± 138.0	6	479.8 ± 79.0	329.8 ± 128.0	—	—	90 ± 17.6
P of difference		> 0.5	> 0.5		> 0.5	> 0.5			0.5
90 Mixed in diet	19	291.8 ± 59.0	219.8 ± 117.0	9	471.6 ± 50.0	456.8 ± 50.8	321.2 ± 69.0	264.9 ± 44.0	66 ± 26.7
0	19	362.0 ± 60.0	234.8 ± 195.0	9	434.4 ± 58.0	469.2 ± 56.7	267.2 ± 65.5	214.6 ± 35.5	120 ± 39.8
P of difference		> 0.5	> 0.5		> 0.5	> 0.5	> 0.5	> 0.5	> 0.5
90 Mixed in diet	22	280.2 ± 52.6	173.8 ± 93.0	12	431.6 ± 15.3	377.8 ± 222.1	351.8 ± 56.1	208.3 ± 12.2	69 ± 29.5
0	22	264.0 ± 120.0	287.0 ± 120.0	12	598.2 ± 240.0	434.8 ± 65.9	280.2 ± 48.2	231.4 ± 34.8	71 ± 37.2
P of difference		> 0.5	> 0.5		> 0.5	> 0.5	> 0.5	> 0.5	> 0.5

[1] Purina chow. [2] Standard deviation. [3] Patellar. [4] Costal.

0056

TABLE 3

Analyses of collagen, determined from hydroxyproline, and of hexosamine in carrageenin granulomata 10 days after subcutaneous injection of carrageenin in guinea pigs. Data represent averages of 5 animals per series

Average values	Daily intake of ascorbic acid	
	90 mg	0 mg
Collagen, gm/100 gm dry tissue	3.8 ± 1.3	4.0 ± 1.4
Hexosamine, mg/100 gm dry tissue	1216 ± 63.6	949 ± 4.1

absolute amounts of collagen in skin were exactly the same as values at operation.

The hexosamine values showed absolute increases after the third postoperative day in both the control and scorbutic groups. The hexosamine values were significantly higher in the control group at the 6th, 9th, and 12th postoperative days than at the day of operation. The scorbutic animals showed increased hexosamine values at the same periods, though with a wider range.

In table 3 are noted the average of analyses of collagen, calculated from hydroxyproline, and of hexosamine determined on specimens from carrageenin granulomata.[4] They were taken 10 days after the subcutaneous injection of carrageenin into guinea pigs receiving daily intakes of 90 mg of ascorbic acid and no ascorbic acid. No difference was noted in the collagen content of the carrageenin tumor for the animals on the 90-mg daily intake of ascorbic acid and those on the scorbutic diet.

The average hexosamine values of specimens from the carrageenin tumor were greater for the animals on 90-mg daily ascorbic acid intake, than for the average of the group deprived of ascorbic acid.

[4] We are indebted to Mr. Leonard Stoloff, Research Director, Seaplant Corporation, New Bedford, Massachusetts, for generously supplying us with a purified extract of Irish moss (*Chondrus crispus*).

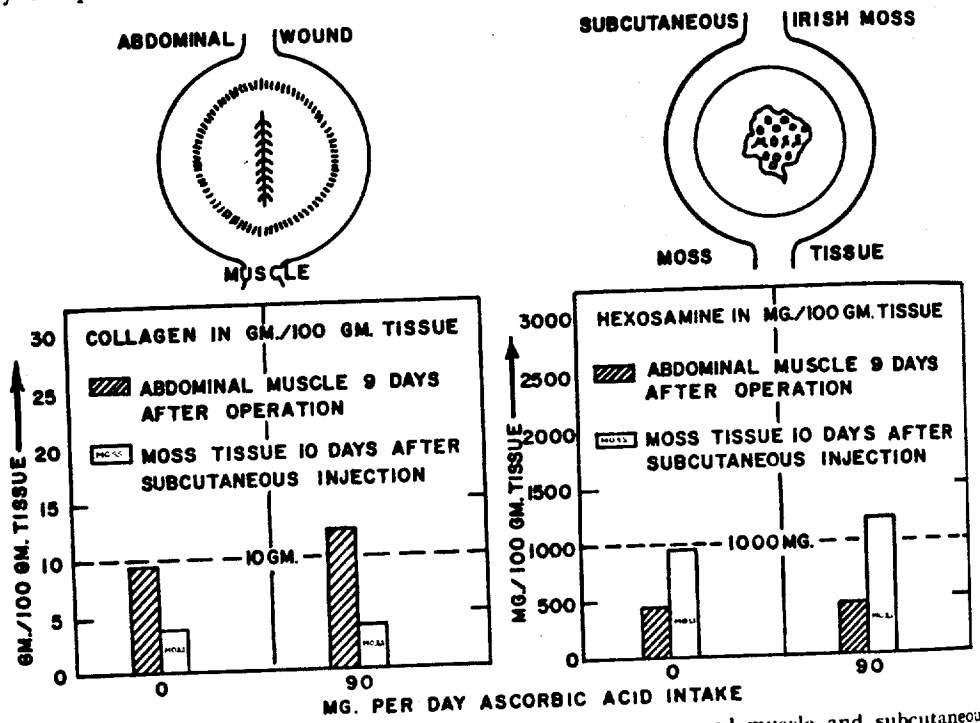


Fig. 3 Comparison of collagen and hexosamine levels in scar wound muscle and subcutaneous carrageenin granuloma at 0 and 90 mg of daily ascorbic acid intake. Determinations were made 9 days after operation and 10 days after subcutaneous injection of the carrageenin. Data represent averages for 5 animals.

In figure 3 is shown a comparison of collagen and hexosamine levels in scar wound muscle and subcutaneous carrageenin granuloma at zero and 90-mg daily ascorbic acid intake. Determinations were made 9 days after operation and 10 days after subcutaneous injection of the carrageenin. The values for collagen are lower in the granuloma tissue than in the scar muscle, both in the scurvy group and in the group receiving optimal vitamin C intake, at equivalent time periods following operation and injection of carrageenin.[5] The levels of hexosamine are the reverse of the collagen levels when comparison is made at equivalent times for the two diets, both hexosamine levels being higher in the granuloma.

Histology of connective tissue. Cross section specimens of the scars were made as they were removed from the animals receiving the varying dietary vitamin C intakes. These were stained with ordinary hematoxylin and eosin stains and with special connective-tissue stain used to demonstrate the regeneration of fibers at various intervals following the operation and for animals on different dietary intake levels. Figure 4 is a cross section of a healing scar taken 10 days postoperatively from a guinea pig who had been receiving 6 mg of ascorbic acid daily, orally, a maintenance diet. The epithelium has healed over completely and the line of incision has filled in with numerous new connective tissue fibers. In contrast to this picture of an active regenerating wound, figure 5 represents a cross section of a scar from a scorbutic animal, 18 days postoperatively. The epithelial layer remains separated where the incision occurred and the derma is ununited. The central portion seems still to contain fibrin and intracellular substances. There are very few, if any, new connective tissue fibers. The dearth of new collagen formation as noted by the special stain employed is most marked when contrasted to the histologic cross section of the healing wound of a guinea pig on a maintenance vitamin C intake. The histologic findings in the guinea pig here presented parallel in every detail histologic findings on wound healing in vitamin C-depleted human subjects, previously observed by one of the authors, Arthur F. Abt[6] (Wolfer et al., '47).

DISCUSSION

A most important finding here reported, is the demonstration that *the highest concentration of ascorbic acid in the wound is in the connective tissue.* This concentration is equal to its concentration in the connective tissue of the tendons and ligaments, simultaneously examined. The exact mechanism related to the increased concentration and build-up of ascorbic acid in wound connective tissue is unknown.

We conclude from our results that hydroxyproline determinations are not an accurate reflection of collagen production; further, that hexosamine is derived largely from plasma, and its concentration in scar tissue is independent of scurvy. The histologic studies corroborate the chemical findings and substantiate the conclusion that ascorbic acid is necessary for the production of new connective tissue.

According to Kellgren ('52), the connective tissues perform at least two important functions. First, they provide a tough framework for the body, giving it cohesion and form, and also a system of levers which allow the exertion of considerable mechanical force; and, secondly, they provide the lubricating materials which enable the various parts of the body to move smoothly and efficiently upon each other. It is also possible that they may play an important part in metabolism, but this possibility still remains unexplored.

According to Stetten ('51), the function of collagen is simply to exhibit a high tensile strength; no other function is known.

The role of collagen in wound healing, and particularly in relation to the healing

[5] Since the manuscript for this paper was submitted for publication, a paper by Chozo Mitoma and Thomas E. Smith has appeared, "Studies on the Role of Ascorbic Acid in Collagen Synthesis," J. Biol. Chem., 235: 426–428, February 1960. Using L-proline-C^{14} as tracer for the formation of collagen in carrageenin granuloma, Mitoma and Smith found that conversion to hydroxyproline was not affected by ascorbic acid deficiency.

[6] "Methods for Detection of Early Signs of Vitamin C Deficiency," investigation under Contract OEMcmr no. 71, Committee on Medical Research, March 25, 1944, responsible investigators, Chester J. Farmer, M.D. and Arthur F. Abt, M.D. Also separately reported.

of wounds in scurvy, has been studied for a considerable period. Wolbach ('37) noted that the chief function assigned to vitamin C in the body is concerned with the formation of colloid intercellular substances. Abt and Farmer ('38) noted that the intercellular substances which appear to be regulated by vitamin C are of mesenchymal origin—the collagen of all fibrous tissue structures, all non-epithelial cement substances, including the intercellular substance of the capillary wall, dentin, cartilage and the matrices of bone.

In relating scurvy to wound healing, Sylvia Bensley ('34) characterized all healing processes by three stages: (1) edema, (2) gelation and (3) fiber production—pointing out that in scurvy the process stopped at the first stage, namely, edema.

Klemperer ('55) notes that the mechanism of deranged collagen formation in scurvy is as yet unclarified, and Goldsmith ('53) agrees that the exact nature of the defect is controversial. Gersh ('52) has noted that in acute scurvy, morphologic collagen shows a decrease, whereas, chemically, no difference exists between that found in non-scurvy animals.

Elster ('50) states that the maintenance of collagen does not require ascorbic acid, and Robertson and Schwartz ('53) note that the formation, in contrast to the maintenance of collagen, requires ascorbic acid.

Lloyd and Sinclair ('53) point out that it is generally agreed that connective tissue is defective in scurvy, though there is disagreement on the nature of the defect, one group noting intactly functioning cells, but that ascorbic acid is ultimately necessary for the setting or fibrillation of collagen and similar substances, while the other group, represented by Fish and Harris ('34), and Ham and Elliott ('38), regards the defect as being in the function of the connective tissue cell.

In recent studies Gould and Woessner ('57) concluded that the collagen formation is impaired as early as 4 days after withdrawal of ascorbic acid. In a subsequent communication Gould ('58) notes that apparently rapid collagen biosynthesis, such as that involved in tissue repair, may be mediated directly by ascorbic acid.

Kodicek and Loewi ('55) reported that collagen formation was impaired in vitamin C deficiency as evidenced by low hydroxyproline values and they further noted, in agreement with our findings, the values for hexosamine in scorbutic granulation tissue did not differ from the values obtained in control animals.

Edwards and Dunphy ('58) note that the hexosamine may be largely derived from plasma glycoproteins.

Weiss ('59) has noted that erstwhile round connective tissue cells elongate along the lines which fibrin strands assume in fresh wounds. Thus the collagen fibers of the new connective tissue of the healing wound are laid down in parallel strands.

Many investigations have been reported concerning the precursor of collagen—termed "procollagen"—since the early experiments of Zacharidès (1900) and Nageotte ('27), who extracted collagen from rat-tail tendon with dilute acid and salt. The latter author termed the soluble fraction "précollagène" and believed it was a soluble precursor of insoluble fibers. Jackson ('58) and Green and Lowther ('59) give most excellent recent reviews of collagen precursors.

Recently, Robertson, Hiwett and Herman ('59) have reported on the relation of ascorbic acid to the conversion of proline to hydroxyproline, and Gross ('59) has noted the effect of vitamin C deficiency on the neutral salt-extractable collagen of skin. He concludes that the deficiency of ascorbic acid either interferes with the synthesis of new collagen in intact skin or causes its destruction and removal as rapidly as it is produced.

The new connective tissue produced by the subcutaneous injection of carrageenin increases rapidly to a maximum 7 to 14 days following injection, but, unlike the connective tissue formed by wounding, is later absorbed after a period of weeks, so that after the 6th week, only a small amount of fatty tissue remains at the injected site. This resorption of connective tissue corresponds to the body response to the presence of a foreign body and differs from the formation of scar tissue after wounding.

Gillman ('57) has pointed out that the fibrosis noted in carrageenin granuloma differs considerably from that usually seen

in healing wounds. In his opinion the two types of fibroses, namely, that seen in scar formation and that produced in granuloma, are quite different. This appears to be a valid criticism and differences in data, obtained from scars produced by abdominal incision and granuloma produced by subcutaneous injection of foreign bodies such as carrageenin, should be recognized. The data obtained from these two types of scar-tissue production may not be comparable.

SUMMARY

1. We have extended previous observations on the concentration of ascorbic acid in scar tissue to a further fractionation of the tissues composing the scar and have found that *the highest concentration of ascorbic acid is in the connective tissue.*

2. The high concentration of ascorbic acid in the connective tissue of the wound parallels the concentrations of ascorbic acid found simultaneously in the connective tissue of tendons and ligaments. The next highest concentration was found in the paravulneral tissue adjacent to the scar and lower amounts occurred in specimens of abdominal muscle distant from the scar as well as samples of muscle taken from the leg and back.

3. From determinations of collagen and hexosamine content of abdominal scar tissue, and of tissue produced in carrageenin granuloma, we have shown that insignificant differences occur between animals with scurvy and those placed on saturation dietary intakes of ascorbic acid. We have also noted that postoperatively, hexosamine values increased, both in the scorbutic and control animals.

4. In histologic cross sections of abdominal incisions, new connective tissue fibers have been demonstrated by special stain in guinea pigs on a maintenance dietary level of ascorbic acid. A dearth of new connective tissue fibers occurred in histologic cross sections of abdominal scars taken from scorbutic animals. These histologic findings parallel those previously made in studies on wound healing in vitamin C-depleted human subjects.

ACKNOWLEDGMENT

Appreciation is expressed to Charles Derr who carried out the greater part of the computations and analytical determinations, as well as for the technical assistance of Carl Karickhoff; also to June Engle for helping to compile the data. We also appreciate the preparation of the photomicrographs by Harry Busey.

LITERATURE CITED

Abt, A. F., and C. J. Farmer 1938 Vitamin C. Pharmacology and therapeutics. J. Am. Med. A., *111:* 1555.

Abt, A. F., S. von Schuching and J. H. Roe 1959a Connective tissue studies. I. Relation of dietary and tissue levels of ascorbic acid to the healing of surgically-induced wounds in guinea pigs. Bull. Johns Hopkins Hosp., *104:* 153.

——— 1959b Connective tissue studies. II. The effect of vitamin C deficiency on healed wounds. Ibid., *105:* 67.

Aschoff, L., and W. Koch 1919 Skorbut: Eine Pathologische—Anatomische Studie. G. Fischer, Jena.

Bensley, S. H. 1934 On the presence, properties and distribution of the intercellular ground substance of loose connective tissue. Anat. Rec., *60:* 93.

Bourne, G. H. 1942 Vitamin C and repair of injured tissues. Lancet, *2:* 661.

Edwards, L. C., and J. E. Dunphy 1958 Wound healing. I. Injury and normal repair. New England J. Med., *259:* 224.

Elson, L. A., and W. T. J. Morgan 1933 A colorimetric method for the determination of glucosamine and chondrosamine. Biochem. J., *27:* 1824.

Elster, S. K. 1950 Effect of ascorbic acid deficiency on collagen content of guinea pig tissues. J. Biol. Chem., *186:* 105.

Fish, E. W., and L. J. Harris 1934 The effects of vitamin C deficiency on tooth structure in guinea pigs. Trans. Roy. Soc. (London), *B233:* 489.

Gersh, J. 1952 Transactions of the Second Conference on Connective Tissues. Josiah Macy, Jr. Foundation, New York, p. 15.

Gillman, T. 1957 *comment on:* Jackson, D. S. The formation and breakdown of connective tissue. Connective Tissue, A Symposium Organized by the Council for International Organizations of Medical Sciences, eds., R. E. Tunbridge, M. Keech, J. F. Delafresnaye and G. C. Wood. Charles C Thomas, Springfield, Illinois, p. 75.

Goldsmith, G. A. 1953 Biochemistry and Physiology of Nutrition. Applications of human nutrition, ed., G. H. Bourne and G. W. Kidder. Vol. II, Ch. 23. Academic Press, Inc., New York, p. 578.

Gould, B. S. 1958 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.

Gould, B. S., and J. F. Woessner 1957 Biosynthesis of collagen. The influence of ascorbic acid on the proline, hydroxyproline, glycine, and collagen content of regenerating guinea pig skin. J. Biol. Chem., *226:* 289.

Green, N. M., and D. A. Lowther 1959 Formation of collagen hydroxyproline *in vitro*. Biochem. J., *71:* 55.

Gross, J. 1959 Studies on the formation of collagen. IV. Effect of vitamin C deficiency on the neutral salt-extractable collagen of skin. J. Exp. Med., *109:* 557.

Ham, A. W., and H. C. Elliott 1938 Bone and cartilage lesions of protracted moderate scurvy. Am. J. Path., *14:* 323.

Hess, A. F. 1920 Scurvy, Past and Present. J. B. Lippincott Co., Philadelphia and London.

Höjer, J. A. 1923–1924 Studies in scurvy. Acta Paediat. 3, Suppl. 3, p. 1.

Jackson, D. S. 1958 Some biochemical aspects of fibrogenesis and wound healing. New England J. Med., *259:* 814.

Kellgren, J. H. 1952 Some concepts of rheumatic disease. Brit. Med. J., *1:* 1093.

Klemperer, P. 1955 The Harvey Lectures, Series XLIX. Academic Press, Inc., New York, p. 109.

Kodicek, E., and C. Loewi 1955 Uptake of (35S) sulfate by mucopolysaccharides of granulation tissue. Proc. Roy. Soc., London, s.B., *144:* 100.

Lloyd, B. B., and H. M. Sinclair 1953 Biochemistry and Physiology of Nutrition. Vitamin C, vol. I., eds., G. H. Bourne and G. W. Kidder. Academic Press, Inc., New York, p. 392.

Martin, C. J., and A. E. Axelrod 1953 Modified method for determination of hydroxyproline. Proc. Soc. Exp. Biol. Med., *83:* 461.

Nageotte, J. 1927 Action des sels neutres sur la formation du caillot artificiel de collagène. Compt. rend. Soc. de biol., *96:* 828.

Neuman, R. E., and M. A. Logan 1950 Determination of collagen and elastin in tissues. J. Biol. Chem., *186:* 549.

Reid, M. E., and G. M. Briggs 1953 Development of a semi-synthetic diet for young guinea pigs. J. Nutrition, *51:* 341.

Robertson, W. van B., J. Hiwett and C. Herman 1959 The relation of ascorbic acid to the conversion of proline to hydroxyproline in the synthesis of collagen in the carrageenin granuloma. J. Biol. Chem., *234:* 105.

Robertson, W. van B., and B. Schwartz 1953 Ascorbic acid and the formation of collagen. J. Biol. Chem., *201:* 689.

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

Roseman, S., and I. Daffner 1956 Colorimetric method for determination of glucosamine and galactosamine. Anal. Chem., *28:* 1743.

Stein, O., and M. Wolman 1958 A histochemical study of wound healing in scorbutic guinea-pigs. Brit. J. Exp. Path., *39:* 418.

Stetten, D., Jr. 1951 Metabolic Interrelations, Transactions of the Third Conference, ed., E. C. Reifenstein, Jr. Josiah Macy, Jr. Foundation, New York, p. 22.

Weiss, P. 1959 Cellular dynamics. Rev. Mod. Physics, *31:* 11.

Wolbach, S. B. 1937 Pathologic changes resulting from vitamin deficiency. J. A. M. A., *108:* 7.

Wolbach, S. B., and P. R. Howe 1926 Intercellular substances in experimental scorbutus. A.M.A. Arch. Path., *1:* 1.

Wolfer, J. A., C. J. Farmer, W. W. Carroll and D. O. Manshardt 1947 An experimental study in wound healing in vitamin C depleted human subjects. Surg. Gynec. Obst., *84:* 1.

Zacharidès, P. A. 1900 Recherches sur la structure du tissue conjonctif, sensibilité du tendon aux acides. Compt. rend. Soc. de biol., *52:* 182.

Anderson, A.K. 1939
Essentials of Physiological Chemistry, 2nd Edition
John Wiley and Sons, Inc., New York, pp. 291-294

C.F.R. 9, Chapter III (USDA M.I.D)
P. 318.7; 319.105; 381.147

Class of substance	Substance	Purpose	Products	Amount
Cooling and retort water treatment agents.	Calcium chloride	To prevent staining on exterior of canned goods.	Any	Sufficient for purpose.
	Citric acid	do	do	Do.
	Dioctyl sodium sulfosuccinate.	do	do	0.05 percent.
	Disodium-calcium ethylenediamine-tetraacetate.	do	do	Sufficient for purpose.
	Disodium ethylenediamine-tetraacetate.	do	do	Do.
	Disodium phosphate	do	do	Do.
	Ethylenediamine-tetraacetic acid.	do	do	Do.
	Isopropanol	do	do	0.002 percent.
	Potassium pyrophosphate.	do	do	Sufficient for purpose.
	Propylene glycol	do	do	Do.
	Sodium bicarbonate	do	do	Do.
	Sodium bisulfate	To inhibit corrosion on exterior of canned goods.	do	0.001 percent.
	Sodium carbonate	To prevent staining on exterior of canned goods.	do	Sufficient for purpose.
	Sodium dodecylbenzene sulfonate.	do	do	0.05 percent.
	Sodium gluconate	do	do	Sufficient for purpose.
	Sodium hexametaphosphate.	do	do	Do.
	Sodium laurylsulfate	do	do	0.05 percent.
	Sodium metasilicate	do	do	Sufficient for purpose.
	Sodium n-alkylbenzene sulfonate (alkyl group predominantly C_{12} and C_{13} and not less than 95 percent C_{10} to C_{18}).	do	do	0.05 percent.
	Sodium nitrite. (The dry nitrite must be decharacterized with 0.05 percent powdered charcoal or 0.03 percent nigrosine. Bulk decharacterized sodium nitrite when in cook room shall be held in a locked container conspicuously labeled "Decharactorized Sodium Nitrite—to be used by authorized personnel only.")	To inhibit corrosion on exterior of canned goods.	do	600 parts per million.
	Sodium pyrophosphate.	To prevent staining on canned goods.	do	0.05 percent.
	Sodium tripolyphosphate.	do	do	Do.
	Zinc oxide	do	do	0.01 percent.
	Zinc sulfate	do	do	Do.
Curing accelerators; must be used only in combination with curing agents.	Sodium Acid Pyrophosphate.	To accelerate color fixing.	Frankfurters, weiners, vienna, bologna, garlic bologna, knockwurst, and similar products.	Not to exceed, alone or in combination with other curing accelerators, the following: 8 ozs. in 100 lbs. of the meat, or meat and meat byproducts, content of the formula; nor 0.5 percent in the finished product.
Curing agents	Ascorbic acid	To accelerate color fixing or preserve color during storage.	Cured pork and beef cuts, cured comminuted meat food product.	75 ozs. to 100 gals. pickle at 10 percent pump level ¾ oz. to 100 lbs. meat or meat byproduct; 10 percent solution to surfaces of cured cuts prior to packaging (the use of such solution shall not result in the addition of a significant amount of moisture to the product).

84-048—73——27

(d) *Cured, water added products.* Products resembling standardized ham and other pork products of the kinds provided for in paragraph (b) or (c) of this section, which do not conform to such provisions because they contain added water not in excess of 10 percent of the weight of the fresh, uncured products, shall bear on their labels the term "Water Added," as a part of the product name, in prominent lettering not less than three-eighths inch in height, and if not placed in a consumer-size package labeled in accordance with this part and Part 317 of this subchapter, shall be marked with the term "Water Added" the full length of the product. However, the Administrator may approve smaller lettering for labels of small packages, such as 4-ounce packages, when he finds that the size and style of the lettering in connection with the product name are such as to insure the prominence of the required terms. The qualifying phrase "Up to 10 percent" or equivalent phrase may be used in labeling such products in connection with the term "Water Added" at the option of the operator of the establishment, provided the qualifying phrase does not detract from the prominence of the term "Water Added."

(e) *Canned products.* The preparation of any canned products such as "Ham," "Pork Shoulder Picnic," or similar products, shall not result in an increase in weight of more than 8 percent over the weight of the fresh uncured article.

(f) *Pressed ham, spiced ham, and similar products.* "Pressed Ham," "Pressed Ham with Natural Juices," "Spiced Ham," and similar products may contain finely chopped ham shank meat to the extent of 25 percent over that normally present in the boneless ham. The weight of the cured chopped ham prior to processing shall not exceed the weight of the fresh uncured ham, exclusive of the bone and fat removed in the boning operation, plus the weight of the curing ingredients and 3 percent moisture.

§ 319.105 Chopped ham.

(a) "Chopped Ham" is the semisolid meat food product, in the form of a compact mass with a limited amount of cooked out juices, which is prepared with ham, curing agents, seasonings, and any of the optional ingredients listed in paragraph (b) of this section, in accordance with the provisions of subparagraphs (1), (2), and (3) of this paragraph.

(1) Fresh ham, cured ham, or smoked ham, or a mixture of two or more of such meat components may be used. The weight of the cured chopped ham prior to processing shall not exceed the weight of the fresh uncured ham and fresh uncured ham shank meat if any is used, exclusive of the bones and fat removed in the boning operations, plus the weight of the curing ingredients and 3 percent moisture.

(2) The curing agents that may be used, singly or in combination, are salt, sodium nitrate, sodium nitrite, potassium nitrate, and potassium nitrite. When sodium nitrate, sodium nitrite, potassium nitrate, or potassium nitrite is used, singly or in combination, the amount thereof shall not exceed that permitted in § 318.7(b)(4) of this subchapter.

(3) The seasonings that may be used, singly or in combination, are salt, sugar (sucrose or dextrose), spice, and flavoring, including essential oils, oleoresins and other spice extractives.

(b) Chopped ham may contain one or more of the following optional ingredients:

(1) Finely chopped ham shank meat (fresh, cured, or smoked, or a combination thereof) to the extent of not more than 25 percent over that normally present in the boneless ham;

(2) Water, for the purpose of dissolving the curing agents, and not in excess of the amount permitted in paragraph (a)(1) of this section;

(3) Monosodium glutamate;

(4) Hydrolyzed plant protein;

(5) Corn syrup solids, corn syrup and glucose syrup, singly or in combination, in an amount not to exceed 2 percent (calculated on a dry basis) of all the ingredients used in preparing the chopped ham;

(6) Disodium phosphate, sodium hexametaphosphate, sodium tripolyphosphate, sodium pyrophosphate, and sodium acid pyrophosphate, singly or in combination, in an amount not to exceed that permitted in § 318.7(b)(4) of this subchapter;

(7) Asorbic acid, sodium ascorbate, isoascorbic acid or sodium isoascorbate in an amount not to exceed that permitted in § 318.7(c)(4) of this subchapter;

(8) Dehydrated onions or onion powder;

(9) Dehydrated garlic or garlic powder.

TABLE I

Class of substance	Substance	Purpose	Products	Amount
Antifoaming agent	Methyl polysilicone	To retard foaming	Soups	10 ppm.
			Rendered fats	10 ppm.
			Curing pickle	50 ppm.
Antioxidants and oxygen interceptors.	BHA (Butylated hydroxyanisole)	To retard rancidity	Various	0.01 percent based on fat content. (0.02 percent in combination with any other antioxidant listed in this table based on fat content.)
	BHT (Butylated hydroxytoluene)	do	do	Do.
	Propyl gallate	do	do	Do.
	Tocopherols	do	do	0.03 percent based on fat content. (0.02 percent in combination with any other antioxidant listed in this Table based on fat content.)
Binders and extenders	Algin	To extend and stabilize product.	do	Sufficient for purpose.
	Carrageenan	do	do	Do.
	Carboxymethyl cellulose (cellulose gum).	do	do	Do.
	Gums, vegetable	do	do	Do.
	Methyl cellulose	To extend and to stabilize product (also carrier).	do	0.15 percent.
	Isolated soy protein	To bind and extend product.	do	Sufficient for purpose.
	Sodium caseinate	do	do	Do.
	Whey (dried)	do	do	Do.
Chilling media	Salt (NaCl)	To aid in chilling	Raw poultry products	700 lbs. to 10,000 gals. of water.[1]
Coloring agents (natural)	Annatto, Carotene	To color products	Various	Sufficient for purpose.
Coloring agents (artificial)	Coal tar dyes (FD&C certified), Titanium dioxide.	To color products to whiten products.	do	Do.
Cooling and retort water treatment agents.	Calcium chloride	To prevent staining on exterior of canned goods.	Salads and spreads	0.5 percent.
			Various	Sufficient for purpose.
	Citric acid	do	do	Do.
	Dioctyl sodium sulfosuccinate	do	do	0.05 percent.
	Disodium-calcium ethylenediamine-tetraacetate.	do	do	Sufficient for purpose.
	Disodium ethylenediamine-tetraacetate.	do	do	Do.
	Disodium phosphate	do	do	Do.
	Ethylenediamine-tetraacetic acid	do	do	Do.
	Isopropanol	do	do	0.002 percent.
	Potassium pyrophosphate	do	do	Sufficient for purpose.
	Propylene glycol	do	do	Do.
	Sodium bicarbonate	do	do	Do.
	Sodium carbonate	do	do	Do.
	Sodium dodecylbenzene sulfonate	do	do	0.05 percent.
	Sodium gluconate	do	do	Sufficient for purpose.
	Sodium hexametaphosphate	do	do	Do.
	Sodium laurylsulfate	do	do	0.05 percent.
	Sodium metasilicate	do	do	Sufficient for purpose.
	Sodium n-alkylbenzene sulfonate (alkyl group predominantly C_{12} and C_{13} and not less than 95 percent C_{10} to C_{16}).	do	do	0.05 percent.
	Sodium nitrite (The sodium nitrite must be decharacterized with 0.05 percent powdered charcoal. Bulk decharacterized sodium nitrite when in cook room shall be held in locked metal bin or container conspicuously labeled "Decharacterized sodium nitrite—To be used by authorized personnel only.").	To inhibit corrosion on exterior of canned goods.	do	600 parts per million.
	Sodium pyrophosphate	To prevent staining on canned goods.	do	0.05 percent.
	Sodium tripolyphosphate	do	do	Do.
	Zinc oxide	do	do	0.01 percent.
	Zinc sulfate	do	do	Do.
Curing agents	Ascorbic acid	To accelerate color fixing and to preserve color in storage.	Various	75 ozs. to 100 gals. pickle at 10% pump level; ¾ oz. to 100 lbs. of poultry product; 10% solution to surfaces of the product prior to packaging (the use of such solution shall not result in the addition of a significant amount of moisture to the product).
	Erythorbic acid	do	do	Do.
	Sodium ascorbate	do	do	87.5 ozs. to 100 gals. pickle at 10% pump level; ⅞ oz. to 100 lbs. of poultry product; 10% solution to surfaces of product prior to packaging (the use of such solution shall not result in the addition of a significant amount of moisture to the product).
	Sodium erythorbate	do	do	Do.
	Citric acid or sodium citrate	Accelerate color fixing	Cured products	May be used in cured products to replace up to 50% of the ascorbic acid or sodium ascorbate that is used.
	Sodium or potassium nitrate	Source of nitrite	do	7 lbs. to 100 gals. pickle; 3½ ozs. to 100 lbs. of poultry product (dry cure); 2¾ ozs. to 100 lbs. of chopped poultry meat.

See footnotes at end of table.

CFR 21, Chapter I (USDA MID)
P. 3.51; 21.101; 125.3

0259

§ 3.50 Status of salt substitutes under the Federal Food, Drug, and Cosmetic Act.

(a) As a result of reported poisonings from salt substitutes containing lithium chloride, under date of March 8, 1949, the Food and Drug Administration announced that it would regard each salt substitute as a new drug within the meaning of section 201 (p) of the Federal Food, Drug, and Cosmetic Act, and that interstate distribution of each salt substitute should be discontinued until a new-drug application had been filed and become effective. Substantial information concerning the safety of many of the ingredients used in salt substitutes has been developed and published since the announcement was made. It is now possible to evaluate the safety of many individual salt substitutes and to determine whether they are new drugs requiring effective applications prior to distribution in interstate commerce.

(b) The Food and Drug Administration no longer regards all salt substitutes as new drugs. Upon request, the Administration will express its opinion whether a new-drug application is necessary for any particular product if complete information concerning its composition and proposed labeling is submitted.

[22 F.R. 10685, Dec. 25, 1957]

§ 3.51 Label declaration of D-erythroascorbic acid when it is an ingredient of a fabricated food.

(a) The article D-erythroascorbic acid (D-araboascorbic acid, D-erythro-3-ketohexonic acid lactone) has sometimes been designated as D-isoascorbic acid. However, this designation is capable of misleading purchasers of food in which it is used as an ingredient because of the similarity of such designation to the chemical name and the common name of vitamin C, which is ascorbic acid. Ascorbic acid (vitamin C) is capable of preventing the deficiency disease scurvy, but D-isoascorbic acid is ineffective for this purpose.

(b) The Joint Committee on Nomenclature of the American Institute of Nutrition and the Society of Biological Chemists has considered this matter, and pursuant to the Committee's recommendation the respective scientific organizations approved a resolution to drop the use of the designation D-isoascorbic acid and to adopt as a common name the name erythorbic acid for D-erythroascorbic acid.

(c) The compound D-erythroascorbic acid is not specified as an ingredient of any food for which a standard has been established. For foods other than those for which standards have been established, section 403 (i) (2) of the Federal Food, Drug, and Cosmetic Act requires that ingredients be listed on labels by their common or usual names. If the label on a food that contains D-erythroascorbic acid designates that ingredient by the name erythorbic acid, the requirement that the label bear the common or usual name of the ingredient will be regarded as having been met.

(Sec. 403, 52 Stat. 1047, as amended; 21 U.S.C. 343) [23 F.R. 5874, Aug. 21, 1958]

§ 3.52 Dimethylsulfoxide (DMSO) preparations; clinical testing and investigational use.

(a) (1) Chronic-toxicity studies with dimethylsulfoxide (DMSO) in animals, including dogs, rabbits, and swine, reported by a consulting laboratory in England and by a number of laboratories in the United States show that the administration of dimethylsulfoxide (DMSO) causes changes in the refractive index of the lens of the eyes of such animals. On the basis of these reports, clinical testing of dimethylsulfoxide (DMSO) preparations was discontinued for a time and later resumed under restricted conditions.

(2) An adequate, controlled human toxicity study (Phase I) involving short-term cutaneous application of 1 gram of dimethylsulfoxide (DMSO) per kilogram of body weight daily for 14 consecutive days has recently been completed. Data obtained, not previously available, show that when dimethylsulfoxide (DMSO) was applied topically to the skin of healthy volunteers, it did not produce adverse effects upon the eyes of the subjects. Mild, apparently reversible, changes were seen suggesting that the drug may have some effect upon the liver and upon the hemopoietic system in some subjects.

(b) A comprehensive evaluation of all available data on dimethylsulfoxide (DMSO) preparations justifies further clinical investigation of the drug in treating certain serious conditions. Although reports concerning the use of dimethylsulfoxide (DMSO) in relatively benign conditions are equivocal regarding its efficacy, short-term clinical use has been established as reasonably safe by adequate Phase I studies. Under appropriate protocols, further short-term clinical investigations in the treatment

§ 121.72 Adoption of regulation on initiative of Commissioner.

(a) The Commissioner upon his own initiative may propose the issuance of a regulation prescribing, with respect to any particular use of a food additive, the conditions under which such additive may be safely used. Notice of such proposal shall be published in the FEDERAL REGISTER and shall state the reasons for the proposal.

(b) Action upon a proposal made by the Commissioner shall, after publication of the notice, proceed as provided in § 121.51 and section 409 of the act.

§ 121.73 Judicial review.

The Secretary of Health, Education, and welfare hereby designates the Assistant General Counsel for Food and Drugs of the Department of Health, Education, and Welfare as the officer upon whom copy of petition for judicial review shall be served. Such officer shall be responsible for filing in the court a transcript of proceedings and the record on which the order of the Secretary of Health, Education, and Welfare is based. The transcript and record shall be certified by the Secretary.

§ 121.74 Procedure for amending and repealing tolerances or exemptions from tolerances.

(a) The Commissioner or any interested person may propose the issuance of a regulation amending or repealing a regulation pertaining to a food additive or granting or repealing an exemption for such additive. Such a proposal by an interested person shall be in writing. If such proposal by an interested person furnishes reasonable grounds therefor, the Commissioner will publish a notice announcing the proposal. Proposals initiated by the Commissioner will likewise be published. Following such publication, the proceedings shall be the same as prescribed by section 409 of the act and the regulations in this part for the promulgation of a regulation.

(b) "Reasonable grounds" shall include an explanation showing wherein the person has a substantial interest in such regulation and an assertion of facts (supported by data if available) showing that new information exists with respect to the food additive or that new uses have been developed or old uses abandoned, that new data are available as to toxicity of the chemical, or that experience with the existing regulation or exemption may justify its amendment or repeal. New data should be furnished in the form specified in § 121.51 for submitting petitions.

§ 121.75 Exemption for investigational use and procedure for obtaining authorization to market edible products from experimental animals.

A food additive or food containing a food additive intended for investigational use by qualified experts shall be exempt from the requirements of section 409 of the act under the following conditions:

(a) If intended for investigational use in vitro or in laboratory research animals, it bears a label which states prominently, in addition to the other information required by the act, the warning:

Caution. Contains a new food additive for investigational use only in laboratory research animals or for tests in vitro. Not for use in humans.

(b) If intended for use in animals other than laboratory research animals and if the edible products of the animals are to be marketed as food, permission for the marketing of the edible products as food has been requested by the sponsor, and authorization has been granted by the Food and Drug Administration in accordance with § 135.3 of this chapter or by the Department of Agriculture in accordance with § 309.20 of Title 9 (9 CFR 309.20), and it bears a label which states prominently, in addition to the other information required by the act, the warning:

Caution. Contains a new food additive for use only in investigational animals. Not for use in humans.

Edible products of investigational animals are not to be used for food unless authorization has been granted by the U.S. Food and Drug Administration or by the U.S. Department of Agriculture.

[36 F.R. 18377, Sept. 14, 1971]

Subpart B—Exemption of Certain Food Additives From the Requirement of Tolerances

§ 121.101 Substances that are generally recognized as safe.

(a) It is impracticable to list all substances that are generally recognized as safe for their intended use. However, by way of illustration, the Commissioner regards such common food ingredients as salt, pepper, sugar, vinegar, baking powder, and monosodium glutamate as safe for their intended use. The lists in paragraph (d) of this section include addi-

tional substances that, when used for the purposes indicated, in accordance with good manufacturing practice, are regarded by the Commissioner as generally recognized as safe for such uses.

(b) For the purposes of this section, good manufacturing practice shall be defined to include the following restrictions:

(1) The quantity of a substance added to food does not exceed the amount reasonably required to accomplish its intended physical, nutritional, or other technical effect in food; and

(2) The quantity of a substance that becomes a component of food as a result of its use in the manufacturing, processing, or packaging of food, and which is not intended to accomplish any physical or other technical effect in the food itself, shall be reduced to the extent reasonably possible.

(3) The substance is of appropriate food grade and is prepared and handled as a food ingredient. Upon request the Commissioner will offer an opinion, based on specifications and intended use, as to whether or not a particular grade or lot of the substance is of suitable purity for use in food and would generally be regarded as safe for the purpose intended, by experts qualified to evaluate its safety.

(c) The inclusion of substances in the list of nutrients does not constitute a finding on the part of the Department that the substance is useful as a supplement to the diet for humans.

(d) Substances that are generally recognized as safe for their intended use within the meaning of section 409 of the act are as follows:

Product	Tolerance	Limitations or restrictions
(1) ANTICAKING AGENTS		
Aluminum calcium silicate	2 percent	In table salt.
Calcium silicate	5 percent	In baking powder.
Calcium silicate	2 percent	In table salt.
Magenesium silicate	do	Do.
*Sodium aluminosilicate (sodium silicoaluminate).	do	
*Sodium calcium aluminosilicate, hydrated (sodium calcium silicoaluminate).	do	Do
Tricalcium silicate	do	
(2) CHEMICAL PRESERVATIVES		
Ascorbic acid		
Ascorbyl palmitate		
Benzoic acid	0.1 percent.	
Butylated hydroxyanisole	Total content of antioxidants not over 0.02 percent of fat or oil content, including essential (volatile) oil content of food.	
Butylated hydroxytoluene	do	
Calcium ascorbate		
Calcium propionate		
*Calcium sorbate		In cheese wraps.
Caprylic acid		
Dilauryl thiodipropionate	Total content of antioxidants not over 0.02 percent of fat or oil content, including essential (volatile) oil content of the food.	
Erythorbic acid	0.1 percent (equivalent antioxidant activity 0.01 percent).	In edible fats or oils:
Gum guaiac	0.1 percent	
*Methylparaben (methyl-p-hydroxybenzoate).		Not in meats or in food recognized as source of vitamin B_1.
Potassium bisulfite		Do.
Potassium metabisulfite		
Potassium sorbate		
Propionic acid		
Propyl gallate	Total content of antioxidants not over 0.02 percent of fat or oil content, including essential (volatile) oil content of the food.	
*Propylparaben (propyl p-hydroxybenzoate).	0.1 percent	
Sodium ascorbate		
Sodium benzoate	0.1 percent	

See footnotes at end of table.

Product	Tolerance	Limitations or restrictions
(2) CHEMICAL PRESERVATIVES—con.		
Sodium bisulfite		Not in meats or in food recognized as source of vitamin B_1.
Sodium metabisulfite		Do.
Sodium propionate		
Sodium sorbate		Do.
Sodium sulfite		
Sorbic acid		
*Stannous chloride	0.0015 percent calculated as tin	Do.
Sulfur dioxide		
Thiodipropionic acid	Total content of antioxidants not over 0.02 percent of fat or oil content, including essential (volatile) oil content of the food.	
Tocopherols		
(3) EMULSIFYING AGENTS		
Cholic acid	0.1 percent	Dried egg whites.
Desoxycholic acid	do	Do.
Diacetyl tartaric acid esters of mono- and diglycerides of edible fats or oils, or edible fat-forming fatty acids.		Do.
Glycocholic acid	0.1 percent	
Mono- and diglycerides of edible fats or oils, or edible fat-forming acids.		
Monosodium phosphate derivatives of mono- and diglycerides of edible fats or oils, or edible fat-forming fatty acids.		
Propylene glycol		Do.
Ox bile extract	0.1 percent	Do.
Taurocholic acid (or its sodium salt)	do	
(5) NUTRIENTS AND/OR DIETARY SUPPLEMENTS [1]		
*Alanine (L- and DL-forms)		
*Arginine (L- and DL-forms)		
Ascorbic acid		
*Aspartic acid (L- and DL-forms)		
*Biotin		
Calcium carbonate		
*Calcium citrate		
*Calcium glycerophosphate		
Calcium oxide		
Calcium pantothenate		
Calcium phosphate (mono-, di-, tribasic).		
*Calcium pyrophosphate		
Calcium sulfate		
Carotene		
*Choline bitartrate		
*Choline chloride	0.005 percent	In table salt as a source of dietary iodine.
Copper gluconate	0.01 percent	
Cuprous iodide		
*Cysteine (L- form)		
*Cystine (L- and DL- forms)		
Ferric phosphate		
Ferric pyrophosphate		
Ferric sodium pyrophosphate		
*Ferrous gluconate		
*Ferrous lactate		In animal feeds.
Ferrous sulfate		
*Glycine (aminoacetic acid)		
*Histidine (L- and DL- forms)		
*Inositol		
Iron, reduced		
*Isoleucine (L- and DL-forms)		
*Leucine (L- and DL-forms)		
*Linoleic acid (prepared from edible fats and oils and free from chick-edema factor).		
Lysine (L- and DL-forms)		
*Magnesium oxide		
*Magnesium phosphate (di-, tribasic)		
*Magnesium sulfate		
*Manganese chloride		
*Manganese citrate		
*Manganese gluconate		
*Manganese glycerophosphate		

See footnotes at end of table.

21.4004 Brominated vegetable oil.

The food additive brominated vegetable oil may be safely used in accordance with the following prescribed conditions:

(a) The additive complies with specifications prescribed in Food Chemicals Codex, First Edition, except that free fatty acids (as oleic) shall not exceed 2.5 percent and iodine value shall not exceed 5.

(b) The additive is used on an interim basis as a stabilizer for flavoring oils used in fruit-flavored beverages, for which any applicable standards of identity do not preclude such use, in an amount not to exceed 15 parts per million in the finished beverage, pending the outcome of additional toxicological studies on which periodic reports at 6-month intervals are to be furnished and final results submitted to the Food and Drug Administration not later than December 1, 1973.

[35 F.R. 12062, July 28, 1970. Redesignated at 38 F.R. 5342, Feb. 28, 1973]

PART 125—LABEL STATEMENTS CONCERNING DIETARY PROPERTIES OF FOOD PURPORTING TO BE OR REPRESENTED FOR SPECIAL DIETARY USES

Sec.
125.1 Definitions and interpretations of terms.
125.2 General label statements.
125.3 Label statements relating to vitamins.
125.4 Label statements relating to minerals.
125.5 Label statements relating to infant food.
125.6 Label statements relating to certain food used in control of body weight or in dietary management with respect to disease.
125.7 Label statements relating to nonnutritive constituents.
125.8 Label statements relating to hypoallergenic food.
125.9 Label statements relating to food for use as a means of regulating the intake of sodium.

AUTHORITY: The provisions of this Part 125 issued under secs. 403, 701, 52 Stat. 1047, as amended, 1055, as amended; 21 U.S.C. 343, 371, unless otherwise noted.

SOURCE: The provisions of this Part 125 appear at 20 F.R. 9646, Dec. 20, 1955, unless otherwise noted.

CROSS REFERENCES: For other regulations in this chapter concerning special dietary foods, see §§ 1.11, 3.9 and 3.32.

§ 125.1 Definitions and interpretations of terms.

(a) The definitions and interpretations of terms contained in section 201 of the Federal Food, Drug, and Cosmetic Act shall be applicable also to such terms when used in the regulations in this part.

(b) Any requirement of the regulations in this part with respect to the quantity of vitamin A means the biologically measured activity of vitamin A and its precursors; but if any such precursor is sold as such or is added to a food the label shall designate such precursor by its common or usual name and not as vitamin A.

(c) Specification in the regulations in this part of a quantity of any vitamin, mineral, or other dietary property as a minimum daily requirement shall not be construed as a determination that such quantity is sufficient for the treatment of any disease resulting from a deficiency in such vitamin, mineral, or other property.

(d) For the purposes of the regulations in this part, the terms "infant," "child," and "adult" mean persons not more than 12 months old, more than 12 months but less than 12 years old, and 12 years or more old, respectively.

§ 125.2 General label statements.

(a) If a food (including food to which any one or more of §§ 125.3 to 125.8, inclusive, is applicable) purports to be or is represented for any special dietary use by man, its label shall bear a statement of the dietary properties upon which such use is based in whole or in part. Such statement shall show the presence or absence of any substance, any alteration of the quantity or character of any constituent, and any other dietary property of such food upon which such use is so based.

(b) If a food (including food to which any one or more of §§ 125.3 to 125.8, inclusive, is applicable) purports to be or is represented for special dietary use by reason of its use for treating any disease resulting from a dietary deficiency in man, its label shall bear, in addition to the information required under paragraph (a) of this section, adequate directions for such use.

§ 125.3 Label statements relating to vitamins.

(a)(1) If a food purports to be or is represented for special dietary use by

man by reason of its vitamin property in respect of:

Vitamin A or its precursors,
Vitamin B_1 (thiamine),
Vitamin C (ascorbic acid),
Vitamin D,
Riboflavin, or
Niacin or niacinamide,

the label (except as provided in this paragraph, with respect to cow's milk and evaporated milk, and unless the exemption under subparagraph (3) of this paragraph applies) shall bear a statement of the proportion of the minimum daily requirement for such vitamin supplied by such food when consumed in a specified quantity during a period of one day. If such purported or represented special dietary use is for persons within two or more age groups for which minimum daily requirements are set forth in paragraph (b), such statement shall include such proportion for each such group; but if such use is for persons irrespective of age groups, such statement may be limited to the proportion of the minimum daily requirement set forth in paragraph (b) of this section for an adult. The quantity specified as above required shall be the quantity customarily or usually consumed during a period of one day, or a quantity reasonably suitable for and practicable of consumption within such period. When such proportion is a whole number and a fraction it may be expressed as the whole number and the fraction may be disregarded. The foregoing requirements of this paragraph shall not apply to cow's milk and evaporated milk which purport to be or are represented for special dietary use by reason of their content of vitamin D; but in the case of cow's milk in which the vitamin D content has been increased by any means, and in the case of evaporated milk with increased vitamin D content as prescribed in the definition and standard of identity for evaporated milk (§ 18.520 of this chapter), the label shall bear a statement of the number of U. S. P. units in a specified quantity of such milk or evaporated milk. In the case of cow's milk in which the vitamin D content is increased to less than 135 U. S. P. units to each quart, the label shall also bear a statement that additional vitamin D should be supplied from other sources.

(2) If a food purports to be or is represented for special dietary use by man by reason of its vitamin property in respect of any vitamin not listed in subparagraph (1) of this paragraph, the label shall bear a statement of the quantity of such vitamin in a specified quantity of such food. The quantity of food specified as required by this section, shall be the quantity customarily or usually consumed during a period of one day, or a quantity reasonably suitable for and practicable of consumption within such period. If the need in human nutrition for such vitamin has not been established, the label shall also bear the statement "The need for _____ in human nutrition has not been established," the blank to be filled in with the name of such vitamin.

(3) If a food purports to be or is represented for special dietary use by man by reason of its vitamin property, and any such use is for treating any disease resulting from a dietary deficiency of any vitamin, the label shall bear a statement of the quantity of such vitamin in a specified quantity of such food. If the represented special dietary use of such food is solely for treating any such disease, such food shall be exempt from the labeling requirements of subparagraphs (1) and (2) of this paragraph when otherwise applicable.

(4) Compliance with the provisions of subparagraphs (2) and (3) of this paragraph shall not be construed as relieving any food which purports to be or is represented for special dietary use by reason of its vitamin property from the application of section 403 (a) and 201 (n) of the act, as in the case where the need for such vitamin in human nutrition is not substantially supported by the opinion of experts qualified by scientific training and experience to determine such needs.

(b) For the purposes of the regulations in this section the following are minimum daily requirements:

(1) For vitamin A, 1,500 U. S. P. units for an infant, 3,000 U. S. P. units for a child, 4,000 U. S. P. units for an adult.

(2) For vitamin B_1 (thiamine), 0.25 milligram (83 U. S. P. units) for an infant, 0.5 milligram (167 U. S. P. units) for a child less than six years old, 0.75 milligram (250 U. S. P. units) for a child six or more years old, 1 milligram (333 U. S. P. units) for an adult.

(3) For vitamin C (ascorbic acid), 10 milligrams (200 U. S. P. units) for an infant, 20 milligrams (400 U. S. P. units) for a child, 30 milligrams (600 U. S. P. units) for an adult.

(4) For vitamin D, 400 U. S. P. units for an infant, child, or adult.

(5) For riboflavin, 0.6 milligram for an infant, 0.9 milligram for a child, 1.2 milligrams for an adult.

(6) For niacin or niacinamide 5.0 milligrams for a child less than 6 years old, 7.5 milligrams for a child 6 or more years old, 10 milligrams for an adult.

[20 F.R. 9646, Dec. 20, 1955, as amended at 22 F.R. 3841, June 1, 1957]

§ 125.4 Label statements relating to minerals.

(a) (1) If a food purports to be or is represented for special dietary use by man by reason of its mineral property in respect of:

Calcium,
Phosphorus,
Iron, or
Iodine,

the label (except as provided in this section with respect to iodized salt, and unless the exemption under subparagraph (3) of this paragraph applies) shall bear a statement of the proportion of the minimum daily requirement for such element supplied by such food when consumed in a specified quantity during a period of one day. If such purported or represented special dietary use is for persons within two or more age groups or other groups having special dietary requirements, for which minimum daily requirements are set forth in paragraph (b) of this section, such statement shall include such proportion for each such group; but if such use is for persons irrespective of such groups, such statement may be limited to the proportion of the minimum daily requirement set forth in paragraph (b) of this section for an adult other than a pregnant or lactating woman. The quantity specified as above required shall be the quantity customarily or usually consumed during a period of one day, or a quantity reasonably suitable for and practicable of consumption within such period. When such proportion is a whole number and a fraction it may be expressed as the whole number and the fraction may be disregarded. The foregoing requirements of this paragraph shall not apply to iodized salt which purports to be or is represented for special dietary use by reason of its iodine content, if the label bears a statement of the quantity or proportion of iodine, or salt thereof, in the iodized salt and the quantity of iodine present therein is not more than 0.02 percent by weight.

(2) If a food purports to be or is represented for special dietary use by man by reason of its mineral property in respect of any element not listed in subparagraph (1) of this paragraph, the label shall bear a statement of the quantity of such element in a specified quantity of such food. Except in the case of foods subject to § 125.9, the quantity of food specified as required in this section shall be the quantity customarily or usually consumed during a period of one day, or a quantity reasonably suitable for and practicable of consumption within such period. If the need in human nutrition for such element has not been established, the label shall also bear the statement "The need for ---------- in human nutrition has not been established," the blank to be filled in with the name of such element.

(3) If a food purports to be or is represented for special dietary use by man by reason of its mineral property, and any such use is for treating any disease resulting from a dietary deficiency of any element, the label shall bear a statement of the quantity of such element in a specified quantity of such food. If the represented special dietary use of such food is solely for treating any such disease, such food shall be exempt from the labeling requirements of subparagraphs (1) and (2) of this paragraph when otherwise applicable.

(4) Compliance with the provisions of subparagraphs (2) and (3) of this paragraph shall not be construed as relieving any food which purports to be or is represented for special dietary use by reason of its mineral property from the application of sections 403 (a) and 201 (n) of the act, as in the case where the need for such element in human nutrition is not substantially supported by the opinion of experts qualified by scientific training and experience to determine such needs.

(b) For the purposes of the regulations in this section, the following are minimum daily requirements:

(1) For calcium (Ca), 750 milligrams for a child or an adult, except a pregnant or lactating woman in which case the minimum daily requirement is 1.5 grams.

(2) For phosphorus (P), 750 milligrams for a child or an adult, except a pregnant or lactating woman in which

case the minimum daily requirement is 1.5 grams.

(3) For iron (Fe), 7.5 milligrams for a child less than six years old, 10 milligrams for a child six or more years old or for an adult, except a pregnant or lactating woman in which case the minimum daily requirement is 15 milligrams.

(4) For iodine (I), 0.1 milligram for a child or an adult.

§ 125.5 Label statements relating to infant food.

(a) If a food (other than a dietary supplement of vitamins and/or minerals alone) purports to be or is represented for special dietary use for infants, the label shall bear, if such food is fabricated from two or more ingredients, the common or usual name of each ingredient, including spices, flavoring, and coloring.

(b) If such food, or any ingredient thereof, consists in whole or in part of plant or animal matter and the name of such food or ingredient does not clearly reveal the specific plant or animal which is its source, such name shall be so qualified as to reveal clearly the specific plant or animal that is such source.

(c) If such use of the food is by reason of its simulation of human milk or its suitability as a complete or partial substitute for human milk, the label shall also bear:

(1) A statement of the percent by weight or weight per unit volume of moisture, protein, fat, available carbohydrate, ash, and crude fiber contained in such food.

(2) A statement of the number of available kilocalories (in the case of food label statements, a kilocalorie is represented by the word "Calorie") supplied by a specified quantity of such food as customarily or usually prepared for consumption.

(3) A statement of the amount of each vitamin-or mineral listed in subparagraph (5) of this paragraph and the amount of other added vitamin(s) and mineral(s) supplied by a specified quantity of such food as customarily or usually prepared for consumption.

(4) The statement "This product should not be used as the sole source of protein in the infant diet" if a quantity which supplies 100 available kilocalories of such food as customarily or usually prepared for consumption contains less than 1.8 grams of protein of a biological quality equivalent to that of casein, or if the amount and biological quality of protein per 100 available kilocalories of such food are such that the quality of protein expressed as a fraction of that of casein multiplied by the amount of protein in grams is less than 1.8, or if the biological quality of protein is less than 70 percent of that of casein.

(i) For the purpose of this subparagraph, the method for determining biological quality of protein shall be the method prescribed on page 800 (secs. 39.166–39.170) under "Biological Evaluation of Protein Quality—Official, Final Action" of "Official Methods of Analysis of the Association of Official Analytical Chemists," 11th edition (1970).

(ii) For the purpose of this subparagraph, the method for determining the amount of protein is to multiply by 6.25 the total nitrogen content in grams, as determined by the method described on page 16 (sec. 2.051) under "Improved Kjeldahl Methods for Nitrate-Free Samples—Official, Final Action" of "Official Methods of Analysis of the Association of Official Analytical Chemists," 11th edition (1970).

(5) If a quantity which supplies 100 available kilocalories of such food as customarily or usually prepared for consumption contains less than the following amounts of vitamins and minerals, a statement that an additional quantity of such vitamin(s) or mineral(s), as the case may be, should be supplied from other sources:

Vitamins and minerals	Unit of measurement	Minimum amounts
Vitamin A	U.S.P. units	250
Vitamin D	do	40
Vitamin E	International units	0.3
Ascorbic acid (vitamin C).	Milligrams	7.8
Thiamin (vitamin B_1).	do	0.025
Riboflavin (vitamin B_2).	do	0.06
Niacin [1]	Milligram equivalents.	0.8
Vitamin B_6	Milligrams	0.035
Folacin	Micrograms	4
Pantothenic acid	Milligrams	0.3
Vitamin B_{12}	Micrograms	0.15
Calcium	Milligrams	50
Phosphorus	do	25
Magnesium	do	6
Iron	do	1
Iodine	Micrograms	5
Copper	Milligrams	0.06

[1] The generic term "niacin" includes niacin (nicotinic acid), niacinamide (nicotinamide), and 1 milligram equivalent for each 60 milligrams of tryptophan in the food.

When a statement prescribed by this subparagraph is required, it shall ap-

pear in immediate proximity to the statement for the appropriate vitamin or mineral required by paragraph (c)(3). The difference in quantity between the amount of vitamin(s) and mineral(s) supplied and the amount required by this subparagraph, expressed on the same basis, must also appear in the same statement.

(6) If such food contains fat at a level supplying less than 15 percent of the total available kilocalories, or linoleic acid (present as a glyceride) at a level supplying less than 2 percent of the total available kilocalories, a statement that an additional quantity of fat or linoleic acid (linoleate), as the case may be, should be supplied from other sources. The requirement of this subparagraph shall not apply to such food which purports to be or is represented for special dietary use by reason of a need for regulating the intake of fat.

(d) The provisions of paragraph (c) of this section shall not apply to whole milk (of cows) or evaporated milk except with respect to ascorbic acid, vitamin D, and iron under paragraph (c)(5) of this section.

(e) A food which purports to be or is represented for special dietary use solely as a food for infants by reason of its simulation of human milk or its suitability as a complete or partial substitute for human milk, and which complies with the provisions of this section, shall be exempt from the effective provisions of §§ 125.3, 125.4, and 125.6 of this Part 125.

[36 F.R. 23555, Dec. 10, 1971; 36 F.R. 24792, Dec. 23, 1971]

§ 125.6 Label statements relating to certain food used in control of body weight or in dietary management with respect to disease.

If a food purports to be or is represented for special dietary use by man by reason of its use as a means of regulating the intake of protein, fat, carbohydrate, or calories, for the purpose of controlling body weight, or for the purpose of dietary management with respect to disease, the label shall bear a statement of:

(a) The percent by weight of protein, fat, and available carbohydrates in such food; and

(b) The number of available calories supplied by a specified quantity of such food.

§ 125.7 Label statements relating to nonnutritive constituents.

If a food purports to be or is represented for special dietary use by man by reason of the presence of any constituent which is not utilized in normal metabolism, the label shall bear a statement of the percent by weight of such constituent, and, in juxtaposition with the name of such constituent, the word "nonnutritive". If such constituent is fibrous plant matter, it shall be considered to be crude fiber and its percent expressed as such. But if such constituent is saccharin or a saccharin salt, the label shall bear, in lieu of such statement and word, the statement "Contains ---- saccharin (or saccharin salt, as the case may be), a nonnutritive artificial sweetener which should be used only by persons who must restrict their intake of ordinary sweets," the blank to be filled in with the percent by weight of saccharin or saccharin salt in such food. The provisions of this section shall not be construed as authorizing the use of saccharin or its salts in any food other than one for use by persons who must restrict their intake of carbohydrates, or as relieving any food from compliance with any requirement of sections 402 (b) or (d), 403 (g), or other provisions of the act.

§ 125.8 Label statements relating to hypoallergenic food.

If a food purports to be or is represented for special dietary use by reason of the decrease or absence of any allergenic property or by reason of being offered as food suitable as a substitute for another food having an allergenic property, the label shall bear:

(a) The common or usual name and the quantity or proportion of each ingredient (including spices, flavoring, and coloring) in case the food is fabricated from two or more ingredients.

(b) A qualification of the name of the food, or the name of each ingredient thereof in case the food is fabricated from two or more ingredients, to reveal clearly the specific plant or animal that is the source of such food or of such ingredient, if such food or such ingredient consists in whole or in part of plant or animal matter and such name does not reveal clearly the specific plant or animal that is such a source.

(c) An informative statement of the nature and effect of any treatment or processing of the food or any ingredient

Proc. Soc. Exp. Biol. Med. 126: 354-356 1967

0284

Interaction of Dimethysulfoxide and Ascorbic Acid on Fibroblasts of Rats. (32443)

J. M. Archer, K. B. Shilkin, J. M. Papadimitriou and M. N-I. Walters.
(Introduced by N. F. Stanley)
Department of Pathology, University of Western Australia, Perth

Despite the current wide employment of dimethylsulfoxide (DMSO) in many scientific disciplines, its basic mechanism of action at the cellular level is ill-understood. It has been shown that DMSO in equivalent concentrations to those used in human therapy damages striated muscle cells(1) and hepatocytes(2) of rats. The endoplasmic reticulum of these cells dilates to form vacuoles; agents which are believed to stabilize cell membranes and so prevent toxic cellular damage are ineffective in the case of DMSO(2). Similar ultrastructural changes in the endoplasmic reticulum have also been observed in fibroblasts in the wounds of scorbutic guinea pigs(3,4). A study was therefore undertaken to assess the relationship of DMSO and ascorbic acid in the production and prevention of ultrastructural abnormalities in the fibroblasts of healing wounds.

Materials and methods. Two parallel linear incisions 2.5 cm in length were made on the shaved abdomen of 16 ether-anaesthetised Wistar rats (200-250 g) through the skin and subcutaneous tissue. The animals were divided equally into 4 groups; all groups had skin biopsies taken after 5 days. Group 1 received an application to the wound site of 90% DMSO 1 hour before biopsy. Groups 2 and 3 received intraperitoneal doses of 100 mg ascorbic acid after 48 hours and again at 7½ hours and 1½ hours before biopsy. In addition DMSO was applied in Group 2 as described above for Group 1. Group 4, which served as controls, was given neither ascorbic acid nor had DMSO applied. A total of 16 healing wounds was examined. Small blocks from these wounds were rapidly immersed in cold osmium tetroxide buffered in S-collodine. After dehydration in graded solutions of ethanol, the tissue was embedded in araldite. Sections were cut on a Huxley ultramicrotome, stained with lead, and examined on a JEM T6 electron microscope at an accelerating voltage of 60 KV. In addition, fibroblasts from DMSO-treated cremaster muscles of Wistar rats were examined as previously described(1).

Results. Fibroblasts from rats receiving neither DMSO nor ascorbic acid (Group 4) or only ascorbic acid (Group 3) appeared normal. Rats receiving DMSO only (Group 1): All fibroblasts from wounds of animals to which DMSO had been applied exhibited some ultrastructural anomaly. The cytoplasm of these cells was swollen and electron lucent. The cisternae of the endoplasmic reticulum were dilated and contained fine fibrillar material (Fig. 1). The cisternal membranes sometimes showed loss of ribosomal material. Mitochondria were also swollen and had fewer and less prominent cristae. The clusters of cytoplasmic ribosomes were diminished. No obvious abnormality of collagen fibrils was seen.

Striated muscle fibres were also swollen and displayed loss of the myofibrillar elements. Mitochondria were diminished in number and those present were swollen. Remnants of the sarcotubular system exhibited gross dilatation producing large electron lucent vacuoles measuring 3-8 μ in diameter (Fig. 2).

In rats receiving both DMSO and ascorbic acid (Group 2) cytoplasmic swelling and dilatation of cisternae of the endoplasmic reticulum were again seen. However, these were much less severe (Fig. 3) than in the group receiving DMSO alone. Myocytic vacuoles were not present although swelling of striated muscle fibres, myofibrillar loss and mitochondrial swelling were present.

Discussion. The ultrastructural changes produced in rat fibroblasts by DMSO in these experiments occur mainly in the endoplasmic reticulum. The cisternae of this organelle dilate and assume a clear vacuolated appearance. There are fewer clusters of ribosomes scattered throughout the cytoplasm, which is

FIG. 1. Fibroblast from wound treated with DMSO. The cytoplasm is swollen and electron lucent, mitochondria are enlarged, the cisternae of endoplasmic reticulum are dilated and free ribosomes are diminished in number. Magnification 8695.

FIG. 2. Striated muscle fibre from rat after DMSO application. The cell is swollen and there is myofibrillar disarray. The elements of the sarcotubular system are grossly dilated. Magnification 8118.

FIG. 3. Fibroblast from animal receiving both DMSO and ascorbic acid. Although the cytoplasm is swollen and mitochondria enlarged the cisternae of ergastoplasm are only slightly dilated. Magnification 2579.

most likely swollen by the accumulation of water. The endoplasmic reticulum is similarly deranged in fibroblasts in wounds of scorbutic guinea pigs. Here the ribosomes lose their orientation to the cisternal membrane and become scattered(3,4). Collagen production is also impaired in this deficiency disease.

Insofar as the ribosome—endoplasmic reticular system is the morphologic counterpart of the protein synthetic pathway, the disturbance of these structures in scorbutus explains the cytologic basis for the lack of manufacture of adequate collagen. Furthermore, after treatment with ascorbic acid there is a reversion to the normal organellar appearance concomitant with the synthesis of new collagen(4). In our experiments, treatment with ascorbic acid also reduces the endoplasmic reticular abnormalities produced by DMSO. The nature of DMSO-induced changes in relation to collagen synthesis has not been assessed in this situation. Collateral evidence raises the possibility that such DMSO-damaged fibroblasts would have impaired protein synthetic ability. Using analyses of collagen formation in rats with experimental granulomata as an index it has been shown that application of DMSO results in lesions containing less of the components of collagen than controls(5). Likewise, the amount of collagen determined by hydroxyproline content, in human radiation scar tissue was less in areas treated by DMSO than in similar untreated areas. Collagen fibrils that had formed before application of DMSO showed no ultrastructural derangement. Although the possibility exists that the decreased content of collagen could be the result of degradation caused by DMSO, the evidence for this is lacking(6). Other human fibroproliferative disorders have benefited from treatment with DMSO(7,8,9). It may well be that in these conditions the improvement results from damage to fibroblasts which cannot then synthesise collagen; the balance of synthesis and degradation is altered to favour the latter.

The nature of the effect of ascorbic acid in partially inhibiting the damage caused by DMSO is unclear. It has been shown that ascorbic acid is concerned with electron transport and is transferred in microsomes(10). DMSO, a dipolar aprotic chemical which

forms powerful hydrogen bonds(11), may interfere with these intracellular mechanisms of electron transfer and this physico-chemical disorganisation may result in the morphological changes described. Ascorbic acid, on the other hand, may partially reverse this effect and so limit the changes due to DMSO.

The large vacuoles in muscle fibres treated with DMSO resemble those arising from sarcotubular elements in hypokalaemic periodic paralysis(12,13). Impairment of the function of this organelle would interfere with muscle contraction and may account for the relief of pain in musculoskeletal disorders described after the application of DMSO(14).

Summary. DMSO causes ultrastructural changes in rat wound fibroblasts of dilation of the endoplasmic reticulum and diminution of ribosomes. Such features are similar to those described in the wound fibroblast of scorbutic guinea pigs. Ascorbic acid partly prevents DMSO damage. DMSO probably acts by interfering with cell membrane electron transfer systems and this may be the basis of its beneficial effect in clinical situations.

1. Walters, M. N-I., Papadimitriou, J. M., Shilkin, K. B., Exp. Molec. Pathol., 1967, v6, 106.
2. Shilkin, K. B., Papadimitriou, J. M., Walters, M. N-I., Aust. J. Exp. Biol. Med. Sci., 1966, v44, 581.
3. Peach, R., J. Ultrastruct. Res., 1962, v6, 579.
4. Ross, R., Benditt, E. P., J. Cell. Biol., 1964, v22, 365.
5. Gries, G., Bublitz, G., Lander, J., Ann. N. Y Acad. Sci., 1967, v141, 630.
6. Frommhold, W., Bublitz, G., Gries, G., ibid., 1967, v141, 603.
7. Scherbel, A. L., McCormack, L. J., Poppo, M. J., Cleveland Clinic Quart., 1965, v32, 47.
8. Scherbel, A. L., McCormack, L. J., Layle, J K., Ann. N. Y. Acad. Sci., 1967, v141, 613.
9. Persky, L., Stewart, B. H., ibid., 1967, v141, 551.
10. Staudinger, Hj., Krisch, K., Leonhauser, S., ibid., 1961, v92, 195.
11. Parker, A. J., Quart. Rev. Chem. Soc., 1962, v2, 163.
12. Shy, G. M., Wanko, T., Rowley, P. T., Engel, A. G., Exp. Neurol., 1961, v3, 53.
13. Howes, E. J., Price, H. M., Pearson, C. H., Blumberg, J. M., Neurology, 1966, v16, 242.
14. Rosenbaum, E. E., Herschler, R. J., Jacob, S. W., J.A.M.A., 1965, v192, 309.

Received July 17, 1967. P.S.E.B.M., 1967, v126.

0287

J Clin Endocrinol 1956 16 653-661

STUDIES ON OBESITY. III. EFFECT OF ASCORBIC ACID ON THE INSULIN-GLUCOSE TOLERANCE CURVE

E. C. ARENDT, M.D., C. M.* AND C. J. PATTEE, M.D., C.M.†
The Clinical Investigation Unit, Queen Mary Veterans Hospital, Montreal, Canada

THE role of ascorbic acid during stress has been emphasized by several investigators (1). Stress not only depletes the body of ascorbic acid but augments the requirements (1, 2). Dugal *et al.* (3, 4) have shown that pretreatment with ascorbic acid will prevent the typical enlargement of the adrenals in the guinea pig when under the influence of stress. These and other experiments (5, 6) indicate that ascorbic acid plays a compensatory role somewhat similar to the one of the adrenocortical hormones, and perhaps prevents release of adrenocorticotropin by the anterior pituitary.

Menten, Sigal and King (7, 8) have shown that in the scorbutic guinea pig (which cannot synthesize vitamin C) there is reduced carbohydrate tolerance. This was evident even before external signs of scurvy were present and was enhanced by the addition of another stressor, namely, the injection of diphtheria toxin. The peak of the glucose tolerance curve moved up and to the right in successive stages of vitamin depletion and was reversible to a certain extent, with only slight change in the fasting blood sugar levels. Animals sacrificed in the prescorbutic stage showed hydropic degeneration of the beta cells of the islets of Langerhans; at a later stage arteriosclerotic changes were evident. Stewart *et al.* (9) investigated the effect of insulin in scorbutic and prescorbutic monkeys and found them relatively insensitive. They interpreted their data as indicating that adrenocortical function is increased rather than decreased in scurvy. Besides this direct effect on the adrenal cortex and the insulin-elaborating mechanism, there is evidence that ascorbic acid is important in the metabolism of fatty acids, and that its absence increases the synthesis of cholesterol (10–13).

Since obese patients are under continuous daily mechanical stress, of greater or lesser degree, it was felt that the body might be partly depleted of ascorbic acid despite an average vitamin intake; the depletion would be enhanced by the fact that the vitamin requirements of the obese must be greater than average, in view of a larger body mass. Analyses of the diets

Received July 30, 1955.
* Formerly Research Fellow. Now with the Montreal General Hospital.
† Director, Clinical Investigation Unit.

of our obese patients showed that, though the majority consumed food high in carbohydrate content, it was deficient in fruits and vegetables. There is wide disagreement as to the existence and manifestation of hypovitaminosis C, but it has been shown recently that blood levels consistent with such a state, or even frank scurvy, are not rare in the North American continent (14). The present study was undertaken to ascertain whether or not ascorbic acid would have any effect on restoring to normal the impaired carbohydrate metabolism of the obese. It was decided to study changes, if any, in the insulin-glucose tolerance curve rather than in the oral glucose tolerance curve, since it was felt that the former test would reflect earlier changes in carbohydrate tolerance. Previous studies on 32 obese patients carried out in our Research Unit showed that all but 1 had an abnormal insulin-glucose tolerance curve (15).

PROCEDURE

Insulin-glucose tolerance curves were obtained according to the procedure described by Engel and Scott (16). The subjects were fasted overnight and kept at rest the following morning. Venous blood was withdrawn and 0.1 unit of regular insulin per kilogram of body weight was injected intravenously. At 30 minutes , or when the first significant symptoms of hypoglycemia appeared (depending on which occurred first), a sample of blood was withdrawn and 0.8 Gm. of glucose per kilogram of body weight was given by mouth. Additional blood samples were taken at 60, 90, 120 and 180 minutes from the time of the initial sample. For comparative purposes, the blood sugar levels are reported as a percentage of the initial blood sugar level, which is taken as 100 per cent.

The patients were then given 500 mg. of ascorbic acid[1] orally every six hours for a minimum period of three weeks, but usually for an average of four weeks. The insulin-glucose tolerance test was then repeated, using the same amounts of insulin and glucose administered during the first test. In 6 patients the test was repeated after a similar period without ascorbic acid; they had been given a reducing diet to see whether a loss of 5 to 15 pounds would affect the tolerance curve.

Blood sugar levels were determined according to the Folin-Wu method adapted to the spectrophotometer after precipitating the proteins with 10 per cent sodium tungstate (17). Blood ascorbic-acid levels were determined according to the method of Heinemann and Hald (18).

RESULTS

The results of the insulin-glucose tolerance tests in 21 obese subjects before and after the oral administration of ascorbic acid are shown in Table 1 and in Figure 1. The degree of overweight varied from 30 to 130 per cent according to the ideal weights listed in the Davenport charts, using 35 years as the optimum age. The average degree overweight was 74.4 per cent and the average duration of the obese state, 14.5 years. All the patients in this study had an abnormal insulin-glucose tolerance curve, *i.e.*,

[1] Ascorbic acid was supplied through the courtesy of Ayerst, McKenna and Harrison, Limited.

TABLE 1. THE EFFECT OF ORAL ASCORBIC ACID ON THE INSULIN-GLUCOSE TOLERANCE OF 21 OBESE PATIENTS

Patient No.	Age and Sex	Weight change	% Initial blood sugar level					Insulin reaction
			30 min.	60 min.	90 min.	120 min.	180 min.	
Group I: Obese patients with abnormal oral glucose tolerance curve								
1	32F*	− 9	46	101	170	203	165	+
2	32M	−14	51	123	169	183	134	+
			72	161	197	169	143	−
3	40F*	0	37	107	155	174	87	?
			48†	114	128	88	88	
4	43M	−16	52	98	166	147	141	−
			39	108	164	164	150	−
5	44M	−12	62	133	184	152	112	−
			53	132	183	168	109	−
6	47M	?	50	123	202	225	98	−
			43	115	182	189	140	−
7	53M	− 6	74	114	178	187	135	−
			70	106	151	166	136	−
8	53M	0	73†	117	166	175	151	−
			47	113	185	211	124	+
			52	100	153	198	191	+
			37	112	229	216	137	−
			50	100	169	150	87	−
Avge.	43	− 8	Before 55	120	189	189	132	
			After 49	111	166	174	129	
			S.D. of differences 10.2	16.4	17.4	19.0	25.5	
			S.E. 3.59	5.8	6.16	6.7	9.0	
			Value of P <.02	<.05	<.02	<.01	<.01	
Group II: Obese patients with normal oral glucose tolerance curve								
9	15M*	0	64	163	163	152	113	−
10	19F*	+ 1	40	128	122	118	107	−
			32	139	150	127	116	−
11	21F*	− 3	46	105	123	128	116	−
			58	84	101	130	138	−
12	32F*	− 6	67	122	153	153	129	−
			47	136	167	163	110	++
13	32F*	− 5	49	99	139	139	112	+
			53	142	217	182	124	−
14	33M	−10	28	113	141	147	97	−
			44	175	219	201	91	−
15	36M	− 5	42	112	152	117	75	−
			52	132	193	198	127	−
16	36F*	− 1	59	103	89	88	70	−
			41	93	140	166	166	−
17	37F*	?	34	103	135	152	152	−
			42	79	119	144	123	−
18	38F*	0	32	75	112	145	121	−
			58	115	157	151	125	−
19	44M	− 4	54	86	146	139	125	−
			30	79	157	171	142	−
20	45F*	− 8	44	80	90	131	112	+
			40	88	161	167	153	++
21	49M	− 1	33	96	119	127	129	+
			53	100	137	167	125	−
			57	103	115	106	115	−
Avge.	33.6	− 4	Before 47	117	160	163	127	
			After 46	102	126	130	112	
			S.D. of differences 23.8	18.3	30.2	32.0	16.3	
			S.E. 2.09	5.07	8.37	8.86	4.51	
			Value of P <.01	<.01	≪.01	<.01	<.01	

* Out-patients.
† After ascorbic acid i.m.

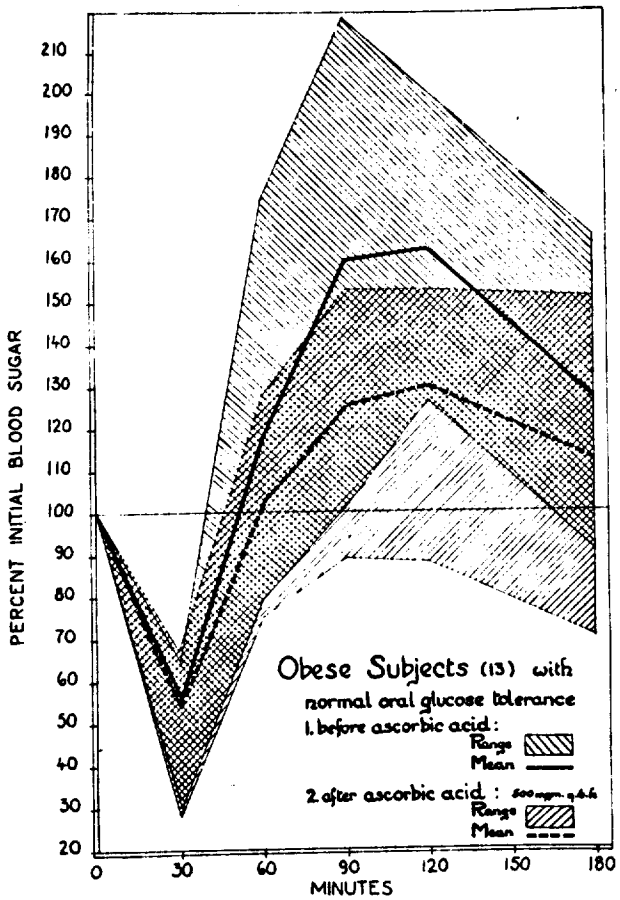


FIG. 1. The effect of 500 mg. of ascorbic acid by mouth every six hours on the insulin-glucose tolerance curve in obesity.

one or more of the individual values fell outside the normal mean ±2 S.D. These data are taken from a previous study of normal patients and are as follows (15):

Mean value	37	96	114	106	92
S.D.	7.7	24.9	21.1	17.0	10.3

The patients were arbitrarily divided into two groups according to whether the oral glucose tolerance curve was normal or not, without regard to ratio of in-patients versus out-patients. The criteria for a normal curve were a fasting blood sugar level below 120 mg. per 100 ml., a peak not higher than 170 mg., and a three-hour value which had decreased to, or near the fasting level. In the group with abnormal oral glucose tolerance curves the change after ascorbic-acid therapy was not as significant as in

the second group. No increased sensitivity to insulin was noted, as shown by the absence of hypoglycemic symptoms or lowered blood sugar levels. The patients in this first group were on the whole ten years older than the patients in the second group with normal oral glucose tolerance curves. In the latter group, a consistent intake of ascorbic acid was questionable, as

TABLE 2. THE EFFECT OF LOSS OF WEIGHT (5–15 POUNDS) ON THE INSULIN GLUCOSE TOLERANCE CURVES OF 6 OBESE PATIENTS

Patient No.	Age and sex	Weight change	% Initial blood sugar level				
			30 min.	60 min.	90 min.	120 min.	180 min.
1	30M	−12	65	127	180	180	91
			65	104	162	182	101
2	32M	−10	72	161	197	169	143
			43	129	175	166	107
3	36M	− 5	52	132	193	198	127
			43	129	169	176	94
4	44M	− 5	50	123	202	225	98
			52	120	182	205	104
5	47M	−15	74	124	178	187	135
			77	100	162	188	163
6	49M	−13	53	100	137	167	125
			55	98	193	145	140
Avge.	39.7	−10	Before 61	126	181	191	120
			After 56	115	166	177	118
			S.D. of differences 11.0	13.4	7.8	10.6	12.65
			S.E. 4.48	5.45	3.18	4.34	5.17
			Value of P <.2	<.05	<.01	<.05	<.02

the greater number of these subjects were studied on an out-patient basis. However, the results were considered significant after statistical analysis.

Tests carried out on 6 in-patients without oral ascorbic acid but having lost 5 to 15 pounds in weight did not show any marked improvement. The results are recorded in Table 2. These tests were made in order to determine whether minor fluctuations in weight might affect the response.

Routine determinations of ascorbic-acid levels in obese patients prior

to the tests did not show any evidence of hypovitaminosis. The values were all within the range of 0.6–2.5 mg. and were considered normal in our laboratory. No toxic effects from the use of large doses of ascorbic acid were observed in our study.

An *in vitro* experiment was performed in order to determine whether ascorbic acid by itself would influence the oxidation-reduction reaction used in testing for the presence of glucose. Various concentrations of ascorbic acid were added to blood samples containing either high or low concentrations of glucose. No effect on glucose values was noted. The data are shown in Table 3.

Using the same time schedule as outlined in the procedure of this study, blood sugar curves were obtained on 2 patients given 500 mg. of ascorbic acid intravenously after a fasting blood sample had been withdrawn. This

TABLE 3. THE *in vitro* EFFECT OF VARIOUS CONCENTRATIONS OF ASCORBIC ACID ON BLOOD SUGAR DETERMINATIONS

Initial blood sugar (mg./100 ml.)	Blood sugar after addition of ascorbic acid in concentrations of:		
	1 mg./100 ml.	4 mg./100 ml.	10 mg./100 ml.
90	91	91	94
320	310	310	314

test was made in order to determine whether ascorbic acid by itself would affect blood sugar determinations *in vivo*. The results were as follows:

Case A: 86, 91, 91, 84, 91, and 89 mg. per 100 ml.
Case B: 84, 85, 89, 85, 89, and 94 mg. per 100 ml.

In Cases 2 and 6, after failure with oral ascorbic acid given for a period of four weeks, it was decided to use an intramuscular preparation (500 mg. every six hours) in order to determine whether there would be a response with parenteral therapy. This had to be discontinued after three days, since the sites of injections became too painful. Only in Case 2 was there some response (Table 1). Two days later this patient's blood sugar curve was 100, 39, 130, 172, 140, and 84 mg. per 100 ml., and after one week, 100, —, 108, 159, 198, and 106 mg. per 100 ml.

The t test was used in the statistical analysis of this paper. The test was applied to each paired observation, before and after ascorbic-acid therapy, or before and after weight loss (Tables 1 and 2). Since our series was not large, it was decided that any value of t would not be considered significant if P lay between 0.05 and 0.02; was probably significant if P lay between 0.02 and 0.01; and was highly significant for P values less than 0.01.

DISCUSSION

Ascorbic acid, dehydroascorbic acid and alloxan resemble each other as to biochemical structure, and each will affect carbohydrate metabolism. Most of the experiments relating to ascorbic acid and carbohydrate metabolism have been carried out in monkeys and guinea pigs, which, like man, cannot synthesize this vitamin. Such experiments point to two possible ways whereby ascorbic acid plays a role, namely, the adrenal and the pancreas. Since stress affects the entire endocrine system, and this system is particularly rich in ascorbic acid, it would not be surprising that a relative deficiency of this substance could affect various metabolic processes. Menten and King (7) have shown that mere deprivation of ascorbic acid in the guinea pig can produce carbohydrate intolerance, even before frank scurvy is present. Since such a state is reversible to a certain extent by the administration of ascorbic acid, it seems possible that this could also be the case in the obese patient. The return of the insulin-glucose tolerance curve to normal or near normal in those of our patients who had a normal oral glucose tolerance curve seems to indicate two things. First, that the insulin-glucose tolerance test is the more sensitive one for the detection of early carbohydrate intolerance and, secondly, that an abnormal curve is reversible at this stage by the administration of large doses of ascorbic acid. Whether smaller doses would be adequate is a matter for further study. Although there may be some improvement in obese patients with marked carbohydrate intolerance, as shown by an abnormal oral glucose tolerance curve as well as an abnormal insulin-glucose tolerance curve, the results are not as significant. The possibility exists that the damage to the pancreas is irreversible or that the stress of obesity and consequent depletion of ascorbic acid has increased the release of adrenocorticotropin by the anterior pituitary, with consequent changes in carbohydrate tolerance.

The dosage of ascorbic acid chosen in our experiment (500 mg. orally every six hours), was based on studies made by Todhunter *et al.* (19). They showed that in patients with a low fasting blood level of ascorbic acid, administration of 50 to 100 mg. did not necessarily raise the blood concentration to 1.3–1.4 mg. per 100 ml.—the threshold at which renal excretion occurs. In contrast, a dose of 400 to 500 mg. not only caused a higher plasma level, but the level was sustained at a plateau above the renal threshold for more than four hours. To saturate a patient with ascorbic acid it is therefore necessary to keep the blood ascorbic acid level at, or above the renal threshold during the greater part of each day.

There is no satisfactory explanation at present for the failure of oral administration of ascorbic acid versus the success of parenteral administration, and for the clinical benefits reported following extremely large doses

versus the lack of effect when used in small doses. Case 2 illustrates the first point. Since the effect lasted for only five days after cessation of parenteral therapy, it is postulated that the obese patient does not store vitamin C and that daily large doses are necessary. There must be individual variations in absorption of vitamin C from the intestinal tract. Under certain conditions the absorption may be definitely abnormal (20), since ascorbic acid administered by subcutaneous injection may be twice as effective as when given by mouth in the protection of guinea pigs against scurvy (21).

CONCLUSIONS

1. The effect of ascorbic acid on the abnormal insulin-glucose tolerance curves in 21 obese subjects is presented. Therapy consisted of 500 mg. of ascorbic acid given orally four times a day for a minimum of three weeks.

2. The effect of such therapy was questionably significant in 8 patients with abnormal oral glucose tolerance curves. In 13 patients with normal oral glucose tolerance curves, there was a significant reversal towards normal of the insulin-glucose tolerance curve, the average readings all falling within the normal range.

3. The possible action of ascorbic acid on carbohydrate metabolism is discussed.

REFERENCES

1. Selye, H.: Stress. Montreal, Acta, Inc., 1950, p. 79.
2. Beck, J. C.; Browne, J. S. L., and Mackenzie, K. R.: The effect of adrenocorticotrophic hormone and cortisone acetate on the metabolism of ascorbic acid, *in* Proceedings of the Second Clinical ACTH Conference, ed. by J. R. Mote. New York, The Blakiston Co., 1951, vol. 1, p. 355.
3. Dugal, L.-P., and Thérien, M.: The Influence of ascorbic acid on the adrenal weight during exposure to cold, *Endocrinology* **44**: 420, 1949.
4. Leblanc, J.: Effects de l'acide ascorbique sur l'activite de la cortico-surrénale, *Laval méd.* **17**: 1068, 1952.
5. Bacchus, H., and Heiffer, M. H.: Urinary corticosteroids and 17-ketosteroids in ascorbic acid-deficient female guinea pigs, *Am. J. Physiol.* **174**: 243, 1953.
6. Khalil, H. H.: Significance of ascorbic acid in the adrenal cortex, *Lancet* **1**: 912, 1954.
7. Menten, M. L., and King, C. G.: The influence of vitamin C level upon resistance to diphtheria toxin. II. Production of diffuse hyperplastic arteriosclerosis and degeneration, in various organs, *J. Nutrition* **10**: 141, 1935.
8. Sigal, A., and King, C. G.: The influence of vitamin C deficiency upon the resistance of guinea pigs to diphtheria toxin; glucose tolerance, *J. Pharmacol. & Exper. Therap.* **61**: 1, 1937.
9. Stewart, C. T.; Salmon, R. J., and May, C. D.: Factors determining the effect of insulin on the metabolism of glucose in ascorbic acid deficiency and scurvy in the monkey, *A. M. A. Am. J. Dis. Child.* **84**: 677, 1952.
10. Ascorbic acid as a coenzyme, *Nutrition Reviews* **11**: 17, 1953.

0287

11. Becker, R. R.: Personal communication.
12. Quastel, J. H., and Wheatley, A. H. M.: An effect of ascorbic acid on fatty acid oxidations in the liver. *Biochem. J.* **28**: 1014, 1934.
13. Willis, G. C.: *Proc. Roy. Coll. Phys. & Surg. Canada* p. 53, 1953.
14. Morris, G. E.: Avitaminosis C in 1953: a clinical study, *Am. Practitioner* **5**: 658, 1954.
15. Arendt, E. C., and Pattee, C. J.: Studies on obesity. I. The insulin-glucose tolerance curve, *J. Clin. Endocrinol. & Metab.* **16**: 367, 1956.
16. Engel, F. L., and Scott, J. L.: The insulin-glucose tolerance test. A modified procedure for the detection of hypoglycemia unresponsiveness in pituitary and adrenal insufficiency, *J. Clin. Invest.* **29**: 151, 1950.
17. Hoffman, W. S.: Photoelometric Clinical Chemistry. New York, W. Morrow & Co., 1941.
18. Heinemann, M., and Hald, M.: Factors that influence the passage of ascorbic acid, *J. Clin. Invest.* **19**: 469, 1940.
19. Todhunter, E. N.; Robbins, R. C., and McIntosh, J. A.: The rate of blood plasma ascorbic acid after ingestion of ascorbic acid, *J. Nutrition* **23**: 309, 1942.
20. Abt, F., and Farmer, C. J.: Vitamin C. Pharmacology and therapeutics, *J.A.M.A.* **111**: 1555, 1938.
21. Hou, H. C.: Comparison of oral and subcutaneous administration of protective doses of ascorbic acid, *Proc. Soc. Exper. Biol. & Med.* **32**: 1391, 1935.

Food Res. 15: 103-106

0329

ASCORBIC ACID CONTENT AND OTHER CHARACTERISTICS OF THE WEST INDIAN CHERRY

CONRADO F. ASENJO AND CARLOS G. MOSCOSO
Department of Chemistry and Nutrition, School of Tropical Medicine, and Department of Plant Breeding, Agricultural Experiment Station, University of Puerto Rico

[Received for publication, November 2, 1949]

The West Indian cherry (*Malpighia punicifolia* L.) is a small, bushy tree which grows to a height of from 10 to 15 feet. It is found in the tropical and semitropical regions of America.

The fruit of the tree is small, weighs approximately four gm., and varies from one-half inch to an inch in diameter. Its color ranges from a light-green, when the fruit is immature, to a yellowish-orange, or a deep, bright red, depending on the stage of maturity, variety of cherry, conditions of soil, and type of fertilizer used. The small seeds in each cherry fluctuate from five to six in number, depending on the variety of the fruit. Approximately 80 per cent of the weight of the fruit is edible.

Fortunately, this tree is extremely hardy; it adapts itself easily to poor soils, and can apparently resist most diseases caused by insect pests. It requires little attention other than at harvest time. In a mature tree, and depending on its variety, a harvest may be expected every 40 days to two months during the bearing season.

Varieties of the tree found so far have not yet been standardized, but experiments are now in progress which should permit the selection of outstanding varieties in the near future.

From observation of the trees in their wild state in various sections of Puerto Rico, it is apparent that the West Indian cherry thrives best on well-drained soils, or sandy loam with abundant rainfall. The size of the fruit and the quantity of the crop may be influenced by the amount of rain; the trees produce a larger and more palatable fruit during a rainy season.

Asenjo and Freire de Guzmán (1946) reported that the West Indian cherry contained from one to three gm. of ascorbic acid per 100 gm. of edible matter, and that the green fruit contained more of the acid than did the ripe. The writers obtained these values by using the 2,6-dichlorophenol indophenol titration, and definitely established the presence of this large amount of ascorbic acid by isolating the same in a crystalline form from the juice.

Later, Mustard (1946) confirmed the above findings when she analyzed several samples of West Indian cherry, grown in Florida, and reported values as high as 4,676 mg. of ascorbic acid per 100 gm. of edible matter of the unripe fruit. She also investigated the ascorbic acid content of several samples of jelly prepared from this cherry, always obtaining values above 450 mg. per 100 gm. of jelly.

It is the purpose of this communication to report some data collected on the ascorbic acid content of the West Indian cherry, as well as on

other chemical characteristics of fruit samples obtained from representative trees belonging to the experimental plantation maintained at the Agricultural Experiment Station of the University of Puerto Rico, at Río Piedras, Puerto Rico.

METHOD OF ANALYSIS

The fruits were collected early in the morning. They were weighed and measured, and the juice expressed and its chemical analysis determined during the following 24 hours.

Total Solids: The residue left after evaporating the juice at 100°C. (212°F.) for 24 hours.

pH: The pH of the juice was determined electrometrically with a glass electrode.

Total Acidity: Ten ml. of juice were titrated with 0.1 N NaOH and phenolphthalein as indicator. The result was expressed on the basis of 100 ml. of juice.

Reducing Sugars: The reducing sugars were determined using Benedict's reagent. Because ascorbic acid will also reduce this solution, a correction was applied; values reported are thus corrected for the ascorbic acid present.

Ascorbic Acid: Ascorbic acid was determined by the method of Bessey and King (2,6-dichlorophenol titration, 1933). The juice was initially diluted with a 2.5 per cent solution of orthophosphoric acid, but subsequent dilutions were made using nine per cent acetic acid.

RESULTS

The ripe fruit was usually larger than the partly ripe, average diameter of each being 2.2 and 1.7 cm., respectively. The weight of the ripe fruit was also greater than that of the green: 4.7 and 3.1 gm., respectively.

The amount of juice obtained, from the partly ripe fruit, by hand expression in a piece of cloth, averaged 55.3 per cent (maximum, 77.9 per cent, minimum, 41.0 per cent); while that obtained from the ripe fruit averaged 69.6 per cent (maximum, 80.2 per cent, minimum, 61.3 per cent). The total amount of juice in the partly ripe fruit averaged 83.3 per cent (maximum, 95.5 per cent, minimum, 74.3 per cent); and in the ripe fruit, 83.4 per cent (maximum, 91.0 per cent, minimum, 78.5 per cent). These values were computed by adding to the expressed juice the amount of juice left as moisture in the residue. The West Indian cherry juice has a beautiful reddish-orange color and an agreeable acid taste.

Results of the chemical analysis are reported (Table 1). Total solids per 100 ml. of juice fluctuated between 4.57 and 7.21, and the pH between 3.1 and 3.3, irrespective of degree of ripeness. Total acidity, expressed as ml. of 0.1 N NaOH necessary to neutralize the acid in 100 ml. of juice, was much higher in the juice from the partly ripe fruit (average, 160.7 ml.) than in that from the completely ripe (average, 135.9 ml.).

The amount of ascorbic acid in the West Indian cherry samples obtained were, with one exception (Sample B-8, ripe), over 1,000 mg. per 100 ml. of juice. The sample which gave the highest value was A-21 (partly ripe, 2,360 mg. per 100 ml.; and ripe, 1,522 mg. per 100 ml.); the one with the lowest content was B-8 (partly ripe, 1,338 mg. per 100 ml.; and ripe, 686

mg. per 100 ml.). In all samples the content of ascorbic acid for the partly ripe fruit was higher than for the ripe, thus confirming the previous observations of Asenjo and Freire de Guzmán (1946). The ascorbic acid per 100 gm. of whole fruit is reported (last column, Table 1).

TABLE 1

Some Chemical Characteristics of the West Indian Cherry Juice

Description	Total solids in juice	Juice	Total acidity of juice, ml. of 0.1 N NaOH per 100 ml. of juice	Reducing sugars in juice as glucose	Ascorbic acid in juice	Ascorbic acid in fruit
	gm./ 100 ml.	*pH*	*ml.*	*gm./ 100 ml.*	*mg./ 100 ml.*	*mg./ 100 gm.*
A-14—partly ripe	5.89	3.3	140.0	3.0	1,613	1,430
A-14—ripe	7.21	3.3	112.4	5.4	1,161	998
A-21—partly ripe	6.60	3.1	208.4	3.7	2,360	1,916
A-21—ripe	7.08	3.2	173.6	5.3	1,522	1,246
B-6—partly ripe	5.84	3.2	156.2	3.0	1,920	1,426
B-6—ripe	5.98	3.3	149.2	3.8	1,456	1,148
B-7—partly ripe	5.13	3.2	133.8	2.8	1,380	1,135
B-7—ripe	4.57	3.2	110.2	3.0	1,051	956
B-8—partly ripe	5.86	3.2	133.8	3.2	1,338	1,277
B-8—ripe	4.66	3.3	81.6	3.6	686	577
B-15—partly ripe	5.45	3.2	192.0	3.5	1,677	1,299
B-15—ripe	5.62	3.1	188.5	3.7	1,325	1,040

OTHER VITAMINS

Determinations were made of the thiamine, riboflavin, and niacin contents of different samples of West Indian cherry juice and the following results obtained:

	No. samples	Min.	Max.	Av.
		µg./ 100 ml.	*µg./ 100 ml.*	*µg./ 100 ml.*
Thiamine (thiochrome method)	6	12	33	24
Riboflavin (microbiological)	5	62	90	73
Niacin (microbiological)	3	470	490	480

These values are within the range of those reported for the same vitamins in the majority of other fruit juices.

DISCUSSION

The West Indian cherry is one of the richest, if not the richest, vegetable source of ascorbic acid known to man. The high production of the fruit, estimated at about two to six pounds per tree per harvest, makes it an important natural source of the above-mentioned acid.

The ascorbic acid produced per tree during one harvest may be estimated, very conservatively, at about 13 gm. Under actual commercial cultivation such a yield could easily be doubled.

There is a possibility that crystalline ascorbic acid could be produced economically by processing the juice of the fruit. Using quite rudimen-

0329

tary methods, Asenjo and Freire de Guzmán (1946) obtained 1.25 gm. of acid from 275 ml. of a sample of juice containing, by analysis, approximately four gm. of ascorbic acid. This figure represents a recovery of only 31 per cent, which could doubtlessly be greatly improved under factory conditions.

Juice from the West Indian cherry could also be used advantageously as an enriching agent. Small amounts added to fruit juices poor in ascorbic acid (one to nine proportion) would produce blends with an ascorbic acid content well over that of the average orange juice, and would prove a very convenient manner of fortifying foods for ascorbic acid without having to resort to use of the synthetic product.

SUMMARY

The ascorbic acid content and other chemical characteristics of six representative types of the West Indian cherry (*Malpighia punicifolia* L.) have been studied. Thiamine, riboflavin, and niacin contents of the juice are also reported.

Possible uses of this fruit are discussed.

REFERENCES

Asenjo, C. F., and Freire de Guzmán, A. R., 1946. The high ascorbic acid content of the West Indian Cherry. Science 103, 219.

Bessey, O. A., and King, C. G., 1933. Distribution of vitamin C in plant and animal tissues, and its determination. J. Biol. Chem. 103, 687.

Mustard, M. J., 1946. The ascorbic acid content of some Malpighia fruits and jellies. Science 104, 230.

Association of Official Analytical Chemists. 1970
Official Methods of Analysis of the Association of
Official Analytical Chemists, 11th Edition
AOAC, Washington, D.C., pp. 777-779

Proc. Soc. Exp. Biol. Med. 79:648:650 1952

0382

Potentiating Effect of Ascorbic Acid on Cortisone-Induced Gluconeogenesis. (19474)

HABEEB BACCHUS, MELVIN H. HEIFFER, AND NORMAN ALTSZULER.
(Introduced by Chester E. Leese.)
From the Department of Physiology, George Washington University, School of Medicine, Washington, D. C.

Previous work from this laboratory suggested that ascorbic acid treatment of rats, mice, and guinea pigs can prevent activation of the pituitary-adrenal axis in response to stressors(1-4). It was observed that the vitamin fails to alter the leukocyte response to cortisone and to adrenocorticotrophin. Furthermore, it was demonstrated that the vitamin does not possess any specific anti-epinephrin action to explain failure of the activation of the pituitary-adrenal axis(4). Those data suggested that the ascorbic acid prevents the activation of the axis by either directly or indirectly rendering the pituitary inert to certain stimuli. Preliminary data indicated that ascorbic acid prolongs the leukocytic effects, and intensifies the gluconeogenic actions, of cortisone. The present communication reports data which indicate that the gluconeogenic action of cortisone is potentiated by ascorbic acid.

Materials and methods. These experiments were conducted on young adult male Swiss mice. The animals were bilaterally adrenalectomized under ether or nembutal anesthesia at least 3 days, and not more than 3 weeks prior to experimentation and maintained on an *ad libitum* fare of Purina Laboratory Chow and 0.9% NaCl solution. At the end of the periods of experimentation the animals were killed by a blow on the head, and the liver extirpated and plunged into a 30% solution of potassium hydroxide; the glycogen was then hydrolyzed by the method of Somogyi *et al.*(6), and the glucose determined by the method of Folin(5).

Experimental designs and results. The first experiment was conducted on male Swiss mice which had been adrenalectomized 3 weeks previously, and were maintained on Chow and saline. Body weight range at time of experiment was 25-30 g. Group I mice received single intraperitoneal injections of ascorbic acid (sodium ascorbate, vit. C, injectable, Roche), 100 mg per 100 g body weight, on the previous evening. Early the next morning these animals received similar amounts of the vitamin. The first injection

TABLE I. Liver Glycogen Concentration in Adrenalectomized Mice Treated with Cortisone and Ascorbic Acid, and with Cortisone and Saline.

Group	No. mice	Treatment	Liver glycogen, mg/1 g liver ± S.E.	P
I	4	A,* C	49.1 ± 3.1	I vs II <.05
II	"	S, C	37.3 ± 2.1	II vs III <.01
III	"	0	20.6 ± 1.75	I vs III <.01

* A = Ascorbic acid; C = Cortisone; S = Saline.

TABLE II. Liver Glycogen Content in Adrenalectomized Mice Maintained on Glucose and Treated with Cortisone and Ascorbic Acid, or with Cortisone and Saline.

Group	No. mice	Treatment	Liver glycogen, mg /10 g body wt ± S.E.	P
I	7	A,* C, G	29 ± 2.7	I vs II <.01
II	7	S, C, G	17 ± 2.4	II vs III <.01
III	5	G only	3 ± 1.2	I vs III <.01

* A = Ascorbic acid; C = Cortisone; S = Saline; G = Glucose.

was made in order to prime the animals. Immediately following the second injection of ascorbic acid the mice received single subcutaneous injections of cortisone (Cortone, Merck), 4.0 mg per 100 g body weight. Group II mice received similar treatment as those of Group I except that in this case vit. C (ascorbic acid) was replaced by intraperitoneal injections of physiological saline. Group III mice received no treatment. The animals were all taken off food following the injections, but were permitted to drink 0.9% NaCl solution. All animals were killed at the end of 4 hours after the injection of cortisone, the controls at the corresponding time, and glycogen determinations conducted.

The data are presented in Table I in which the liver glycogen concentration is presented as milligrams of glucose per 1 g of liver. It is seen that the liver glycogen content of the animals receiving cortisone is higher than that in the animals receiving no treatment. The liver glycogen content in the animals which received cortisone and vit. C (Group I) is significantly greater than that of the animals which received cortisone and saline (Group II) ($P < 0.05$).

The second experiment was conducted on mice weighing 20 to 25 g. The procedure suggested by Venning *et al.*(7) for assay of corticoids was adopted in this experiment. Group I mice (adrenalectomized) received the previous evening the priming dose of ascorbic acid as those in the previous experiment, and were allowed to feed *ad libitum* overnight. The following morning these animals received 7 injections of a menstruum containing cortisone and glucose subcutaneously, the total dose of cortisone amounting to 70 μg, of glucose, 70 mg. These animals also received 2 injections of ascorbic acid, one at the time of the first cortisone injection, and the other at the time of the third cortisone-glucose injection, the amount of ascorbic acid being 100 mg/100 g body weight. Group II animals were treated like those of Group I, except that they received saline solution in place of the intraperitoneal injections of ascorbic acid. The animals of Group III received 7 injections of glucose, amounting to 70 mg, but received no cortisone, or ascorbic acid. The 7 injections mentioned above were given in the course of 6 hours, the animals being killed one hour after the last injection, *i.e.*, a total period of 7 hours after the first injection of the menstruum.

The data are presented in Table II in which the liver glycogen, as glucose, is presented as milligrams per 10 g of body weight of the animals. This is the method recommended by Venning *et al.*(7). It is observed that the liver glycogen content of Group II animals is 466% greater than that of the animals which received no cortisone (Group III); the livers of Group I mice contain 806% more glycogen than those of Group III animals. The liver glycogen content in the vitamin-cortisone mice is significantly greater ($P < 0.01$) than that of the cortisone-saline animals.

Discussion. The data obtained from the 2 experiments indicate that ascorbic acid treatment of adrenalectomized mice supplied with small and large doses of cortisone increases the deposition of glycogen in the liver. This deposition of glycogen in the livers of the cortisone treated animals was probably due to gluconeogenesis. It is unlikely that the glucose supplied to the mice in the second experiment was converted to glycogen stores in

the liver: it is observed that the glycogen values in the animals receiving glucose alone were extremely low. Venning(7) had shown that in such glucose-treated animals, the glycogen deposition following cortical hormones was due to gluconeogenesis. The ability of ascorbic acid to enhance the liver glycogen deposition must therefore be ascribed to an increase in the gluconeogenesis due to the ascorbic acid in association with the cortisone, since the vitamin alone fails to enhance gluconeogenesis in adrenalectomized mice (unpublished data). McKee *et al.*(8) reported that liver glycogen deposition following the injection of cortical hormones into scorbutic guinea pigs was lower than that in normal animals. This observation could conceivably have been due to the general debility in the scorbutic animals. In the present study it is observed that vitamin treatment to animals on otherwise good ascorbic acid balance (the mouse requires little or no ascorbic acid in the diet) is capable of enhancing the glycogen deposition. This finding may be related to the action of the vitamin in normal intact animals exposed to stressors, in which case the alarm reaction is prevented, and survival is increased (9). It was shown that this ability of the vitamin requires the presence of the adrenal cortex, or of circulating cortical hormones(10).

It is possible that the vitamin "synergizes" with the existent cortical hormones in intact animals, and in animals supplied with cortical hormones. Data supporting this postulate come from the observation that the vitamin is capable of prolonging the leukocyte effects of cortisone(11). This prolongation, in addition to the demonstrated potentiation, of the action of cortisone by ascorbic acid suggests a synergism of ascorbic acid with cortisone. This synergism may explain the non-activation of the pituitary-adrenal axis in ascorbic acid-treated intact animals exposed to stress. It is possible that through this synergism the titer of cortical hormones is maintained in the blood, hence increasing the threshold of the anterior pituitary, according to the mechanism proposed by Sayers and Sayers(12). The relation of this possible synergism to normal adrenal secretion is being investigated.

Summary. The action of ascorbic acid treatment on the gluconeogenic action of cortisone was investigated in adrenalectomized mice. The deposition of glycogen in the liver is enhanced in animals treated with ascorbic acid in addition to cortisone. This observation is discussed in relation to the action of the vitamin in preventing the activation of the pituitary-adrenal axis in animals exposed to stressors. It is suggested that the vitamin may be synergistic with cortisone in some of its actions.

This study was aided by a Grant from the National Institutes of Health. The authors acknowledge the interest of Doctor Chester E. Leese. The ascorbic acid (Vit. C, Injectable, Na ascorbate, Roche) was supplied through the generosity of Doctor Roger A. Lewis, Hoffmann-La Roche, Nutley, N. J. Doctor Elmer Alpert, Merck and Co., Rahway, N. J., kindly supplied the cortisone (Cortone, Merck).

1. Bacchus, H., *et al.*, *Science*, 1951, v113, 269, 367.
2. ———, PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 167.
3. Bacchus, H., and Altszuler, N., In press.
4. Bacchus, H., Altszuler, N., and Heiffer, M. H., Data to be published.
5. Folin, O., *J. Biol. Chem.*, 1920, v41, 367.
6. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, v100, 485.
7. Venning, E., Kazmin, V. E., and Bell, J. C., *Endocrinology*, 1946, v38, 79.
8. McKee, R. W., Cobbey, T. S., and Geiman, Q. M., *Endocrinology*, 1949, v45, 21.
9. Dugal, L. P., and Therien, M., *Endocrinology*, 1949, v44, 420.
10. Bacchus, H., Toompas, C. A., and Heiffer, M. H., *Fed. Proc.*, 1951, v10, 7.
11. Bacchus, H., Altszuler, N., and Heiffer, M. H., manuscript in preparation.
12. Sayers, G., and Sayers, M. A., *Endocrinology*, 1947, v40, 265.

Received March 10, 1952. P.S.E.B.M., 1952, v79.

Endocrinology 51:302-305 1952

0385

DECREASED EXCRETION OF CORTISONE METABOLITES IMMEDIATELY FOLLOWING ASCORBIC ACID TREATMENT

HABEEB BACCHUS, NORMAN ALTSZULER AND MELVIN H. HEIFFER

Department of Physiology, The George Washington University, School of Medicine, Washington, D. C.

IN THE course of studying the role of ascorbic acid in adrenal cortex function it was observed that the vitamin is capable of prolonging the hematologic actions of exogenous and of endogenous cortical hormones (Bacchus, Altszuler, Heiffer, 1952a, b). The data were interpreted as indicating that the ascorbic acid in some manner may be responsible for either a diminished excretion or a diminished inactivation of these hormones. At any rate, detectable amounts of the hormones persisted in the animals which received simultaneous ascorbic acid treatment.

The present paper reports data which indicate that the excretion of 17-ketosteroids in the first 24-hour urine sample following cortisone is diminished by ascorbic acid treatment. This diminished excretion of the metabolites corresponds very well with the observed period of prolongation of hematologic effects noted above.

MATERIALS AND METHODS

Female adrenalectomized Wistar rats were the animals of choice in this experiment. It is generally accepted that the chief source of 17-ketosteroids in the female is the adrenal cortex (Heard, 1948; Selye, 1952). In the adrenalectomized female, however, the ovary may produce small amounts of 17-ketosteroids (Dorfman, 1948). The animals were bilaterally adrenalectomized under ether anesthesia at least three weeks prior to experimentation and were maintained on an ad libitum fare of 0.9% NaCl and Purina Laboratory Chow throughout the period of study.

Group I rats received single intraperitoneal injections of 100 mg. ascorbic acid (Na ascorbate, Roche) per 100 grams body weight. Fifteen minutes later the animals were given single subcutaneous injections of 2 mg. cortisone acetate (Merck) per 100 grams body weight. Group II rats received saline injections (instead of ascorbic acid) and the same dosage of cortisone as above. Group III rats received single intraperitoneal injections of ascorbic acid at the same dosage as above, while Group IV received saline solution only. Immediately after the injections the animals were placed in individual metabolism cages and supplied with food and fluid. A urine sample of the first 24 hours was collected from each animal. The 17-ketosteroids in the urine were determined by the method of Drekter, *et al.*, (1952) in which dehydroisoandrosterone was used as standard.

Received for publication April 15, 1952.

The results are expressed as 17-ketosteroids as mg. of dehydroisoandrosterone per the 24-hour urine sample.

RESULTS

The data in Table I indicate that the 17-ketosteroid excretion in adrenalectomized female rats which received no cortisone is relatively low. Ascorbic acid did not affect this pattern. The excretion of 17-ketosteroids in the animals which received saline plus cortisone is significantly higher ($P = <0.05$) than that in the rats which were treated with ascorbic acid and cortisone. The data may be interpreted to suggest that ascorbic acid depresses the excretion of 17-ketosteroids following cortisone treatment.

TABLE I. EXCRETION OF 17-KETOSTEROIDS IN THE 24-HR. URINE SAMPLE FOLLOWING INJECTION INTO FEMALE ADRENALECTOMIZED RATS OF SALINE, ASCORBIC ACID, SALINE PLUS CORTISONE, OR ASCORBIC ACID PLUS CORTISONE

Group	No. rats	Treatment	17-KS as mg. dehydroisoandrosterone in first 24-hr. urine sample ±S.E.*
I	8	Ascorbic acid plus cortisone	0.128 ±0.015
II	8	Saline plus cortisone	0.500 ±0.157
III	4	Ascorbic acid }	0.075 ±0.011**
IV	3	Isotonic saline }	

* $S.E. = \sqrt{\frac{\Sigma(x-\bar{x})^2}{n(n-1)}}$

** Values were grouped together since they were similar.

DISCUSSION

It is probable that most of the 17-ketosteroids observed in these urines were derived from the injected cortisone. This is in consonance with generally accepted facts (Sprague, Power, Mason, Albert, Mathieson, Hench, Kendall, Slocumb, and Polley, 1950; Selye, 1952). The diminished excretion suggests that the level of cortisone is maintained in the blood in animals which received ascorbic acid along with cortisone. This finding may explain the prolongation to 24 hours of the hematologic (eosinopenia, lymphopenia, and polymorphonuclear leukocytosis) actions of cortisone in animals with such treatment (Bacchus, Altszuler, and Heiffer, 1952 a, b). It may be noted that the hematologic actions of adrenocorticotrophin injections into non-adrenalectomized rats is also prolonged to 24 hours by simultaneous ascorbic acid treatment (Bacchus, Altszuler, and Heiffer, 1952 b). Preliminary data indicate that a similar pattern as above may be present in the excretion of 17-ketosteroids in such animals.

The above findings may provide a reasonable explanation for previous observations to the effect that non-specific stressors (e. g. epinephrin,

trauma, cold) fail to activate the pituitary-adrenal axis in ascorbic acid treated intact rats, mice and guinea pigs (Bacchus et al., 1951; Bacchus, Heiffer and Altszuler, 1952; Bacchus and Altszuler, 1952). Recent work by Eisenstein and Shank (1952) confirms the observation made by Bacchus et al., (1951) that ascorbic acid pretreatment prevents the depletion of adrenal ascorbic acid and adrenal cholesterol following epinephrin. All of the above data are the basis of a tentative hypothesis that ascorbic acid maintains the level of cortical hormones in the blood (for a longer time, hence their manifestations) and in this manner increases the threshold for pituitary stimulation. The mechanism proposed by Sayers (1947) involves a regulatory cycle whereby a high level of cortical hormones in the blood depresses pituitary-adrenotrophic function, whereas a low titer stimulates pituitary-adrenotrophic function. The suggested mechanism of action of ascorbic acid described above is in consonance with this theory of Sayers (1947).

(The observation that ascorbic acid excess diminishes the excretion of cortisone metabolites (17-ketosteroids) prompts reevaluation of the data of Clayton and Prunty (1951) and of Nadel and Schneider (1951) who reported increased excretion of cortical hormone metabolites in scurvy. Those authors interpreted their data as indicating adrenal hyperactivity in scurvy. Such interpretation is in disagreement with the findings of McKee, et al., (1949) who reported low liver glycogen values in such animals, and of Treager, et al., (1950) who reported normal responses to ACTH in scurvy patients. It is suggested that the increased 17-ketosteroids excretion observed by those authors in scurvy may be due, not to adrenal hyperactivity, but rather to the absence of ascorbic acid, which maintains the levels of circulating hormones. Studies are being conducted on the mechanism of action of ascorbic acid in this respect.)

SUMMARY

The action of ascorbic acid on the excretion of the 17-ketosteroid metabolites of injected cortisone was investigated in adrenalectomized female rats. The data indicate that ascorbic acid significantly diminishes the excretion of 17-ketosteroids in the first 24-hour urine sample following the treatments. Since it was observed that similar vitamin treatment prolongs the action of such doses of cortisone for 24 hours, the present findings are interpreted as indicating that the vitamin serves to maintain the levels of circulating cortisone.

ACKNOWLEDGEMENT

This study was aided by a grant from the National Institutes of Health. The ascorbic acid (Vitamin C, Injectable) was obtained from the Hoffman-

La Roche Co. through the generosity of Doctor R. A. Lewis. The Cortone was kindly supplied by Doctor Elmer Alpert, Merck Co., and the dehydroisoandrosterone by Doctor E. Henderson of the Schering Corporation. Doctor P. K. Smith of the Pharmacology Department kindly availed us some facilities for the chemical determinations.

REFERENCES

BACCHUS, H. ET AL.: *Science* **113**: 269, 367. 1951.

BACCHUS, H. AND N. ALTSZULER: *Endocrinology*. **51**: 1. 1952.

BACCHUS, H., M. H. HEIFFER AND N. ALTSZULER: *Endocrinology*, **51**: 94. 1952.

BACCHUS, H., N. ALTSZULER AND M. H. HEIFFER: In press 1952 (a) and (b).

CLAYTON, B. E. AND F. T. G. PRUNTY: *Brit. Med. J.*, ii: 927. 1951.

DORFMAN, R. I.: *The Hormones*, Acad. Press, Ed. G. Pincus and K. V. Thimann, Vol. 1. 1948; pp. 467.

DREKTER, I. J., A. HEISLER, G. R. SCISM, G. STERN, S. PEARSON, AND T. H. MCGAVACK: *J. Clin. Endocrinol. and Metab.* **12**: 55. 1952.

EISENSTEIN, A. B. AND R. W. SHANK: *Fed. Proc.* **11**: 207. 1952.

HEARD, R. D. H.: *The Hormones*, Acad. Press, Ed. G. Pincus and K. V. Thimann, Vol. 1. 1948 pp. 549.

NADEL, E. M. AND J. J. SCHNEIDER: *J. Clin. Endocrinol.*, **11**: 791. 1950.

SAYERS, G. AND M. A. SAYERS: *Endocrinology* **40**: 265. 1947.

SELYE, H.: *First Annual Report on Stress*, Acta, Inc., Montreal, 1951.

SPRAGUE, R. G., H. M. POWER, H. L. MASON, A. ALBERT, D. R. MATHIESON, P. S. HENCH, E. C. KENDALL, D. H. SLOCUMB AND H. F. POLLEY: *Arch. Int. Med.* **85**: 199. 1950.

TREAGER, H. S., G. J. GABUZDA, N. ZAMCHECK AND C. S. DAVIDSON: *Proc. Soc. Exper. Biol. and Med.* **75**: 517. 1950.

Amer. J. Clin. Nutr. 23: 905-912 1970

0432

THE AMERICAN JOURNAL OF CLINICAL NUTRITION
Vol. 23, No. 7, July, 1970, pp. 905-912
Printed in U.S.A.

Vitamin C Supplementation Related to Physiological Response to Exercise in Smoking and Nonsmoking Subjects

DONALD A. BAILEY,[1] P.E.D., ALBERT V. CARRON,[2] ED.D., RICHARD G. TEECE,[3] AND HARALD J. WEHNER[4]

IT HAS BEEN demonstrated that in the presence of vitamin C deficiency there is an impairment of physical efficiency, working capacity, and athletic performance. Further, there are reports that when the deficiency is corrected through vitamin C supplementation, physiological adjustment to heavy exercise can be enhanced (1–4), although there is one conflicting account (5). However, it remains debatable as to whether vitamin C supplementation aids healthy subjects meet the additional stress imposed by exercise or heavy physical work. A number of studies have indicated no augmentation of effect (4–9) although this has not been totally confirmed (10–14).

Other reports appear in the literature indicating that plasma levels of vitamin C are lower in cigarette smokers as compared with nonsmokers (15–18); however, there is no universal agreement regarding these results either (19). In the present investigation, healthy smokers and nonsmokers were given vitamin C supplementation to detect whether differences occur in their relative physiological adjustment to heavy exercise. A double-blind technique was adopted in which either vitamin C or placebo was given to groups of smokers and nonsmokers. The resulting physiological response was evaluated by measuring oxygen utilization and respiratory adjustment parameters, before, during, and after a treadmill run of progressively increasing intensity.

METHODS AND MATERIALS

Forty male subjects (mean age, 24.5 years; standard deviation, 3.5) participated in the experiment. Twenty subjects were smokers (mean age, 24.1 years; standard deviation, 2.7). For the purpose of this study, smokers were defined as those individuals who smoked between 10 and 20 cigarettes/day. Twenty subjects were nonsmokers (mean age, 24.8 years; standard deviation, 4.0). It should be noted that the majority of subjects were students in physical education who were probably more active than the normal population. All subjects were volunteers with varying amounts of experience on the treadmill.

Upon reporting to the laboratory on the first morning of the experiment a nonfasting venous blood sample was taken and each volunteer was required to run on a motor-driven treadmill. The subject was then given 2 g vitamin C or a placebo in capsule form. As specified by the double-blind protocol, neither the subjects nor the investigators knew the capsule contents. Drug administration was continued daily for 5 days. On the 5th morning, after the final ascorbic acid or placebo supplementation, a second blood sample was taken and the treadmill test was repeated at the same hour as the first test.

[1] Associate Professor, School of Physical Education, University of Saskatchewan, Saskatoon, Saskatchewan. [2] Assistant Professor, School of Physical Education, University of Saskatchewan. [3] Research Assistant, School of Physical Education, University of Saskatchewan. Present address: Instructor in Physical Education, University of Saskatchewan Regina Campus, Regina. [4] Director of Medical Services, Hoffmann-La Roche Ltd., Montreal, Quebec.

TABLE I

Mean plasma vitamin C concentrations (mg/100 ml) in smoking and nonsmoking subjects

	Non-smokers	Number of subjects	Smokers	Number of subjects
Presaturation	0.66	20	0.61	20
After 5 days				
Placebo	0.58	10	0.66	10
Vitamin C	1.23	10	1.50	10
Significance	P 0.05		P 0.05	

The treadmill test involved the minute-to-minute collection of data from a subject for 5 min of standing preexercise, followed by 9 min of continuous running on a motor-driven treadmill at level grade (3 min at 3 miles/hr, 3 min at 6 miles/hr, and 3 min at 9 miles/hr) and 10 min of standing recovery. Heart rate was monitored by means of chest lead electrocardiograph (EKG) fluid column electrodes. Minute ventilation ($\dot{V}_I$) was determined using a high speed dry gas meter (Parkinson-Cowan CD4) on the inspired side, and oxygen uptake ($\dot{V}_{O_2}$) was determined by analysis of the mixed air (Beckman E2 O_2 analyzer and Godart Capnograph CO_2 analyzer). Respiratory rate was measured as the pressure change in the breathing valve using a pressure transducer (Statham P23). A breathing valve of the type described by Bannister and Cormak (20) was used but with a large inner diameter (dead air space, 120 cc) to minimize the effect of circuit resistance. At a constant flow rate of 250 liters/min the maximum resistance of the circuit was 12 mm of H_2O.

Respiratory adjustment and oxygen utilization data could be evaluated continuously using the instruments described above. The mean minute ventilation (ml/min) per kilogram body weight ($\dot{V}_I$/kg) was determined at specific work levels and at specific oxygen uptake levels. The ratio of tidal volume to vital capacity (V_T–V_C), expressed as a percent value, was calculated at specific levels of work as well. The mean oxygen uptake (ml/min) per kilogram body weight ($\dot{V}_{O_2}$/kg) was calculated for both work levels and the heart rate levels. Oxygen pulse, defined as the amount of oxygen taken up per pulse beat ($\dot{V}_{O_2}$/HR) was also determined at specific work levels.

Thus, values for $\dot{V}_I$/kg, V_T–V_C, $\dot{V}_{O_2}$/kg, and $\dot{V}_{O_2}$/HR were obtained at rest, at 3, 6, and 9 miles/hr, and at recovery, before and after vitamin C or placebo supplementation. In addition, $\dot{V}_I$/kg was calculated at an oxygen uptake of 30 and 40 ml/kg body wt and $\dot{V}_{O_2}$/kg was calculated at a heart rate of 150 and 160 beats/min.

RESULTS

Plasma Vitamin C Concentration

Plasma concentration of vitamin C was determined using a calorimetric 2,6-dichlorophenolindophenol method as described by Varley (21). Mean plasma vitamin C concentrations expressed in milligrams per 100 milliliters plasma for smoking and nonsmoking subjects before and after the 5-day exposure to vitamin C are presented in Table I. Initially there is no significant difference between smokers and nonsmokers. After the 5-day supplementation period, the plasma concentrations of those subjects who were given vitamin C rose to saturation levels and are significantly higher than those smokers and nonsmokers given placebos.

Respiratory Adjustment and Oxygen Utilization

The mean values of all the parameters of respiratory adjustment and oxygen utilization before and after treatment are presented in Tables II and III. To test the statistical significance of the differences in the before and after response of smokers versus nonsmokers in vitamin C-saturated and unsaturated states, a three factor (2 × 2 × 2) analysis of variance was used. Taking into consideration the confounding of one factor, the design followed the form outlined by Winer (22) for a three-factor analysis in which there are repeated measures on one factor. The results of the analyses of variance performed on all parameters at all work levels are also contained in Tables II and III.

TABLE II

Summary of mean values and three-factor analysis of variance results for respiratory adjustment parameters secured during a progressive treadmill test conducted before and after a double-blind 5-day ascorbic acid–placebo exposure on smoking and nonsmoking subjects

	Ventilation volume ($\dot{V}_I$) at specific work levels in ml/kg body wt per min (STPD)										Ventilation volume ($\dot{V}_I$) at specific oxygen uptakes in ml/kg body wt per min (STPD)				V_T-V_C at specific work levels as a percentage									
	Rest, mean		3 mph, 3rd min		6 mph, 3rd min		9 mph, 3rd min		Recovery, mean		30 ml $\dot{V}_{O_2}$		40 ml $\dot{V}_{O_2}$		Rest, mean		3 mph, 3rd min		6 mph, 3rd min		9 mph, 3rd min		Recovery, mean	
	PL	ASC	PL	ASC	PL	ASC	PL	ASC	PL	ASC	PL	ASC	PL	ASC	PL	ASC	PL	ASC	PL	ASC	PL	ASC	PL	ASC
Smokers																								
Before	144	150	273	278	693	708	1,094	1,135	332	332	525	514	804	779	16.2	16.5	23.0	22.2	36.3	34.1	39.3	37.9	22.3	22.8
After	140	153	268	267	682	674	1,094	1,075	305	322	537	501	813	752	16.1	17.3	23.3	21.7	34.5	36.1	38.2	39.6	20.7	22.8
Nonsmokers																								
Before	134	134	268	267	673	652	1,024	1,020	283	300	507	488	749	762	14.7	14.1	21.5	18.8	36.1	33.3	40.2	38.1	22.0	21.4
After	134	130	258	243	675	627	1,040	1,023	280	280	509	496	744	740	15.0	14.1	20.4	19.5	35.1	31.3	40.2	39.8	22.0	20.6
Source of variation	df	MS	*F*	MS	*F*	MS	*F*	MS	*F*	MS	*F*	MS	*F*	MS	*F*	MS	*F*	MS	*F*	MS	*F*	MS	*F*	MS	*F*
B: placebo–ascorbic acid	1	245		238		4,790		0		1,436		7,763	1.62	15,792		0		44.7	1.22	65.8	1.54	7.9		0.2	
C: smokers–nonsmokers	1	3,976	2.74	3,075	1.54	21,550	1.70	105,415	2.87	27,195	*5.00*	7,508	1.58	28,956	1.74	85.9	*4.12*	123.9	3.38	34.0		14.0		7.3	
BC: interaction	1	673		480		7,315		2,289		0		227		4,234		10.8		1.6		46.9	1.10	7.9		27.0	
error between	36	1,449		1,995		12,675		36,722		5,433		4,738		16,636		20.8		36.6		42.6		34.0		30.0	
A: before–after	1	36		3,001	*9.57*	6,073	*4.62*	2,000		4,307	*7.49*	99		80		1.2		0.4		9.5	1.57	7.0	1.35	7.9	3.60
AB: interaction	1	4		490	1.56	3,038	2.31	6,771	1.10	0		410		3,645	1.23	0.4		1.1		9.5	1.56	24.6	*4.70*	1.2	
AC: interaction	1	8		353	1.13	589		7,722	1.26	208		154		858		0.2		0		12.9	2.13	1.5		0.7	
ABC: interaction	1	140		58		27		2,856		1,353	2.35	1,224	2.58	470		1.9		8.0	1.79	27.6	*4.55*	1.1		6.8	
error within	36	278		313		1,316		6,139		574		473		2,957		3.3		8.3		6.1		5.2		2.2	
Total	79																								

F values <1 are not reported. *F* values that are italicized are significant beyond $P = 0.05$

0432

TABLE III

Summary of mean values and three-factor analysis of variance results for oxygen utilization parameters secured during a progressive treadmill test conducted before and after a double-blind 5-day ascorbic acid–placebo exposure on smoking and nonsmoking subjects

	Oxygen uptake ($\dot{V}O_2$) at specific work levels in ml/kg body wt per min (STPD)										Oxygen uptake ($\dot{V}O_2$) at specific heart rates in ml/kg body wt per min (STPD)				Oxygen pulse ($\dot{V}O_2$/HR) at specific work levels in ml/beat (STPD)									
	Rest, mean		3 mph, 3rd min		6 mph, 3rd min		9 mph, 3rd min		Recovery, mean		Heart rate 150		Heart rate 160		Rest, mean		3 mph, 3rd min		6 mph, 3rd min		9 mph, 3rd min		Recovery, mean	
	PL	ASC	PL	ASC	PL	ASC	PL	ASC	PL	ASC	PL	ASC	PL	ASC	PL	ASC	PL	ASC	PL	ASC	PL	ASC	PL	ASC
Smokers																								
Before	5.3	5.7	15.1	16.0	36.0	36.8	47.6	49.1	11.8	11.8	32.3	33.2	36.6	37.7	4.5	4.9	10.7	11.6	17.3	17.6	19.7	20.7	7.4	7.2
After	5.5	5.7	14.9	15.2	35.4	36.9	47.9	49.6	11.2	12.0	33.0	35.5	38.5	39.9	4.8	5.1	11.1	11.5	17.5	18.6	20.2	21.2	7.1	7.4
Nonsmokers																								
Before	5.5	5.4	14.9	14.9	36.9	36.9	50.5	48.4	11.3	11.2	33.7	32.1	38.2	36.1	5.1	4.5	11.3	10.9	17.8	18.2	21.1	20.5	7.2	6.9
After	5.9	5.1	14.9	14.0	36.9	35.4	51.0	49.6	11.2	10.9	34.8	35.8	39.1	39.6	5.4	4.9	11.7	11.6	18.3	18.8	21.8	21.8	7.3	7.3

Source of variation	df	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F
B: placebo–ascorbic acid	1	0.17		0.30		1.25		0.21		0.17		8.86		0.87		0.21		0.95		6.66		2.26		0.10			
C: smokers–nonsmokers	1	0.24		7.78	1.86	1.29		37.39	1.32	6.35	1.87	7.51		0.12		0.43		0.88		4.97		15.61	1.26	0.21			
BC: interaction	1	2.54	2.54	5.77	1.38	18.90	1.23	56.33	1.99	1.85		19.49		19.74		3.90	*4.39*	4.15	1.18	0.25		7.70		0.21			
error between	36	1.00		4.19		15.36		28.35		3.40		58.87		57.61		0.22		3.52		7.15		12.38		1.97			
A: before–after	1	0.08		4.26	2.28	5.39	1.60	7.10		0.81	1.86	76.97	*10.04*	91.51	*9.37*	1.95	*8.99*	2.61	2.12	5.83	*5.83*	11.28	*8.98*	0.31	1.52		
AB: interaction	1	0.86	2.63	2.49	1.33	0.65		0.89		0.24		22.29	2.91	10.78	1.10			0.03		1.12	1.12	0.43		0.62	3.03		
AC: interaction	1			0.02		1.23		1.28		0.02		4.17		0.07		0.03		0.71		0.01		1.19		0.41	1.98		
ABC: interaction	1	0.23		0.14		5.57	1.65	0.29		1.37	3.12	1.17		6.70		0.04		0.76		0.48		0.62		0.02			
error within	36	0.33		1.87		3.37		7.18		0.44		7.67		9.77		0.89		1.23		1.00		1.26		0.20			
Total	79																										

F values <1 are not reported. *F* values that are italicized are significant beyond $P = 0.05$.

0432

Minute Ventilation

The mean $\dot{V}_I$ values expressed in milliliters per kilogram body weight for those subjects who were given the placebo and those given ascorbic acid are shown in Table IV. For any given level of work, or at any specific oxygen uptakes, no major differences are apparent between vitamin C-saturated and placebo subjects after the 5-day experiment. The results of the analyses of variance in Table II statistically substantiate this observation in that none of the F values comparing means for the two groups are statistically significant ($P > 0.05$). Mean values for smoking and nonsmoking subjects after the 5-day exposure are also presented in Table IV. The $\dot{V}_I$/kg is higher for smokers than nonsmokers at all levels; however, the difference is statistically significant ($P < 0.05$) at recovery only, as shown by the results of the analyses of variance in Table II.

Tidal Volume/Vital Capacity

Table V contains the mean V_T–V_C values after the 5-day treatment for vitamin C-saturated and placebo subjects. For any given level of work, no significant differences between means exist ($P > 0.05$) as verified by the results of the analyses of variance shown in Table II. When comparing smokers and nonsmokers, V_T–V_C is significantly higher for smoking subjects at rest only.

TABLE IV

Mean values for minute ventilation, $\dot{V}_I$/kg in ml/min, secured on a progressive treadmill test after a 5-day exposure to vitamin C or placebo

	Placebo	Vitamin C	Smokers	Nonsmokers
Mean rest	137	142	147	132
At 3 mph	263	255	268	251
At 6 mph	679	656	678	651
At 9 mph	1,067	1,049	1,085	1,031
Mean recovery	293	301	314	280
At $\dot{V}_{O_2}$/kg, 30 ml	523	499	519	503
At $\dot{V}_{O_2}$/kg, 40 ml	779	746	782	742

TABLE V

Mean values for the ratio of tidal volume to vital capacity (V_T–V_C in %) secured on a progressive treadmill test after a 5-day exposure to vitamin C or placebo

	Placebo	Vitamin C	Smokers	Nonsmokers
Mean rest	15.5	15.7	16.7	14.5
At 3 mph	21.9	20.6	22.6	20.0
At 6 mph	34.8	33.7	35.3	33.2
At 9 mph	39.2	39.7	38.9	40.0
Mean recovery	21.4	21.7	21.7	21.3

TABLE VI

Mean values for oxygen uptake ($\dot{V}_{O_2}$/kg in ml per min) secured on a progressive treadmill test after a 5-day exposure to vitamin C or placebo

	Placebo	Vitamin C	Smokers	Nonsmokers
Mean rest	5.7	5.4	5.6	5.5
At 3 mph	14.9	14.6	15.1	14.5
At 6 mph	36.2	36.2	36.2	36.2
At 9 mph	49.5	49.6	48.8	50.3
Mean recovery	11.2	11.5	11.6	11.1
At heart rate 150	33.9	35.7	34.3	35.3
At heart rate 160	38.8	39.8	39.2	39.4

Oxygen Uptake

Table VI contains the mean $\dot{V}_{O_2}$ values for vitamin C-saturated and placebo subjects after the 5-day exposure. At any given level of work, or at any specific heart rate, no major differences are apparent between the vitamin C and placebo groups. This observation is substantiated by the results of the analyses of variance in Table III in which none of the corresponding F values is statistically significant ($P > 0.05$). In comparing mean values for smoking and nonsmoking subjects after the 5-day exposure, the same holds true, that is, there is no significant difference in the mean $\dot{V}_{O_2}$/kg at any level.

Oxygen Pulse

Table VII contains the final mean $\dot{V}O_2$/HR values for vitamin C-saturated and placebo groups. For any given level of work it can be seen that no distinct differences are apparent, nor are there any substantial differences between smokers and nonsmokers. The results of the analyses of variance in Table III support these observations in that none of the F values comparing means of treatment versus placebo subjects and smoking versus nonsmoking subjects is statistically significant ($P > 0.05$).

DISCUSSION

The mean plasma vitamin C levels of smokers and nonsmokers obtained in the present and other recent investigations are contained in Table VIII. The mean nonsaturated level of 0.66 mg vitamin C for the 20 nonsmoking subjects compares to previous results. However, the mean level for the 20 smokers although lower than the value for the nonsmokers was not significantly lower and is somewhat higher than the levels for smokers reported by other investigators. The relatively high plasma vitamin C levels for smokers in the present study may be due to the fact that the smokers who made up the sample were young, smoked less, and were probably more active than the smoking subjects used in other investigations.

Plasma concentrations taken after 5 days of vitamin C supplementation and immediately before the final treadmill run clearly indicate a saturation level for those subjects given vitamin C as opposed to the placebo subjects as shown in Table I. Thus, four distinct groups (nonsaturated nonsmokers, nonsaturated smokers, saturated nonsmokers and saturated smokers) were compared on their response to a treadmill run in terms of respiratory adjustment and oxygen utilization.

None of the 24 separate analyses of variance performed on parameters of respiratory adjustment and oxygen utilization at various levels of work revealed significant differences for the mean responses of vitamin C-saturated and placebo subjects (Tables II and III). This is interpreted as providing evidence that daily supplementation of 2 g vitamin C had no effect

TABLE VII

Mean values for oxygen pulse ($\dot{V}O_2$/HR in ml per beat) secured on a progressive treadmill test after a 5-day exposure to vitamin C or placebo

	Placebo	Vitamin C	Smokers	Nonsmokers
Mean rest	5.1	5.0	5.0	5.2
At 3 mph	11.4	11.6	11.3	11.7
At 6 mph	17.9	18.7	18.1	18.6
At 9 mph	22.0	21.5	20.7	21.8
Mean recovery	7.2	7.4	7.3	7.3

TABLE VIII

Comparative plasma vitamin C concentrations of smoking and nonsmoking males, mg/100 ml plasma

Investigator	Nonsmokers			Smokers			
	Plasma vitamin C	Mean age	Number of subjects	Plasma vitamin C	Mean age	Number of subjects	Number of cigarettes/day
Calder et al. (15)	0.91		91	0.52		31	15 or more
Brook and Grimshaw (18)	0.62	40	32	0.44	40	22	1–14 and 15 or more
Pelletier (17)	0.72	34	5	0.29	29	5	27–30
Present study	0.66	24	20	0.61	25	20	10–20

on respiratory adjustment and oxygen utilization before, during, or after exercise. These results are in agreement with the findings of a number of studies (4–9) and support the contention that the ingestion of vitamin C affords healthy subjects no extra benefit in meeting the additional stress imposed by exercise regardless of their smoking habits.

The results between smoking and nonsmoking subjects are almost as definitive as those between vitamin C-saturated and placebo subjects. Significant differences were found in only 2 of the 24 separate analyses of variance performed on parameters of respiratory adjustment and oxygen utilization. This number could reasonably occur by chance in terms of the probability level ($P = 0.05$) selected for use in the study. These results are in agreement with the findings of Juurop and Muido (23) comparing healthy smoking and nonsmoking subjects for oxygen utilization and respiratory variables but in disagreement with several other investigations (24, 25). The literature indicates an increasing interest in the effect of smoking on cardiorespiratory function in healthy individuals (23–28) and the lack of unanimity clearly calls for further research in this area.

The purpose of this study, however, was to investigate whether supplementation of vitamin C had a differing effect on the physiological adjustment to heavy exercise of healthy smoking and nonsmoking subjects. The results provide no support for this postulate.

SUMMARY

Twenty smoking and twenty nonsmoking males (mean age, 24.5 years; standard deviation, 3.5) were required to run continuously on a level-grade, motor-driven treadmill for 3 min at 3 miles/hr, 3 min at 6 miles/hr, and 3 min at 9 miles/hr, before and after a 5-day, 2 g/day, vitamin C or placebo exposure. Following the standard double-blind protocol, 10 smoking and 10 nonsmoking subjects were given vitamin C daily while the remainder received the placebo. Plasma vitamin C levels were measured before and immediately following the 5-day treatment period and were found to fall within normal limits. Further, the vitamin C concentration of subjects on vitamin supplementation rose to saturation levels. The purpose of the study was to determine the effect of vitamin C saturation on respiratory adjustment and oxygen utilization before, during, and after exercise in smokers and nonsmokers. Measurements were made of minute ventilation ($\dot{V}_I$), the ratio of tidal volume to vital capacity (V_T–V_C), oxygen uptake ($\dot{V}O_2$), and oxygen pulse ($\dot{V}O_2$/HR) at the various work levels as well as during pre-exercise and recovery. By employing a 2 × 2 × 2 factorial analysis of variance design, calculations were made of the differences in the before and after response of placebo versus vitamin C-saturated subjects in smokers and nonsmokers. The results of the various analyses performed on all parameters in all levels revealed no significant differences between the mean response of subjects given vitamin C and those given the placebo, whether they were smokers or nonsmokers. This was interpreted as providing evidence that daily supplementation of 2 g vitamin C had no effect on respiratory adjustment and oxygen utilization before, during, and after exercise in smoking and nonsmoking subjects.

REFERENCES

1. Medical Research Council. Vitamin C subcommittee of the accessory food factors committee. *Lancet* 1: 853, 1948.
2. Crandon, J. H., et al. Ascorbic acid deficiency in experimental and surgical subjects. *Proc. Nutr. Soc. Engl. Scot.* 12: 273, 1953.
3. Editorial. Food for the athlete. *Lancet* 2: 152, 1948.
4. Keys, A. Physical performance in relation to diet. *Federation Proc.* 2: 164, 1943.
5. Johnson, R. E., F. Sargent and P. F. Robinson. Effects of variation in dietary vitamin C on the

physical well being of manual workers. *J. Nutr.* 29: 155, 1945.

6. Keys, A., and A. F. Henschel. Vitamin supplementation of U.S. Army rations in relation to fatigue and the ability to do muscular work. *J. Nutr.* 23: 259, 1942.
7. Henschel, A. et al. Vitamin C and ability to do work in hot environments. *Am. J. Trop. Med. Hyg.* 24: 259, 1944.
8. Fox, F. W. et al. Vitamin C requirements of native mine laborers. *Brit. Med. J.* 2: 143, 1940.
9. Rasch, P. J. et al. Effects of vitamin C supplementation on cross country runners. *Sportartzliche Praxis* 5: 10, 1962.
10. Brunner, H. Vitamin C experiences with redoxon in the Swiss Army in Frauenfeldt. *Schweiz. Med. Wochschr.* 71: 715, 1941.
11. Hoffink, A. W. J. H. Vitamin C and work. Studies on the influence of work and of vitamin C intake on the human organism. *Verhandel. Ned. Inst. Prevent. Geneesk.* 4: 176, 1946.
12. Hoffink, A. W. J. H. Researches on the influence of vitamin C administration on the human organism, in particular in connection with the working capacity. *Acta Brev. Neerl. Physiol. Pharmacol. Microbiol.* 14: 62, 1946.
13. Hoogerwerf, A. et al. The influence of vitamin C administration on the mechanical efficiency of the human organism. *Intern. Z. Angew. Physiol. Einschl. Arbeitsphysiol.* 20: 164, 1963.
14. Prokop, L. Die Wirkung von Naturlichen Vitamin C auf O_2 Utilisation und Kreislaufokanomie. *Z. Aerztl. Fortbil.* 49: 2, 1960.
15. Calder, J. H. et al. Comparison of vitamin C in plasma and leucocytes of smokers and non-smokers. *Lancet* 1: 556, 1963.
16. Durand, C. M. et al. Hypovitaminose C latente et tabac. *Concours Med.* 84: 4081, 1962.
17. Pelletier, O. Smoking and vitamin C levels in humans. *Am. J. Clin. Nutr.* 21: 1259, 1968.
18. Brook, M., and J. J. Grimshaw. Vitamin C concentration of plasma and leukocytes as related to smoking habit, age, and sex of humans. *Am. J. Clin. Nutr.* 21: 1254, 1968.
19. Hogler, F. Zur Frage der Nikotinschadigung. *Wien. Med. Wochschr.* 93: 309, 1943.
20. Bannister, R. G. et al. Two low resistance, low dead space respiratory valves. *J. Physiol., London* 124: 4, 1954.
21. Varley, H. *Practical Clinical Biochemistry* (3rd ed.) London: Heinemann, 1962, p. 528–529.
22. Winer, B. J., *Statistical Principles in Experimental Design*, New York: McGraw-Hill, 196. p. 672.
23. Juurop, A. et al. Acute effects of cigaret smoking on O_2 consumption, pulse rate, breat ing rate and blood pressure in working o ganisms. *Acta Physiol. Scand.* 11: 48, 1946.
24. Krumholtz, R. A. et al. Cardio-pulmonar function in young smokers: A comparison pulmonary function measurements and som cardio-pulmonary responses to exercise betwe a group of young smokers and a comparabl group of non-smokers. *Ann. Internal Med.* 60: 603, 1964.
25. Chevalier, R. B., J. A. Bowers, S. Bonduran and J. C. Ross. Circulatory and ventilatory effects of exercise in smokers and non-smokers. *J. Appl. Physiol.* 18: 357, 1963.
26. Blackburn, H. et al. Comparison of cardiovascular and related characteristics in habitual smokers and non-smokers. *Ann. N. Y. Acad. Sci.* 90: 277, 1960.
27. Shapiro, W. et al. Effects of smoking and athletic conditioning on ventilatory mechanics, including observations of the reliability of the forced expirogram. *Am. Rev. Respirat. Diseases* 85: 191, 1962.
28. Glassford, R. G. et al. Smoking and physical fitness: a preliminary report. *Can. Family Physician* 15: 60, 1969.
29. Crandon, J. H. et al. Experimental human scurvy. *New Engl. J. Med.* 223: 353, 1940.
30. Farmer, C. J. Some aspects of vitamin C metabolism. *Federation Proc.* 3: 179, 1944.
31. Vitamin supplements and performance capacity. *Nutr. Abstr. Rev.* 8: 312, 1950.
32. Vitamin C Requirements of Human Adults. *Med. Res. Council Spec. Rept.* no. 280, p. 179, 1953.

Ind. Eng. Chem. Anal. Ed. 13, 89

0472

Determination of Ascorbic Acid in Citrus Fruit Juices

ROBERT BALLENTINE
Physiology Laboratory, Princeton University, Princeton, N. J.

THE chemical methods for the determination of ascorbic acid (vitamin C) have been mainly based on the method of Tillmans, Hirsch, and Hirsch (*5*) employing 2,6-dichlorophenolindophenol or on some modification of the iodometric titration. In general the iodometric methods have been subject to criticism since they lack specificity and a sharp end point, although Stevens (*3*) has shown that by employing a double back-titration in the presence of a high concentration of hydrogen ions the iodine end point may be made very sharp. Further, Tauber and Kleiner (*4*) have demonstrated that the iodometric method is adequate for the determination of ascorbic acid in citrus fruit juices, since interfering substances are absent. However, in common with all iodometric methods, the reagents employed in Stevens' method change their titer with time, requiring frequent standardization, and the double back-titration increases the volumetric error.

In the method presented here, a direct titration with iodate in acid-iodide gives an end point comparable in sharpness with that of the above double back-titration method, and in addition avoids the necessity for frequent standardization. Only one accurately prepared solution is required, and this is stable over long periods of time.

In addition to the iodate titration described below, the ascorbic acid content of the citrus juices was determined by the method of Stevens (*3*). The juices were also titrated by the Mack and Tressler (*1*) modification of the Tillmans method (*5*). The indophenol dye, recrystallized from the Eastman product, was standardized by the method of Menaker and Guerrant (*2*). All the reagents used in the three methods were referred to a single primary standard solution, 0.1 *N* potassium iodate.

REAGENTS. Standard iodate, 0.1 *N* (0.0167 *M*), contains 3.567 grams of potassium iodate per liter. For use this stock solution is diluted to 0.01 *N*.

Potassium iodide, 10 per cent solution fresh daily.

Sulfuric acid, 2 *N*.

Starch indicator is made fresh daily from improved Lintner's soluble starch, using 1 gram per 100 ml. with 2 grams of potassium iodide added.

PROCEDURE. Add to 5 ml. of citrus fruit juice 1 ml. of 10 per cent potassium iodide and 2 ml. of 2 *N* sulfuric acid. Titrate the resulting solution with 0.01 *N* iodate, adding the reagent dropwise near the end point. It is best not to add the starch until very near the end point.

1 ml. of 0.01 *N* iodate = 0.88 mg. of ascorbic acid.

Results and Discussion

A preliminary comparison of the three methods employed for the determination of ascorbic acid was carried out on solutions of synthetic, crystalline ascorbic acid (Merck). The results, presented in Table I, show that the indophenol and the iodate titration are in agreement, although the absolute amounts recovered were slightly high. On the other hand, the double back-titration of Stevens gave slightly low values for the ascorbic acid content.

Analyses on citrus juices are recorded in Table II. The results obtained by the Stevens and iodate methods are compared with the ascorbic acid content as determined by the Mack and Tressler indophenol titration as a standard. Stevens' method gives variable results that differ rather widely from those determined by the indophenol method, being up to 12 per cent too high. On the other hand, the ascorbic acid content as determined by the iodate method agrees with that found by the indophenol titration. On the basis of these results the iodate method can be recommended for routine determinations of ascorbic acid in citrus juices.

The iodate titration has the following advantages over the double back-titration method of Stevens: increased accuracy, stability of reagents, greater simplicity and rapidity, and more reproducible results.

TABLE I. DETERMINATION OF ASCORBIC ACID

Ascorbic Acid Taken	Indophenol Titration: Ascorbic acid found	Indophenol Titration: Recovery	Stevens' Iodine Titration: Ascorbic acid found	Stevens' Iodine Titration: Recovery	Iodate Titration: Ascorbic acid found	Iodate Titration: Recovery
Mg.	*Mg.*	%	*Mg.*	%	*Mg.*	%
1.75	1.81	103.4	1.80	102.8	1.77	101.7
2.00	2.09	104.5	1.88	94.0	2.08	103.9
2.00	2.09	104.5	1.90	95.0	2.07	103.6
3.00	3.10	103.3	2.96	98.6	2.97	99.0

TABLE II. ANALYSES OF CITRUS JUICES

Material	Indophenol Titration	Stevens' Iodine Titration	Stevens' Iodine Titration: Difference from indophenol titration	Iodate Titration	Iodate Titration: Difference from indophenol titration	Iodate Titration: Difference from Stevens' iodine titration
	G./100 ml.	*G./100 ml.*	%	*G./100 ml.*	%	%
Lemon juice, 1	0.0460	0.0470 0.0471	2.1	0.0460 0.0461	0.0	−2.1
Lemon juice, 2	0.0427	0.0465 0.0461	8.4	0.0434 0.0438	2.1	−6.2
Lemon juice, 3	0.0462	0.0460	−0.5	0.0462	0.0	0.5
Lemon juice, 4	0.0396	0.0397	0.2	0.0392	−1.0	−1.2
Lemon juice, 5	0.0416	0.0458 0.0448	8.8	0.0424	1.9	−6.4
Orange juice, 1	0.0416	0.0420 0.0420	0.9	0.0419 0.0417	0.5	−0.4
Orange juice, 2	0.0388	0.0390 0.0388	0.2	0.0387 0.0387	−0.3	−0.5
Orange juice, 3	0.0399	0.0346 0.0344	1.7	0.0342 0.0341	0.6	−1.1
Grapefruit juice, 1	0.0424 0.0430	0.0461 0.0470	9.8	0.0446 0.0442	4.0	−4.5
Grapefruit juice, 2	0.0370 0.0370	0.0412 0.0416	11.8	0.0372 0.0378	1.4	−9.4

Literature Cited

(1) Mack, G. L., and Tressler, D. K., *J. Biol. Chem.*, **118**, 735 (1937).
(2) Menaker, M. H., and Guerrant, N. B., IND. ENG. CHEM., Anal. Ed., **10**, 25 (1938).
(3) Stevens, J. W., *Ibid.*, **10**, 269 (1938).
(4) Tauber, H., and Kleiner, I. S., *J. Biol. Chem.*, **108**, 563 (1935).
(5) Tillmans, J., Hirsch, P., and Hirsch, W. Z., *Z. Untersuch. Lebensm.*, **63**, 1 (1932).

1957, J. Nutrition 63(4) 601-609

EFFECT OF ASCORBIC ACID IN PANTOTHENIC ACID DEFICIENCY [1]

JOSEPH J. BARBORIAK AND WILLARD A. KREHL
Yale Nutrition Laboratory, Department of Biochemistry, Yale University School of Medicine, New Haven, Connecticut

(Received for publication July 16, 1957)

INTRODUCTION

Several authors have reported that the inclusion of ascorbic acid (Daft, '51; Daft and Schwartz, '52; Everson et al., '54) or of related compounds (Hundley and Ing, '53) in a pantothenic acid (PA)-deficient diet alleviates the signs of PA deficiency. Similar suppression of the PA-deficiency syndrome has been observed with deficient diets supplemented with antibiotics (Lih and Baumann, '51; Sauberlich, '52; Guggenheim et al., '54). As the antibiotics are believed to act by inducing changes in intestinal microflora (Johansson et al., '53), it was decided to study whether ascorbic acid acts through the same mechanism. A high dose of sulfathiazole was used to suppress the growth of intestinal micro-organisms. The effect of antibiotics in preventing the PA-deficiency syndrome was tested with a new antibiotic, hygromycin.[2] The properties and therapeutic spectrum of this drug were described by Pittenger et al. ('53) and by Mann et al. ('53). Hygromycin is a broad-spectrum antibiotic and in preliminary investigations it showed some growth-promoting properties.

EXPERIMENTAL

Two series of experiments were conducted, using male weanling rats of the Sprague-Dawley strain with average initial

[1] This study was supported by a grant-in-aid from The National Vitamin Foundation, Inc.

[2] Kindly supplied by Dr. O. K. Behrens, the Eli Lilly Research Laboratories.

weights of about 45 gm. In the first series, each of the 5 groups consisted of 8 animals; in the second series, 10 animals for each of the 6 groups were used. The animals were housed in individual metal screen-bottom cages in an animal room maintained at 70°F and 40 to 45% relative humidity.

Food and water were supplied ad libitum and the rats were weighed at regular intervals. The first series was terminated after 52 days, the second series after 31 days.

The composition of the control PA-deficient diet was as follows: vitamin-free casein, 25%;[3] corn oil, 5%; sucrose, 65.6%; mineral salts mixture, 4%;[4] choline Cl, 0.2%; and cystine, 0.2%. Vitamins were supplied in milligrams per 100 gm of diet as follows: thiamine, 1.0; riboflavin, 1.0; pyridoxine, 1.0; nicotinic acid, 10.0; *i*-inositol, 20; *p*-aminobenzoic acid, 20.0; folic acid, 0.1; biotin, 0.1; menadione, 1.0; and α-tocopherol acetate, 10.0. Vitamins A and D were supplied in the form of halibut oil and Drisdol.[5] The experimental rations were prepared by suitable modifications of the control diet; 2% of the sucrose was replaced by the weight equivalent of either ascorbic acid or citric acid or phthalylsulfathiazole (sulfathiazole). In one diet 4% of sucrose was replaced by 2% ascorbic acid plus 2% sulfathiazole, and the hygromycin diet was prepared by supplementing the control diet by 100 mg hygromycin per kilogram of diet in the first series and 200 mg hygromycin per kilogram of diet in the second series. The animals which died during the experiment and those that were sacrificed at the end of the experiment were autopsied, their adrenals weighed, and together with testes, preserved in buffered formaldehyde for histological examination. Serum protein distribution was studied with the help of paper electrophoresis using Spinco-Durrum cells,[6] barbiturate buffer of pH 8.6, ionic strength 0.075, constant current of 10 mA per cell, and 16-hour runs. The strips were stained in bromphenol blue and evaluated in Spinco Analytrol.

[3] Labco, Borden Company.
[4] Hegsted et al., '41.
[5] Winthrop-Stearns, Inc.
[6] Beckman, Spinco Division, Palo Alto, California.

0544

RESULTS

The weight gains computed separately for the first 10 days of the experiment and for the following three weeks are summarized in table 1. In the first series, only the data for the first 31 days are treated statistically, because after this period, an appreciable number of animals succumbed, especially in the group receiving ascorbic acid plus sulfathiazole.

During the initial 10 days the animals grew quite rapidly, gaining daily from 2.2 gm in the group fed ascorbic acid plus sulfathiazole, to 4.1 gm in the group receiving the hygromycin supplement. After this period the growth rate declined and the group fed ascorbic acid plus sulfathiazole in the first series even lost some weight. Only the groups fed ascorbic acid or hygromycin showed more substantial weight gains, ranging from 20 to over 40 gm for the following three weeks. During the final 20 days of experiment the group receiving ascorbic acid continued to gain weight, in contrast to the group receiving hygromycin whose growth rate declined toward the end of the experiment.

At the end of about three weeks, external signs of pantothenic acid deficiency, such as thinning of the fur, porphyrine whiskers, and arched back were noted in some animals in the groups receiving ascorbic acid plus sulfathiazole. During the following week, these deficiency signs appeared in a few animals in all of the other groups with the exception of the ascorbic acid and the hygromycin groups. After about 45 days, even in these two groups a few animals showed the deficiency signs.

Upon autopsy some differences between the individual groups with respect to the condition of certain vital organs were noticed. In the second series, where the animals were sacrificed after 31 days, most rats in the groups receiving ascorbic acid plus sulfathiazole did not have descended testes. Similar defects were found in about one half of the animals of the control group and in the group fed 2% citric acid. The animals in the hygromycin group were normal in this respect. An extensive production of intestinal gas was observed in the ascorbic acid plus sulfathiazole group as well as in the hygro-

mycin group. Much less gas was noted in the controls and in the sulfathiazole groups.

On macroscopical examination, most of the adrenal glands seemed to be normal, light brown in color, with the exception of two dark brown glands found in the group fed ascorbic acid plus sulfathiazole. The fat tissue in which the adrenals are normally imbedded was found only in the ascorbic acid and hygromycin groups. Few cases of lung infection and lung tumors were detected; however, no relation to the diet fed was apparent.

In the first series where the animals were sacrificed after 52 instead of 31 days, results similar to those of the second series were obtained. The adrenals were more seriously affected, however, being dark brown in color; in a few animals in the ascorbic acid plus sulfathiazole and the hygromycin groups they were markedly hypertrophied and hemorrhagic.

Histological examination of the adrenal glands of animals in the ascorbic acid plus sulfathiazole group revealed excessive capillary congestion of the zona reticularis; in a few cases necrotic areas between the zona reticularis and fasciculata were also observed. A few instances of capillary congestion in zona reticularis were also noted in the control group and the group receiving citric acid or hygromycin. Many animals showed a marked degeneration or loss of interstitial tissue in the testes. Again, as in the second series, the group on ascorbic acid plus sulfathiazole was most seriously affected. However, about one half of the animals in the other groups also showed this condition. Similarly, spermiogenesis was affected in all groups and this defect did not seem to be related to any special diet.

The relative weight of the adrenal glands e.g. the weight of glands per 100 gm body weight, is sometimes used as a criterion of the degree of pantothenic acid deficiency. In our experiment, the highest relative weight of adrenals was found in the ascorbic acid plus sulfathiazole and the sulfathiazole groups, namely, 50.7 and 45.6 mg respectively. The hygromycin group had the lowest average value, 26.2 mg. There was

TABLE 1

Effect of various supplements on the weight gains of pantothenic acid-deficient rats

SERIES	DAYS	PA-DEFICIENT CONTROLS	ASCORBIC ACID	SULFA-THIAZOLE	AC + ST [1]	HYGROMYCIN	CITRIC ACID
		gm	*gm*	*gm*	*gm*	*gm*	*gm*
I	1 – 10	27 ± 4.1[2]	34 ± 2.9	25 ± 2.1	24 ± 3.4	41 ± 2.0[3]	
	11 – 31	9 ± 3.5	31 ± 9.1[2]	11 ± 2.5	8 ± 3.6	20 ± 3.3[2]	
	32 – 52	23	61	0.4	——	13	
II	1 – 10	26 ± 3.0	26 ± 3.2	25 ± 1.6	22 ± 2.2	31 ± 2.2	27 ± 2.1
	11 – 31	17 ± 3.4	36 ± 7.7[2]	0.7 ± 2.7[3]	3 ± 2.0[3]	41 ± 3.5[2]	16 ± 3.5

[1] Ascorbic acid and sulfathiazole.
[2] Mean ± standard error of the mean.
[3] Significant at 0.1% level.

TABLE 2

Effects of supplements to a pantothenic acid-deficient diet on serum protein distribution

SUPPLEMENT	NO. OF SAMPLES	ALBUMIN	GLOBULINS			
			α_1	α_2	β	γ
		%	%	%	%	%
None						
Pantothenic acid-deficient control	8	36 ± 6.2[1]	19 ± 3.8	10 ± 1.6	23 ± 4.6	12 ± 3.3
Citric acid	6	48 ± 8.3[2]	17 ± 3.8	8 ± 3.4	19 ± 3.1	8 ± 2.4
Sulfathiazole	5	46 ± 2.7[2]	20 ± 2.1	9 ± 1.8	20 ± 4.0	5 ± 1.3[3]
Ascorbic acid	7	33 ± 6.7	19 ± 2.3	12 ± 3.0	19 ± 1.9	17 ± 4.9[3]
Ascorbic acid and sulfathiazole	4	38 ± 5.9	18 ± 5.3	12 ± 0.6	19 ± 4.0	13 ± 3.6
Hygromycin	8	52 ± 2.8[2]	18 ± 2.6	8 ± 2.3	14 ± 2.8[2]	8 ± 1.4[3]

[1] Mean ± standard error of the mean.
[2] Significant at the 0.1% level.
[3] Significant at the 0.5% level.

0544

practically no difference between the relative weights of the adrenals in the other three groups, the values being 31.4, 30.0, and 29.2 mg for the control, citric acid and ascorbic acid groups respectively.

The results of the electrophoretic study are summarized in table 2. The highest average value for serum albumin was found in the hygromycin group, namely 52%. The lowest value, 33%, was seen in the ascorbic acid group. The differences are statistically significant ($P > 0.01$). The percentages of α_1-, α_2-, and β-globulins did not vary markedly from one group to another, staying in the range from 17 to 20% for α_1-, 8 to 12% for α_2-, and 19 to 23% for the β-globulins. An exception was the hygromycin group which had the rather low β-globulin value of 14%. The differences in the γ-globulin percentages were more pronounced. The sulfathiazole group showed the lowest value, 5%, and the ascorbic acid groups the highest, 17%. The differences were again statistically significant ($P > 0.01$).

DISCUSSION

In confirmation of Daft's ('51) findings, the inclusion of 2% ascorbic acid in the PA-deficient diet prevented the PA-deficiency syndrome in most cases. When fed simultaneously with sulfathiazole, however, the ascorbic acid failed to show such a beneficial effect. As sulfathiazole is believed to inhibit the activity of intestinal microorganisms (Wright and Welch, '44) it can be concluded that the favorable influence of ascorbic acid is a secondary one, mediated through changes in the intestinal microflora. This suggestion is supported by the findings that glucuronolactone, which when fed in 2% concentration in a PA-deficient diet also prevents the deficiency signs, is ineffective when given parenterally (Hundley and Ing, '53). In agreement with reports of Lih and Baumann ('51), Sauberlich ('52) and Guggenheim et al. ('54) the antibiotic agent prevented the PA-deficiency syndrome. This protection, however, was only temporary, and after about 45 days of experiment the signs of deficiency appeared.

0544

The prevention of deficiency signs, noted in rats receiving 2% ascorbic acid seemed to be a lasting one, (Daft, '51), as compared with the only temporary protection offered by hygromycin. This observation, considered along with the differences in pathology of some vital organs and in serum protein distribution, suggests that even if these two substances seem to alleviate the signs of PA deficiency by changes in the intestinal microflora, the underlying mechanisms are probably not identical.

Pantothenic acid deficiency has been reported to lower the resistance of animals to infections. The primary cause of death in many cases is a lung infection (Zucker et al., '54; Seronde, '54). Axelrod and Pruzansky ('54) reported a decreased production of antibodies in PA-deficient rats. This finding could, at least partially, explain the lower resistance to the infections.

Although antibodies are believed to belong mainly to the γ-globulin type of proteins, Axelrod and Pruzansky ('54) did not find any difference between the vitamin-supplemented controls and the deficient animals with respect to serum γ-globulin levels. In our experiments, the variation between the individual groups with respect to the γ-globulin concentration was considerable. As the principal effects of the administered substances are believed to be some qualitative or quantitative changes (or both) in microflora, the results of our experiments suggest possible connections between the microbiological conditions of the intestinal tract and the processes regulating the synthesis of blood proteins, especially the γ-globulins.

SUMMARY

The influence of ascorbic acid, phthalylsulfathiazole, ascorbic acid and sulfathiazole combined, citric acid, and an antibiotic, hygromycin, on the physiological effects of a pantothenic acid-deficient diet were investigated in experiments with male weanling albino rats. The concentration of hygromycin was 100 or 200 mg per kilogram of diet; each of the other test substances was used at a 2% level.

The presence of either ascorbic acid or hygromycin in the pantothenic acid-deficient diet delayed or suppressed the appearance of deficiency signs. Sulfathiazole, fed simultaneously with ascorbic acid, counteracted the beneficial effect of the ascorbic acid. The addition of citric acid did not seem to influence significantly the effect of the basal deficient diet. The histological picture of the adrenals and testes, as well as the distribution of blood serum proteins, were affected markedly by the supplements. It is concluded that the favorable effect of ascorbic acid is probably a secondary one, mediated through changes in the intestinal microflora, and that these changes may not be identical with those observed when feeding hygromycin.

ACKNOWLEDGMENTS

The authors wish to thank Dr. R. J. Floody, Hoffmann-La-Roche, Inc. for the generous supply of vitamins and Dr. R. D. Coghill, Abbott Laboratories, Inc. for the halibut liver oil. We are indebted also to Dr. A. D. Whedon for the histological examination of tissues, and to Miss Charleen Sibley for technical assistance.

LITERATURE CITED

AXELROD, A. E., AND J. PRUZANSKY 1954 Role of Vitamins in Antibody Production. Nutrition Symp. Ser. 8: National Vitamin Foundation, Inc.

DAFT, F. S. 1951 Effect of vitamin C on pantothenic acid-deficient rats. Federation Proc., *10:* 380.

DAFT, F. S., AND K. SCHWARZ 1952 Prevention of certain B-vitamin deficiencies with ascorbic acid or antibiotics. Ibid., *11:* 200.

EVERSON, G., L. NORTHROP, N. Y. CHUNG AND R. GETTY 1954 Effect of ascorbic acid on rats deprived of pantothenic acid during pregnancy. J. Nutrition, *54:* 305.

GUGGENHEIM, K., S. HALEVY, I. HARTMANN, AND R. ZAMIR 1954 The effect of antibiotics on the metabolism of certain B-vitamins. Ibid., *50:* 245.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459.

HUNDLEY, J. M., AND R. B. ING 1953 Prevention of pantothenic acid deficiency with glucuronolactone and other compounds related to ascorbic acid. Federation Proc., *12:* 417.

JOHANSSON, K. R., G. E. PETERSON AND E. C. DICK 1953 Effect of dietary aureomycin upon intestinal microflora and the intestinal synthesis of vitamin B_{12} in the rat. J. Nutrition, *49:* 135.

0544

LIH, H., AND C. A. BAUMANN 1951 Effect of certain antibiotics on the growth of rats fed diets limiting in thiamine, riboflavine, or pantothenic acid. Ibid., *45:* 143.

MANN, R. L., R. M. GALE AND F. R. VANABELE 1953 Hygromycin. II. Isolation and properties. Antibiotics Ann. 1953-54: 167. Med. Encyclop., Inc., New York, N. Y.

PITTENGER, R. C., R. N. WOLFE, M. M. HOEHN, P. N. MARKS, W. A. DAILY AND J. M. MCGUIRE 1953 Hygromycin. I. Preliminary studies on the production and biological activity of a new antibiotic. Antibiotics Ann. 1953-54: 157, Med. Encyclop., Inc., New York, N. Y.

SAUBERLICH, H. E. 1952 Effect of aureomycin and penicillin upon the vitamin requirement of the rat. J. Nutrition, *46:* 99.

SERONDE, J. JR. 1954 Resistance of rats to inoculation with corynebacterium pathogenic in pantothenic acid deficiency. Proc. Soc. Exp. Biol. Med., *85:* 521.

WRIGHT, L. D., AND A. D. WELCH 1944 Folic acid, biotin and pantothenic acid deficiency and the liver storage of various vitamins in rats fed succinylsulfathiazole in highly purified rations. J. Nutrition, *27:* 55.

ZUCKER, T. F., AND L. M. ZUCKER 1944 Pantothenic acid deficiency and loss of natural resistance to a bacterial infection in the rat. Proc. Soc. Exp. Biol. Med., *85:* 517.

Acta Anat (Basel) 73: 278-80, 1969

0548

Acta anat. 73: 278–280 (1969)

Raja Peary Mohan College, Uttarpara, Hooghly, West Bengal, India

Failure of ascorbic acid in the prevention of adrenal hypertrophy and ovarian inhibition in reserpinized rats

N. R. BARDHAN and A. CHATTERJEE

Introduction

The role of ascorbic acid in the prevention of adrenal cortical hypertrophy (CHATTERJEE [1964], CHATTERJEE *et al.* [1966]) and gonadal inhibition (DEB *et al.* [1963], CHATTERJEE *et al.* [1965]) in several nonspecific stressful situations has been documented. Reserpine (Serpasil, Ciba), an ataractic drug, as well as a stressor, has been shown to induce both adrenal cortical hypertrophy (CHATTERJEE [1965a]) and gonadal inhibition (KHAZAN *et al.* [1960]), possibly through its action on the hypothalamus.

The object of the present experiment is to ascertain whether ascorbic acid has any influence at all in the normalisation of the altered adrenal and gonadal physiology in reserpinized female rats.

Materials and methods

18 healthy female adult albino rats were used in the experiment. The rats were divided into three groups, group 1 and 2 containing 7 animals each and 4 in group 3. Before experimentation, regularity of oestrous rhythms of all the animals was verified. The animals in group 1 received reserpine at a dose level of 0.25 mg/kg/day by the subcutaneous route for seven consecutive days. The animals of group 2 simultaneously received both the subcutaneous injection of reserpine (0.25 mg/kg/day) and an intramuscular injection of ascorbic acid (1 g/kg/day) at different sites for the same period of experimentation. Animals in group 3 were taken as controls. All the rats were allowed to eat a balanced vitaminized diet and drink water *ad libitum*. Vaginal smears of all the animals were routinely recorded. At the termination of the experiment the animals were sacrificed. At autopsy, the adrenal glands and uteri were taken and compared gravimetrically. The ovaries were processed for histologic examination.

Results

Gravimetric data in the animals of groups 1 and 2 showed an increase in adrenal weight, as well as a decrease in uterine weight when compared to the controls of group 3 (table I). Histologic examination of the ovaries revealed persistent corpora lutea and follicular atresia in the animals of groups 1 and 2.

Table I. Organ weights of normal, reserpine-treated and reserpine with ascorbic acid-treated rats

Treatment	Body weight (g)		Adrenal weight		Uterine weight	
			Actual weight (mg)	Relative weight (mg/100 g)	Actual weight (mg)	Relative weight (mg/100 g)
Control (Group 3)	139.2 ± 5.05	(4)	28.3 ± 0.88	20.3 ± 0.36	204.3 ± 14.64	148.2 ± 7.36
Reserpine (Group 1)	117.5 ± 2.4	(7)	39.3 ± 0.4	33.5 ± 1.0	97.0 ± 5.1	86.1 ± 4.5
Reserpine with ascorbic acid (Group 2)	112.0 ± 3.6	(7)	37.8 ± 0.9	33.1 ± 1.7	83.3 ± 4.5	76.3 ± 4.1

Averages ± S. E. Number of animals between brackets.

Discussion

From the above experiment it is definitely observed that the induced physiological alteration of the adrenal glands and gonads of female reserpinized rats can not be normalized by a chronic administration of ascorbic acid in high doses. Several experimental documents are in favour of the concept that ascorbic acid is able to normalize the altered physiology of adrenal glands (CHATTERJEE [1964], CHATTERJEE *et al.* [1966]) and gonads (DEB *et al.* [1963], CHATTERJEE *et al.* [1965]) in some stressful situations. In his recent paper CHATTERJEE [1965 b] has shown that cortisone, which prevents an increased adenohypophyseal ACTH activity by "feed-back" mechanism, is able to counteract the inhibitory property of reserpine in the pituitary-gonadal axis. Ascorbic acid has been found to prevent, to some extent, the hyperactivity of the adenohypophyseal corticotrophs, but its exact mechanism of action is not yet clearly known. Therefore, in this instance, the mechanism of reserpine action in the inhibition of the pituitary-gonadal axis and stimulation of the pituitary-adrenal axis possibly differs from other stressful situations where ascorbic acid was found to have a definite value in counteracting the altered physiology of the pituitary-adrenal-gonadal axis.

Acknowledgements

Thanks are due to Mr. BIMAL SHANKAR PAUL for his devoted technical assistance. The encouraging co-operation of Principal K. C. GUPTA is greatly appreciated. The

0548

drug, reserpine (Serpasil, Ciba) and ascorbic acid (Celin, Glaxo) used in this experiment were kindly supplied by "Ciba of India Ltd." and "Glaxo Laboratories (India) Private Ltd., Bombay", respectively.

Summary

Ascorbic acid therapy, which was known to have a definite value in the physiology of the adrenals and gonads in stressful situation, failed to counteract the altered physiology of the pituitary-adrenal-gonadal axis in reserpinized female rats.

Résumé

On sait qu'un traitement par l'acide ascorbique exerce une action favorable sur les fonctions des surrénales et des gonades dans ces situations de «stress». Cette médication est sans effet sur les fonctions de l'axe hypophyse-surrénale-gonades de rattes soumises à l'action de la réserpine.

Zusammenfassung

Die Ascorbinsäuretherapie, die für die Nebennieren- und Gonadenfunktion in Streß-Situationen von einem bestimmten Wert ist, hatte keine Wirkung auf die veränderte Physiologie der Hypophysen-Nebennieren-Gonaden-Achse bei weiblichen Ratten, die unter Reserpinmedikation standen.

References

CHATTERJEE, A.: Prevention of adrenal cortical hypertrophy in diabetic rats by the use of ascorbic acid. Endokrinologie *47:* 19–23 (1964). – Effect of reserpine on adrenal cortical activity in rats. Endokrinologie *48:* 265–268 (1965a). – Role of cortisone in the prevention of gonadal inhibition in reserpinized rats. Endocrinology *77:* 395–398 (1965b).

CHATTERJEE, A. and BARDHAN, N.R.: The influence of ascorbic acid on the rat adrenal in stilbestrol induced stress. Naturwissenschaften *53:* 110–111 (1966).

CHATTERJEE, A. and DEB, C.: The role of ascorbic acid in the inhibition of estrous cycle in hypervitaminosis A. Anat. Anz. *116:* 6–10 (1965).

DEB, C. and CHATTERJEE, A.: Role of ascorbic acid in the inhibition of estrous cycle in alloxan diabetes. Endocrinology *72:* 159–160 (1963).

KHAZAN, N.; SULMAN, F.G. and WINNIK, H.Z.: Effect of reserpine on pituitary-gonadal axis. Proc. Soc. exp. Biol., N.Y. *105:* 201–204 (1960).

Received October 6th, 1966

Author's address: Dr. N. R. Bardhan and Prof. Dr. A. Chatterjee, Raja Peary Mohan College, *Uttarpara, Hooghly, West Bengal* (India)

Brit. J. Nutrition 13: 389-400, 1959.

0561

Some blood coagulation studies in normal and scorbutic guinea-pigs

By P. BARKHAN

Department of Medicine, University of Cambridge

and A. N. HOWARD

Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council

(*Received* 3 *January* 1959—*Revised* 9 *May* 1959)

Haemorrhage is a feature of severe ascorbic-acid deficiency in both primates and guinea-pigs. The scorbutic picture in man is usually accompanied by a deficiency of other vitamins and nutrients, and the haemorrhagic state may reflect more than a lack of ascorbic acid. There is much evidence to indicate a capillary abnormality in scurvy (Findlay, 1921; Wolbach & Howe, 1926; Wolbach & Bessey, 1942; Lee & Lee, 1947), and the haemorrhage is generally believed to result from this vascular alteration. Other evidence indicates some change in the blood-clotting mechanism. Sullivan, Gangstad & Link (1943) observed a prolonged one-stage prothrombin time with scorbutic guinea-pig plasma. This finding was confirmed by Marx & Bayerle (1943), who in addition found an increase in the fibrinogen concentration. Both abnormalities disappeared after the administration of ascorbic acid.

To our knowledge there is no information of possible changes at the thromboplastic level of blood coagulation. In guinea-pigs, which we have used in these experiments, it is possible to produce a pure ascorbic-acid deficiency under controlled conditions. We have therefore studied some aspects of the coagulation mechanism in scorbutic and normal guinea-pigs with a view to extending the observations of these earlier workers.

0561

METHODS

Animals and diet

Male guinea-pigs of about 250 g body-weight were obtained from Allington Farm, Ministry of Supply, Porton, Wilts, and fed initially on the semisynthetic diet no. 13 described by Reid & Briggs (1953). Vitamins A, D, E and K were not mixed in the diet, but were provided by two drops of halibut-liver oil, four drops of a solution of α-tocopheryl acetate (5 % (w/v) in arachis oil) and two drops of a solution of 2-methyl-1,4-naphthoquinone (0·25 % (w/v) in arachis oil) given orally to each animal once weekly. The diet contained 2 g ascorbic acid/kg and the normal intake of ascorbic acid was 50–70 mg/day.

Avitaminosis C

On reaching 300 g body-weight guinea-pigs were placed in one of two groups. A scorbutic group was fed on the diet just described with the ascorbic acid omitted; a normal control group was allowed to continue on the complete diet. On the scorbutogenic diet guinea-pigs grew normally for the first 10–12 days and then lost weight. Death usually occurred after 22–28 days when the animals reached a body-weight of 190 g. Animals for coagulation studies were killed at various stages of the deficiency but no moribund animals (less than 200 g body-weight) were used. The food intake dropped progressively after 10–12 days to about one-third of the intake of normal animals. It was considered, however, that no deficiency other than that of vitamin C developed during the experimental period.

Hypovitaminosis C

Another series of guinea-pigs was placed on the scorbutogenic diet, and after 10 days dosed with different levels of ascorbic acid for 3 months. The dose levels given were 0·4, 0·8 or 1·6 mg/day in 1 % (w/v) citric acid. Some animals received the equivalent dose only three times or twice a week. Guinea-pigs receiving 1·6 mg/day showed normal growth (mean 480 g/120 days); with 0·8 mg/day growth was retarded (mean 200 g/120 days); those receiving 0·4 mg/day did not grow but just maintained their body-weight.

Collection and treatment of blood

The chest and abdominal cavities were opened under deep ether anaesthesia. The supra-diaphragmatic portion of the inferior vena cava was exposed and blood aspirated from it with a no. 20 B.D. steel needle and an all-glass paraffined syringe. It was generally possible to obtain 5–10 ml blood in this way, although in some of the scorbutic animals, owing to their small body-weight, only less could be aspirated. One portion of the blood was citrated (for plasma) by transferring it to a siliconed glass tube containing a volume of a solution of trisodium citrate (38 g/l.) equal to one-tenth the volume of the blood and gently mixing the contents. Another portion was allowed to clot at 37° for several hours and the serum separated. Both plasma and serum were used fresh whenever possible, but many samples were stored at −20° for several days or weeks before testing.

Coagulation techniques

Platelet count. The direct method described previously (Barkhan, 1957) was used.

Clot retraction and lysis. A visual inspection was made after incubation of the clotted blood in glass tubes for 1–3 h at 37°. Lysis was determined by inspection of the clot for dissolution.

Plasma one-stage prothrombin time. (*a*) Method of Quick (1939). The thromboplastin was prepared from normal guinea-pig brains. (*b*) Stypven method. The method used was that described by Barkhan, Tomlin & Archer (1957). Russell's viper venom (Stypven preparation of Burroughs Wellcome Ltd) was used as thromboplastin, and guinea-pig brain lipid as venom cofactor.

Blood thromboplastin formation. It was measured by the method of Biggs & Douglas (1953) as modified by Bell & Alton (1954). For convenience, brain lipid of constant composition was used instead of platelet suspensions in all assays unless platelet activity was to be tested. The lipid material (platelet substitute) was prepared from normal guinea-pig brain. Reagents were diluted about 1 h before being tested. Since maximum thromboplastic activity was generated within 6 min in almost all instances, the test was usually not carried out beyond that time. The 1st min reading was not recorded as the amount of thromboplastin formed after 1 min was usually negligible.

Antiheparin activity. The test systems consisted of 0·1 ml normal platelet-poor plasma, 0·1 ml saline or platelet suspension (approximately $1{\cdot}5 \times 10^6/mm^3$), 0·1 ml saline or heparin (0·1 μg) and 0·1 ml thrombin (2 units of the material supplied by the Lister Institute for Preventive Medicine, London).

Terminology. The nomenclature used for the various coagulation factors and activities is that of Biggs & McFarlane (1957).

RESULTS

Except where specified, the results quoted for scorbutic blood refer to those from guinea-pigs fed on the diet free from ascorbic acid for 17–23 days. Adsorbed plasma refers to plasma adsorbed on aluminium hydroxide.

Clot reaction and lysis

Samples from the normal and the scorbutic animals appeared to retract equally well and the clots maintained their integrity at 37° for at least 5 h after formation, which excluded any gross defect in clot retraction and lysis in the blood of scorbutic guinea-pigs. On the assumption that there is no qualitative change in the platelets of scorbutic animals, their clot retraction might be expected to be even better than normal because of the increased platelet concentration (see below); this difference would not necessarily be evident from visual inspection.

Platelet count

A significant increase (50–80%) in the whole-blood platelet count was found in animals receiving no ascorbic acid, 0·4 mg/day, or 0·8 mg/day dosed twice weekly

(Table 1). In the group completely deprived of ascorbic acid there was an increase after only 10 days. Animals receiving 1·6 mg/day, or 0·8 mg/day dosed daily or thrice weekly did not differ significantly from controls receiving 60 mg/day.

Plasma one-stage prothrombin time

With brain thromboplastin. Plasma prothrombin times were lengthened by 25% in guinea-pigs kept on a diet free from ascorbic acid for 19–24 days (Table 1). No significant change was observed in animals deprived of ascorbic acid for 10 days or dosed with amounts higher than 0·4 mg/day.

Table 1. *Effect of ascorbic-acid intake on the whole-blood platelet count and plasma one-stage prothrombin times in guinea-pigs*

Mean ascorbic-acid intake (mg/day)	Frequency of of dosing	No. of days	No. of animals	Platelets† ($\times 10^{-3}$/mm^3)	Prothrombin time† (sec)	Percentage difference from normal animals: Platelets	Percentage difference from normal animals: Prothrombin time
60	Daily	19–24	8	491 ± 39	26·1 ± 0·6	—	—
1·6	Daily	100–120	8	476 ± 38	26·6 ± 0·6	− 3	+ 2
0·8	Daily	100–120	5	428 ± 28	24·2 ± 0·7	−13	− 7
0·8	Thrice weekly	100–120	3	559 ± 25	26·2 ± 0·5	+10	+ 4
0·8	Twice weekly	100–120	4	755 ± 47**	26·0 ± 0·7	+54	− 4
0·4	Daily	100–120	2	733 ± 119*	29·5 ± 0·5*	+49	+13
0	—	10	5	900 ± 177*	28·2 ± 0·4	+83	+ 8
0	—	19–24	8	870 ± 76***	32·7 ± 1·5**	+77	+25

Significance of difference from normal animals: * $0·05 > P > 0·01$; ** $0·01 > P > 0·001$; *** $P < 0·001$.

† Mean value with its standard error.

Table 2. *Effect of saline, and of serum and adsorbed plasma from normal guinea-pigs, on the plasma one-stage prothrombin times of scorbutic guinea-pigs*

Plasma sample no.	Prothrombin time (sec): Saline	Adsorbed plasma	Serum
1	32	36	28
2	34	38	30
3	33	32	27
4	35	35	27
5	44	48	32

A few samples of plasma from scorbutic guinea-pigs with rather prolonged prothrombin times were further examined for the effect of adding either serum or adsorbed plasma from normal guinea-pigs to the test system (Table 2). Only the addition of serum shortened the clotting times to within the normal range. When serum was added to plasma from normal animals, the plasma prothrombin time was usually unaltered, but with some specimens it was shortened by 1–2 sec.

With Stypven. Plasma from four scorbutic guinea-pigs showing lengthened plasma

0561

prothrombin times with brain thromboplastin was further examined with Stypven (Table 3). The clotting times were somewhat lengthened when no lipid cofactor was added. With lipid, however, the plasma from scorbutic animals was as active as that from normal ones.

Table 3. *Mean values with their standard errors for Stypven clotting times of plasma from normal and scorbutic guinea-pigs*

Source of plasma	No. of animals	Stypven clotting times (sec)	
		Saline	Lipid
Normal animals	3	12·7 ± 0·2	7·0 ± 0·3
Scorbutic animals	4	14·8 ± 0·4**	6·6 ± 0·4

** $0{\cdot}01 > P > 0{\cdot}001$.

Effect of ascorbic acid and vitamin K. To investigate whether ascorbic acid was involved *per se* in the plasma prothrombin times, ascorbic acid was added to plasma from scorbutic guinea-pigs to achieve a normal physiological concentration (0·01 mg/ml), and plasma was also taken from scorbutic guinea-pigs injected intramuscularly with ascorbic acid (0·5–1·0 g neutralized with $NaHCO_3$) 1 h before blood was taken. No shortening of the plasma prothrombin times was noted (Table 4), which indicates that ascorbic acid had no direct effect on prothrombin activity.

Table 4. *Plasma one-stage prothrombin times and whole-blood platelet counts of scorbutic guinea-pigs injected with ascorbic acid shortly before they were bled and killed or with vitamin K several days before*

Animal no.	Vitamin injected	Prothrombin time (sec)	Platelets ($\times 10^{-3}$/mm^3)
1	C	32	866
2		32	—
3		32	828
4	K	38	813
5		34	897
6		30	1880

As prolonged prothrombin times also occur in vitamin K deficiency, it was thought that the prolonged clotting times of plasma from scorbutic animals might be caused by a conditioned deficiency of vitamin K, which might occur if the intestinal absorption of vitamin K is impaired in scurvy. Vitamin K (100 μg sodium 2-methyl-1,4-naphthohydroquinone diphosphate in 0·1 ml saline) was therefore injected intramuscularly into scorbutic guinea-pigs for several days before they were bled and killed. The treatment had no effect on the plasma prothrombin times, which indicates that vitamin K was not involved (Table 4).

As was expected, the administration of ascorbic acid 1 h before death and of vitamin K for several days produced no change in the elevated whole-blood platelet count (Table 4).

Blood thromboplastin activity

Normal animals. Components from normal guinea-pig blood used for thromboplastin formation behaved like those from man and would substitute for human components. Human thromboplastin also reacted as well with guinea-pig plasma as with human plasma. Clotting times when guinea-pig thromboplastin was added to human plasma were, however, slightly longer (1–2 sec) than when it was added to guinea-pig plasma.

The thromboplastic activity of blood from normal guinea-pigs (animals receiving 60 mg ascorbic acid/day) is shown in Table 5. The mean minimum clotting time was 11·9 sec, reached after 3–4 min incubation. When a normal incubation mixture with minimum substrate clotting time of 12 sec was diluted 1:1, 1:3 or 1:4 with saline, the clotting times were 15, 19 and 25 sec respectively. In the absence of lipid or platelets, minimum clotting times were generally about 25 sec or more and were reached after longer periods of incubation (8–10 min).

Table 5. *Thromboplastic activity of serum and adsorbed plasma from normal and scorbutic guinea-pigs*

(Brain lipid was used as platelet substitute. In the statistical comparison of groups, the minimum substrate clotting times of groups 2–6 were compared with that of group 1)

Group no.	Mean ascorbic-acid intake (mg/day)	No. of days	No. of animals	Substrate clotting time (mean value and range) (sec) — Incubation time: 2 min	3 min	4 min	5 min	6 min	Minimum
1	60	19–24	29	18 (10·5–33)	14 (10·5–25)	12·5 (9·5–19·5)	13 (10–21)	13·5 (10·5–21)	11·9 (9·5–14·5)
2	1·6	100–120	4	19·5 (13–30)	14 (11–21)	12·5 (11–15·5)	12·5 (11·5–14·5)	—	11·9 (11–14)
3	0·8	100–120	12	26 (16–37)	18 (11–25)	14·5 (11–22)	15 (11–23)	15 (10·5–17·5)	12·8 (10·5–16·5)
4	0·4	100–120	2	20 (17·5–23)	18 (16–20)	16 (15–17)	16·5 (15·5–17·5)	18·5 (18–19)	16·0 (15–17)
5	0	10	5	†	†	†	†	†	15·8*** (14–18)
6	0	19–24	18	24 (12·5–45)	19 (12·5–37)	18 (11·5–37)	18 (12·5–37)	20 (13–37)	17·0*** (11·5–37)

*** $P < 0·001$. † Figures not available.

Animals with different levels of ascorbic-acid intake. As shown in Table 5, animals receiving 0·8 mg or more ascorbic acid daily had thromboplastic activities approximately the same as controls receiving 60 mg/day. Those receiving 0·4 mg/day or no ascorbic acid showed reduced thromboplastic activity, and a difference was seen after only 10 days of complete deficiency. Animals on a completely deficient diet for 19–24 days had a mean minimum clotting time of 17·0 sec compared with 11·9 sec for normal animals. If it is assumed that a clotting time of 11·9 sec represents 100% thromboplastin formation, 17 sec would represent 25–50% of normal. Although the difference was highly significant ($P < 0·001$) there was a wide scatter of values and about 50% of these scorbutic animals had values in the normal range. All had haemorrhages in the muscles adjacent to the knee joints (but not intra-articular), and some had blood in the caecal lumen. It was not possible therefore to correlate the extent of the haemorrhage and thromboplastic activity. But it should be noted that many of the

specimens were stored at −20° for variable periods and could have improved on storage as is known to occur in the plasma thromboplastin antecedent (PTA) syndrome (Rosenthal, Dreskin & Rosenthal, 1955).

Separate assay of plasma and serum from scorbutic guinea-pigs. To find whether the defect in thromboplastin formation was due to factors in the plasma or serum, plasma and serum from fourteen scorbutic animals were assayed with serum and adsorbed plasma from normal animals respectively. The results are shown in Table 6. Serum from normal and adsorbed plasma from scorbutic animals gave a system significantly more active ($0{\cdot}05 > P > 0{\cdot}01$) than when both were from scorbutic animals, but the mean minimum clotting time was still longer than normal. Plasma from normal animals added to serum from scorbutic ones gave a lower mean clotting time than when both were from scorbutic animals, but the difference was not significant ($P > 0{\cdot}05$). It is to be noted, however, that out of the fourteen systems examined eight showed a reduction in clotting time greater than or equal to 1 sec, the remainder being unchanged. It was therefore concluded that there was a deficiency of a factor or factors in both serum and adsorbed plasma.

Effect of ascorbic acid and vitamin K. Ascorbic acid in vitro (0·01 mg/ml test system), or given by injection 1 h before the animals were bled and killed or vitamin

Table 6. *Thromboplastic activity of serum and adsorbed plasma from scorbutic guinea-pigs assayed respectively with adsorbed plasma and serum from normal guinea-pigs*

(Brain lipid was used throughout as platelet substitute)

	Minimum substrate clotting times (sec)		
Animal no.	Group 1. Serum + adsorbed plasma from scorbutic animals	Group 2. Serum from scorbutic animals + adsorbed plasma from normal ones	Group 3. Adsorbed plasma from scorbutic animals + serum from normal ones
1	21	21	14·5
2	16	16	14
3	21	20	15
4	37	17	16·5
5	17	17	14
6	14	13·5	13·5
7	14·5	13	13·5
8	16·5	15	12
9	14·5	12	14
10	14	14	15
11	14	14	14·5
12	12	10·5	10·5
13	15	13	11·5
14	16	13	12
Mean	17·3	14·9	13·6

Group 4. Adsorbed plasma + serum from twenty-nine normal animals (see Table 5), mean value = 11·9.

Statistical comparison of groups

Groups compared	1 *v.* 2	1 *v.* 3	1 *v.* 4	2 *v.* 3	2 *v.* 4	3 *v.* 4
Significance rating	—	*	***	—	***	**

* $0{\cdot}05 > P > 0{\cdot}01$. ** $0{\cdot}01 > P > 0{\cdot}001$. *** $P < 0{\cdot}001$.

Table 7. *Effect of injection of ascorbic acid shortly before death or vitamin K for several days before death on the thromboplastic activity of serum and adsorbed plasma from scorbutic guinea-pigs*

Vitamin injected	No. of animals	Substrate clotting time (mean value and range) (sec)				
		Incubation time				
		2 min	3 min	4 min	5 min	Minimum
None	18	24 (12·5–45)	19 (12·5–37)	18 (11·5–37)	18 (12·5–37)	17 (11·5–37)
C	3	23·5 (15·5–40)	19·5 (14·5–26)	17 (15·5–19·5)	17·5 (16·0–19·5)	16 (14·5–16·5)
K	2	34 (30–38)	23 (23)	19·5 (22–24)	19·5 (18–21)	19·5 (18–21)

Table 8. *Effect of ascorbic acid in vitro on the thromboplastic activity of serum and adsorbed plasma from scorbutic guinea-pigs*

Animal no.	Addition to test system	Substrate clotting time (sec) Incubation time			
		2 min	3 min	4 min	5 min
1	Saline	34	24	16	16
	Ascorbic acid	34	20	15	16
2	Saline	33	26	29	29
	Ascorbic acid	40	26	28	35
3	Saline	25	24	22	24
	Ascorbic acid	46	26	22	22
4	Saline	29	22	25	27
	Ascorbic acid	31	24	22	29

K injected for several days before (as described in the section on prothrombin times), produced no improvement in the thromboplastic activity of adsorbed plasma and serum from scorbutic animals (Tables 7 and 8).

Thromboplastic activity of platelets from normal and scorbutic animals

Serum and adsorbed plasma from five scorbutic animals, showing clearly subnormal thromboplastic activities with brain lipid, were further tested by replacing it with platelets from normal or scorbutic animals (Table 9). Somewhat greater activity was found in the platelet-containing systems, although total activity was still relatively low. In this respect platelets from scorbutic animals did not differ from those from normal ones.

On several occasions the reaction between platelets from normal and scorbutic animals and serum and adsorbed plasma from normal animals was studied, the same serum and adsorbed plasma being used for each comparison. The results (Table 10) showed that platelets from scorbutic animals were as active as those from normal ones.

The antiheparin activity of pooled platelets from normal animals and from scorbutic ones was examined as shown in Table 11. No differences were observed between those from scorbutic and normal animals.

0561

Table 9. *Comparison of thromboplastic activities of serum and adsorbed plasma from scorbutic guinea-pigs assayed with brain lipid or with platelets from scorbutic or normal guinea-pigs*

(Five animals/group. Concentration of platelets in the incubation mixture approximately 350000/ mm³)

Addition to serum and adsorbed plasma from scorbutic animals	Substrate clotting time (mean value and range) (sec)				
	Incubation time				
	2 min	3 min	4 min	5 min	Minimum
Brain lipid	36 (31–44)	29 (20–32)	24 (17–38)	25 (17–37)	23 (20–37)
Platelets from scorbutic animals	24 (19–35)	20 (19–21)	23 (16–30)	23 (15–28)	19 (15–20)
Platelets from normal animals	30 (25–34)	19 (17–21)	21 (14–25)	23 (14–30)	18 (14–19)

Table 10. *Comparison of thromboplastic activity of platelets from normal and from scorbutic guinea-pigs with serum and adsorbed plasma from normal guinea-pigs*

Addition to test system	No. of animals	Substrate clotting time (mean value and range) (sec)				
		Incubation time				
		2 min	3 min	4 min	5 min	Minimum
Platelets from normal animals	4	14 (9·5–21)	10·5 (10·5–11)	10·5 (10–11)	11 (9·5–12)	10 (9·5–11)
Platelets from scorbutic animals	3	13 (9–15)	9 (8·5–10)	10 (9·5–11)	11 (9·5–11)	9 (8·5–9·5)
Saline	—	47	41	34	35	29

Table 11. *Antiheparin activity of platelets from normal and scorbutic guinea-pigs*

Addition to test system (ml)				
Platelets* from normal animals	Platelets* from scorbutic animals	Saline	Heparin†	Clotting time (sec)
—	—	0·2	—	13·5
—	—	0·1	0·1	19
0·1	—	—	0·1	13·3
—	0·1	—	0·1	13·3

* *c.* $1·5 \times 10^6$/mm³. † 1 μg/ml.

DISCUSSION

Most of the current knowledge of mammalian blood coagulation has been derived from observations on human blood. In the interpretation of our results we assume that there is a similarity between the guinea-pig and the human test systems. We are not aware of any definitive comparative study, but the reactions of the blood from normal guinea-pigs in the tests are sufficiently similar to those of human blood to warrant tentative interpretation in terms of human blood coagulation theory, at least on a

qualitative basis. If we are only concerned with finding out whether there is a difference between normal and scorbutic guinea-pigs, the validity of the foregoing assumption is irrelevant since the reference standard for the scorbutic animal is the normal animal. The validity of the assumption is relevant, however, when we attempt to interpret any difference that is found; here reference has to be made to a blood-coagulation theory based mainly on human blood.

Our investigation indicated some depression of the coagulation reaction of blood from scorbutic guinea-pigs. It was most clearly seen in the group completely deprived of vitamin C and was evident at the prothrombic and thromboplastic levels. The prolonged plasma one-stage prothrombin times were corrected by serum, but not by adsorbed plasma, from normal animals. The defect does not, therefore, involve factor V which is present in adsorbed plasma, or prothrombin which is absent from normal serum.

Factor X (Koller, 1955), which may also be identical with the Stuart or Prower factor (Denson, 1958), was not involved as the Stypven time was normal; factor X deficiency is associated with a prolonged Stypven time (Biggs, 1956; Dische, 1958). A deficiency of factor VII (Koller, Loeliger & Duckert, 1951) appears to be the most likely explanation since this factor is present in serum, but not in adsorbed plasma, from normal animals.

The blood thromboplastin defect cannot be explained by a factor VII deficiency, however, since factor VII does not take part in thromboplastin formation (Ackroyd, 1956). It is therefore necessary to postulate the deficiency of another factor. Both the serum and adsorbed plasma showed decreased thromboplastic activity. It could be caused by a deficiency of plasma thromboplastin antecedent (Rosenthal *et al.* 1955) or a combined deficiency of antihaemophilic globulin and Christmas factor. It is clear that further work is necessary before the defect can be fully characterized. In contrast to the prothrombic defect, lowered thromboplastic activity was found in only half the scorbutic animals. Interpretation of this fact should be cautious, however, as most specimens were stored for varying periods, and it is well known that samples from patients with PTA syndrome can revert to normal on storage (Rosenthal *et al.* 1955; Biggs, Sharp, Margolis, Hardisty, Stewart & Davidson, 1958).

Ascorbic acid *per se* played no part in either the prothrombic or thromboplastic defect since, when added in vitro or injected shortly before the animals were bled and killed, it caused no improvement. Likewise, vitamin K was not directly involved since adequate amounts were provided in the diet and supplementary injections of the vitamin had no effect.

All scorbutic animals examined had haemorrhages in the muscles adjacent to the knees and some also in the viscera. But it has not so far been established whether they were causally related to the coagulation defects. Although the plasma prothrombin times were prolonged, such an increase (25 %) by itself would not be expected to produce haemorrhage. Likewise, the thromboplastic defect was relatively mild. Although haemorrhage by itself can cause a depression of coagulation factors (Chalmers, Bigelow & Desforges, 1954) this depression occurs only when blood loss is large. It would seem an unlikely explanation, as gross haemorrhage was not observed.

No definite explanation can be given for the consistent increase in platelets. Fibrinogen is also known to be raised (Marx & Bayerle, 1943; Howard, 1958), and the two effects may be a secondary response to tissue damage and chronic haemorrhage. It is noteworthy that human platelets are rich in ascorbic acid (Barkhan & Howard, 1958). As yet, there is no evidence that this high concentration is connected with any haemostatic function. In the present work we have found both the clot-retracting and thromboplastic activities of platelets from scorbutic animals to be normal. It is tempting to speculate that the petechial haemorrhages characteristic of thrombocytopaenic purpura and also of scurvy in man are in some way related. The defect in scurvy has been attributed primarily to vascular alteration (Wolbach & Bessey, 1942), but platelets may play a more important part in vascular integrity than is at present realized. The nature of the interaction between blood-clotting factors and the vascular wall is a fundamental aspect of haemostasis which is only beginning to be understood (Roos, 1957).

SUMMARY

1. Some aspects of the mechanism of blood coagulation were investigated in thirty normal and twenty-five scorbutic guinea-pigs.

2. In scorbutic animals, there was an increase in the platelet concentration (50–80%) and in plasma one-stage prothrombin times (25%). The blood thromboplastic activity was also impaired. Stypven times and the clot retraction and thromboplastic activities of platelets were normal.

3. The prothrombic and thromboplastic defects were found in animals that received no ascorbic acid for 10–23 days or 0·4 mg/day for 100 days, but not in those receiving 0·8 mg/day or more.

4. The defects appeared to be multifactorial but their exact nature was not established. Correction experiments suggested a deficiency of factor VII as well as of a thromboplastic factor or factors of serum and adsorbed plasma.

5. Ascorbic acid injected shortly before death, vitamin K for several days before death and ascorbic acid in vitro produced no correction.

6. Small haemorrhages, consistently in muscles around the knees (but not intra-articular) and sometimes visceral, were present in all the scorbutic animals. It was not established whether they were causally related to the coagulation defects.

Miss Sheila Tomlin and Mr K. Day gave valuable technical assistance. This work was done during the tenure by P. B. of an Elmore Research Studentship of the University of Cambridge.

REFERENCES

Ackroyd, J. F. (1956). *Brit. J. Haemat.* **2**, 397.
Barkhan, P. (1957). *J. clin. Path.* **10**, 26.
Barkhan, P., Tomlin, S. C. & Archer, R. K. (1957). *J. comp. Path.* **67**, 358.
Barkhan, P. & Howard, A. N. (1958). *Biochem. J.* **70**, 163.
Bell, W. N. & Alton, H. G. (1954). *Nature, Lond.*, **174**, 880.
Biggs, R. (1956). *Brit. J. Haemat.* **2**, 412.
Biggs, R. & Douglas, A. S. (1953). *J. clin. Path.* **6**, 23.
Biggs, R. & MacFarlane, R. G. (1957). *Human Blood Coagulation and its Disorders*, 2nd ed. Oxford: Blackwell Scientific Publications.
Biggs, R., Sharp, A. A., Margolis, J., Hardisty, P. M., Stewart, J. & Davidson, W. M. (1958). *Brit. J. Haemat.* **4**, 177.
Chalmers, T. C., Bigelow, F. S. & Desforges, J. F. (1954). *J. Lab. clin. Med.* **43**, 511.
Denson, K. W. (1958). *Brit. J. Haemat.* **4**, 313.
Dische, F. E. (1958). *Brit. J. Haemat.* **4**, 201.
Findlay, G. M. (1921). *J. Path. Bact.* **24**, 446.
Howard, A. N. (1958). *Brit. J. Nutr.* **12**, 346.
Koller, F. (1955). *Rev. Hemat.* **10**, 362.
Koller, F., Loeliger, A. & Duckert, F. (1951). *Acta haemat.* **6**, 1.
Lee, R. E. & Lee, N. Z. (1947). *Amer. J. Physiol.* **149**, 465.
Marx, R. & Bayerle, H. (1943). *Biochem. Z.* **315**, 366.
Quick, A. J. (1939). *Proc. Soc. exp. Biol., N.Y.*, **42**, 788.
Reid, M. E. & Briggs, G. M. (1953). *J. Nutr.* **51**, 341.
Roos, J. (1957). *Thrombosis et Diathesis Haemorrhagica*, **1**, 471.
Rosenthal, R. L., Dreksin, H. & Rosenthal, N. (1955). *Blood*, **10**, 120.
Sullivan, W. R., Gangstad, E. O. & Link, K. P. (1943). *J. biol. Chem.* **151**, 477.
Wolbach, S. B. & Bessey, O. A. (1942). *Physiol. Rev.* **22**, 233.
Wolbach, S. B. & Howe, P. R. (1926). *Arch. Path.* **1**, 1.

J. Sci. Food Agr. 22 (4): 173-175

058

Determination of Cysteine Hydrochloride, Ascorbic Acid and Potassium Bromate in Admixture in Bread Improver Formulations

by Stella Barrett, A. G. Croft and A. W. Hartley

Spillers Research and Technology Centre, Station Road, Cambridge

(*Revised manuscript received 7 October, 1970*)

A method of selective extraction is proposed for the determination of cysteine, ascorbic acid and potassium bromate when present in admixture. Other ingredients and various diluent materials that might be encountered in commercial bread improvers have been shown to cause negligible interference.

Suitable titrimetric methods are recommended for each additive after the extraction procedure has been carried out.

Introduction

In the production of bread, the lengthy fermentation stage can be replaced by a mechanical dough development at the mixing stage. For optimum results, ascorbic acid or ascorbic acid plus potassium bromate are used as oxidants. The process, known as the Chorleywood Bread Process,[1,2] has been described in numerous published reports. An alternative process which does not require high speed mixers is the Activated Dough Development Process (A.D.D.),[3,4] in which the physical changes in the dough are brought about by the addition of a reducing agent as well as ascorbic acid and potassium bromate. The reducing agent used in the U.S.A. and Eire for this purpose is L-cysteine hydrochloride, but the use of this additive in bread production is not, at present, permitted in the U.K.

The improving effect of L-cysteine hydrochloride is achieved by levels within the range 10–80 ppm in the bread, and formulations have been proposed in which various levels of ascorbic acid and potassium bromate are used together with L-cysteine hydrochloride. For convenience of addition of these improvers in the bakery, it is usual to blend the additives with yeast nutrients and fat in a pre-mix with a cereal, soya flour or milk powder base, which may be called a bread improver. In such a bread improver, intended to be used at the rate of 2 or 3 lb/280 lb of flour (355–535 g/50 kg), the oxidants and reductants mentioned may be present at levels between 0·1 and 1·0%.

In anticipation of a possible change in the bread regulations permitting the use of L-cysteine, it was necessary to develop an analytical method for the determination of L-cysteine, ascorbic acid and potassium bromate in admixture, and also in the presence of cereals and other ingredients.

Experimental

Method for L-cysteine

Several methods were considered for the determination of L-cysteine. These included: (a) oxidation to cystine or cysteic acid,[5] (b) reaction with silver or mercuric ions,[6] (c) alkylation of the thiol group with iodoacetamide or *N*-ethylmaleimide,[7] and (d) reaction of the thiol group with an organomercurial compound.[8] The last method was chosen as being suitably specific and convenient for routine use. The reaction can be followed by spectrophotometry or by an amperometric technique, but a method based on the work of Wronski,[9] using titrimetry, was adopted because of its simplicity. A solution of *o*-hydroxymercuribenzoic acid is used as titrant with thiofluorescein as indicator. This indicator is deep blue in alkaline solution. The *o*-hydroxymercuribenzoic acid reacts preferentially with the thiol group of cysteine, the end-point being reached when excess of reagent decolorises the indicator.

Separation of cysteine hydrochloride from potassium bromate

If an aqueous extract is made of a complex bread improver there is oxidative loss of cysteine and ascorbic acid by reaction with potassium bromate in solution. There may also be some loss caused by the reaction of cysteine with proteins present in the base material. The solubilities of the three active ingredients were tested in several anhydrous solvents including methanol, ethanol, isopropanol and acetone. Methanol was chosen as a selective solvent for cysteine and ascorbic acid, leaving the potassium bromate largely undissolved. When dry nitrogen was passed through the methanol to de-aerate it, improved recoveries of cysteine and particularly of ascorbic acid were obtained. The proposed method of separation is shown in Fig. 1. Ascorbic acid is determined by titration with 2,6-dichlorophenol indophenol[10] on a portion of the methanol extract, and potassium bromate is determined in the residue by titration of the iodine liberated from acidified potassium iodide.[11]

Negligible amounts of interfering substances accompany the ascorbic acid and cysteine hydrochloride in the methanolic filtrate. Zinc sulphate and sodium hydroxide are used to clarify the aqueous extract of the residue before titration of the bromate.

Analytical procedure

The whole operation, including the titrations, should be carried out as rapidly as possible without unnecessary delays and away from direct sunlight. It is advisable to use amber glassware where possible. The procedure recommended is as follows.

Extraction of the improver

Reagent

Add 10 g of anhydrous sodium sulphate to each litre of A.R. methanol and de-aerate immediately before use by bubbling dry nitrogen through for 15 min (<0·5% water).

Method

Weigh 2·50 (±0·02) g sample, or sufficient to contain between 2·5 and 15 mg of the active ingredients, and transfer to a 50 ml beaker. Add 20 ml of dry methanol and mix thoroughly with a glass rod for about 30 sec. Transfer to a 12·5 cm No. 1 Whatman filter paper, previously wetted with methanol. Collect the filtrate in a 50 ml volumetric flask. Wash the beaker with 3 × 10 ml portions of dry methanol and add these washings to the filtration. When filtration is complete retain the filter paper and contents and the beaker for the bromate analysis. Make the filtrate up to 50 ml with methanol.

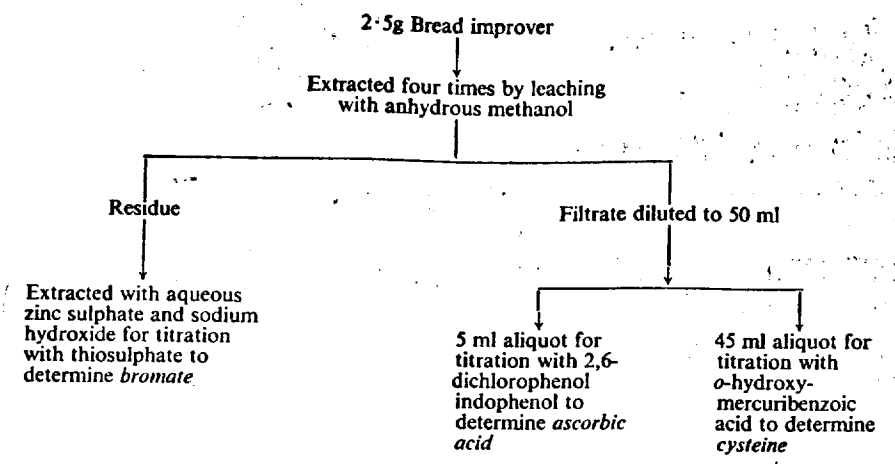


FIG. 1. *Separation scheme for cysteine hydrochloride, ascorbic acid and potassium bromate in bread improver*

Ascorbic acid analysis

Reagents

Metaphosphoric acid reagent.—Add 15 g metaphosphoric acid and 40 ml of glacial acetic acid to 800 ml of cold distilled water, and leave to dissolve overnight. Make up to 1 litre with distilled water, filter and store in the refrigerator. Prepare this reagent freshly each week.

Indophenol reagent.—Dissolve 50 mg of 2,6-dichlorophenol indophenol in distilled water containing 42 mg of sodium bicarbonate and make up to 500 ml with distilled water. Keep in a cool, dark place and observe the precautions noted in the A.O.A.C. method for the determination of ascorbic acid with this reagent.[10]

Standard ascorbic acid reagent.—Dissolve 50 mg of ascorbic acid and make up to 250 ml with distilled water.

Method

Take 5 ml of the standard ascorbic acid solution and 5 ml of the sample filtrate in separate flasks. Add 20 ml of the metaphosphoric acid reagent to each. Titrate each solution with indophenol reagent to a pink colour that lasts for at least 10 sec after shaking. Carry out a reagent blank by titrating 20 ml of metaphosphoric acid reagent with indophenol in the same way.

$$\% \text{ ascorbic acid in sample} = 0{\cdot}4 \times \frac{(A_2 - A_3)}{(A_1 - A_3)}$$

where A_1, A_2 and A_3 are titres for standard solution, sample extract and reagent blank, respectively.

Cysteine hydrochloride analysis

Reagents

0·5 N Ammonium hydroxide.—Dilute 23 ml of 0·880 ammonia to 1 litre with distilled water.

0·002 N *o*-Hydroxymercuribenzoic acid.—Dissolve 0·641 g of the reagent in 50 ml 0·1 N sodium hydroxide and make up to 1 litre with distilled water.

It should be noted that *o*-hydroxymercuribenzoic acid is a Schedule I poison.

0·5% Thiofluorescein (obtainable from Eastman Kodak Ltd.) in acetone.—Prepare 10 ml at a time and store in amber glass in a refrigerator. The indicator is stable for at least one month.

Standard cysteine hydrochloride.—Dissolve 50 mg of L-cysteine hydrochloride monohydrate in distilled water and dilute to 50 ml in a volumetric flask.

Method

Take 10 ml of the standard cysteine solution in one flask and add 20 ml of water and 45 ml of methanol. Transfer the remaining 45 ml of sample extract to a second flask, washing the volumetric flask with 30 ml of water and adding this to the sample extract. To both solutions add two drops of thiofluorescein indicator followed by 2 ml of 0·5 N ammonium hydroxide. Titrate immediately and rapidly with the *o*-hydroxymercuribenzoic acid solution to the disappearance of the blue colour. Also carry out a reagent blank titration with 30 ml of distilled water, 45 ml of methanol, 2·0 ml 0·5 N ammonium hydroxide and two drops of thiofluorescein in the same way.

$$\% \text{ L-cysteine hydrochloride monohydrate in sample} = 0{\cdot}445 \times \frac{(C_2 - C_3)}{(C_1 - C_3)}$$

where C_1, C_2 and C_3 are titres for standard solution, sample extract and reagent blank, respectively.

Potassium bromate analysis

Reagents

5 N Sulphuric acid
Potassium iodide
Starch indicator

0·01 N Sodium thiosulphate.—Dissolve 2·482 g of sodium thiosulphate pentahydrate in distilled water and make up to 1 litre.

0·18 N Zinc sulphate solution.—Dissolve 51·7 g of zinc sulphate heptahydrate in distilled water and dilute to 1 litre.

Hyflo Supercel diatomaceous filter-aid.

Standard potassium bromate.—Dissolve 50 mg of potassium bromate (A.R.) in water and dilute to 50 ml in a volumetric flask.

Method

Transfer the filter paper and residue from the sample extraction to a 250 ml beaker and add the washing from the 50 ml extraction beaker, using 10 ml of water. Add 10 ml of zinc sulphate solution, 10 ml of 0·18 N sodium hydroxide solution and 2 g of Hyflo Supercel. Mix the contents of the beaker, breaking up the filter paper with a glass rod. Set aside for 10 min before continuing with the analysis. Filter the mixture through a No. 40 Whatman filter paper on a Buchner funnel.

TABLE I

Recoveries of ascorbic acid, cysteine hydrochloride and potassium bromate from various mixtures

Bread improver base[a]	Ascorbic acid			Cysteine hydrochloride			Potassium bromate		
	Added, mg	Found, mg	Recovery, %	Added, mg	Found, mg	Recovery, %	Added, mg	Found, mg	Recovery, %
Soya flour	5·75	5·20	90·4	4·86	4·15	85·5	6·95	6·38	91·7
Soya flour	6·29	5·70	90·6	9·67	8·82	91·3	9·62	9·26	96·2
Soya flour	9·77	9·10	93·1	6·93	6·17	89·1	5·61	5·25	93·7
Wheat flour	4·01	4·00	99·8	7·03	6·42	91·3	4·53	4·41	97·4
Wheat flour	8·32	8·09	97·2	5·62	5·44	96·7	9·12	8·68	95·1
Milk powder	6·11	5·76	94·3	8·18	6·91	84·4	9·80	9·43	96·2
Milk powder	9·01	8·88	98·3	4·94	4·19	84·9	8·30	8·14	98·0
Sucrose	5·46	5·36	98·1	5·41	4·81	89·0	4·24	3·99	94·1
Soya:wheat:fat[b]	5·66	4·93	87·2	7·08	6·79	96·0	8·02	7·73	96·4
Soya:wheat:fat[b]	6·20	6·07	97·8	6·28	6·02	95·8	7·38	7·11	96·4
Soya+minerals[c]	5·29	4·83	91·3	8·24	7·57	91·9	5·96	5·41	90·9
Soya:wheat:fat +minerals[c]	9·28	8·56	92·2	4·39	4·12	93·7	4·24	4·05	95·4
Range of recoveries, %		87·2—99·8			84·4—96·7			90·9—98·0	
Average recovery, %		94·2			90·8			95·1	
Standard deviation		4·0			4·3			2·2	

[a] The weighed ingredients were added to 2·5g of the bread improver base shown and analysed immediately after mixing
[b] A mixture of enzyme-active soya flour, wheat flour and fat in the ratio 4:5:1
[c] Minerals added to soya flour and to the soya:wheat:fat mixture[b] at the rate 0·05g ammonium chloride and 0·35g calcium sulphate to 2g of the bread improver base

washing the beaker with 2 × 15 ml of distilled water and adding these to the filtration. Retain the filtrate.

To a second flask add 10 ml of standard potassium bromate solution and 50 ml of distilled water. To both flasks add 2 ml of 5N sulphuric acid, 1 g of potassium iodide, and starch indicator. Titrate with the thiosulphate solution to the disappearance of the starch/iodine colour. Determine a reagent blank by titrating 60 ml of water together with 2 ml of sulphuric acid, 1 g of potassium iodide, and starch indicator.

$$\% \text{ potassium bromate in sample} = 0{\cdot}4 \times \frac{(B_2 - B_3)}{(B_1 - B_3)}$$

where B_1, B_2 and B_3 are titres for standard solution, sample extract and reagent blank, respectively.

Results

All three determinations gave sharp end-points and reproducible titrations when the precautions noted above were observed.

The technique was checked with varying amounts of the three additives in the presence of other ingredients that might be expected to be present in commercially available bread improvers. When cereals, soya flour or milk powder were present, some methanol-soluble material was carried through to yield a turbid solution on the addition of water, but this did not interfere either with the cysteine or with the ascorbic acid titrations. The results of analyses of typical mixtures are given in Table I.

Discussion

For most determinations, recoveries better than 90% were obtained. There does not appear to be any indication of serious interference by one ingredient in the determination of another. Recoveries of ascorbic acid and cysteine are equally good in the presence of high or low levels of potassium bromate. Failure to achieve recoveries better than 98% is probably due to a combination of minor errors caused by the incomplete selectivity of the solvent, atmospheric oxidation and the effect of light.

The methods outlined are suitable for the determination of ascorbic acid, cysteine hydrochloride and potassium bromate when present together in bread improvers. The precision and accuracy are considered to be satisfactory for commercial purposes.

References

1. Chamberlain, N., Collins, T. H., & Elton, G. A. H., *Br. Bak. Ind. Res. Ass. Rep.*, 1961, No. 59, and 1962, No. 62
2. Collins. T. H., *Br. Bak. Ind. Res. Ass. Rep.*, 1964, No. 74
3. Henika, R. G., & Zenner, S. F., *Baker's Dig.*, 1960, 34 (3), 36
4. Axford, D. W. E., & Elton, G. A. H., *Chemy Ind.*, 1960, p. 1257
5. Sarkar, B. C. R., & Sivaraman, R., *Analyst, Lond.*, 1956, **81**, 668
6. Benesch, R. E., Lardy, H. A., & Benesch, R., *J. biol. Chem.*, 1955, **216**, 663
7. Alexander, N. M., *Analyt. Chem.*, 1958, **30**, 1292
8. Hamilton, L. D. G., 'Analytical Methods of Protein Chemistry', (Eds. Alexander, P., & Block, R. J.), Vol. II (London & New York: Pergamon Press Ltd.)
9. Wronski, M., *Analyst, Lond.*, 1963, **88**, 562
10. 'Official Methods of Analysis', 1965, (10th Edn), Para. 39. 040 (Washington: A.O.A.C.)
11. Howe, M., *Cereal Chem.*, 1951, **28**, 132

Lancet I, 1102-5, 1920

059

SCURVY:

WITH SPECIAL REFERENCE TO PROPHYLAXIS IN THE ROYAL NAVY.[1]

BY P. W. BASSETT-SMITH, C.B., C.M.G., F.R.C.P.,
SURGEON-CAPTAIN, R.N.

THE subject of scurvy is of particular interest to all who have to maintain the health and well-being of our sailors.

Past History.

In the days of the old sailing ships, with voyages of extended duration, the danger of scurvy was much greater than it is now; little was known of the ætiology of the disease and catering difficulties were much more marked. Dr. Lind and Sir Gilbert Blane, the pioneers of naval hygiene, were both very active in studying and in endeavouring to eradicate this scourge of the navy. Fresh fruit and vegetables, as prophylactic and curative agents for scurvy, had been known to the Dutch as far back as 1564, and these had been successfully employed in the fleet by Sir Robert Hawkins in 1600, but the use of orange and lemon juice had been more or less forgotten until Dr. Lind insisted on their value. He, however, recommended *preserved* juice for use on long voyages. The fresh fruit juice was concentrated by heat to a thick syrup: as this was done in glazed earthenware vessels soluble lead salts were formed, and Lind drew attention to this danger, which he referred to as "death in the pot." Trotter strongly opposed the use of this form of preserved juice, as it was said to undergo fermentation and become mouldy. Unfortunately its issue to the crews in Cook's famous voyage of 1773-1774 did not prevent scurvy breaking out.

It was Gilbert Blane's teaching and powerful influence which brought Lind's recommendation into official use. Sir Gilbert Blane in 1757 pointed out the importance of cleanliness, sufficient ventilation, and absence of damp in ships, and the need of a supply of lemon juice for the prevention of scurvy. It was, however, not until 1796, when he was one of the Commissioners of the Navy Board, that the use of lemon juice was ordered as an essential part of the naval dietary, and this quickly resulted in the practical disappearance of scurvy from the Service afloat. In 1782 two-thirds of an ounce of lemon juice per man per diem protected the whole ship's company of H.M.S. *Suffolk* during a voyage of 23 weeks without touching port. In a discussion on the probable causes of the disease, which he attributed to the lack of a "certain element which is necessary for growth and repair," Blane foreshadowed the present deficiency theory.

From that time to the present lime juice has been a standing ration, but its efficacy has disappeared.

The reason for this has been ably shown by Mrs. Alice Henderson Smith. As originally prepared the lime juice was made from sweet limes, *Citrus medica*, with lemons, imported chiefly from Spain. In 1793 war stopped these supplies, but in 1802 delivery was resumed, and scurvy, which had obtained a temporary hold, was again almost eliminated. About 1860, by the development of the cultivation of limes in the West Indies, a large quantity was made available, and the contracts entered for the navy caused these, the sour lime, *Citrus medica var acida*, to supersede the sweet limes and lemons formerly in use, and for a time the new lime juice was believed to be better than the old. In Ross's polar expedition the original lemon juice had been issued, but Sir George Nares, in 1875, was provided with the new West Indian lime juice. The general conditions of the two expeditions were very similar, but while the former escaped scurvy, Sir George Nares's men suffered severely from it, in spite of the regular issue of the lime juice, thus demonstrating the failure of the new preparation as a prophylactic. The sister ship in the latter expedition, the *Investigator*, was supplied with lemon juice, and for 27 months after leaving home she had few cases, though the crew suffered great hardships. In three and a half years she had only three deaths from scurvy, whereas Nares had the same number in one year.

The evidence is clear that lemon juice is far more effective than lime juice in preventing scurvy, and this has been fully confirmed by the large number of laboratory experiments carried out at the Lister Institute, London, and elsewhere, which have determined exactly their relative values for the purpose. Lemon juice and sweet limes have therefore been found to repair a deficiency which, if not rectified, induces scurvy, and the consumption of these juices is curative for the same disease. This is in accordance with modern teaching, which attributes the condition to a deficiency of a certain accessory factor known as scurvy vitamine. This factor is found in living vegetable (and secondarily in animal) tissue; in largest amount in fresh fruit, green vegetables, and growing pulses, and, to a less extent, in roots and tubers. Young vegetables contain more than old. It is present in small amount in fresh meat and milk. It has not been found in yeast, fats, dry cereals, and pulses, but exists to a certain extent in canned tomatoes. The antiscurvy food factor is sensitive to high temperatures, and if exposed to them it rapidly loses its efficacy; the presence of alkali is also detrimental.

Recent History.

Much has been written of the prevalence of scurvy during and since the war among the combatant forces and in the civil populations chiefly affected in war areas. Of the former, those in Mesopotamia suffered very severely, and Colonel W. H. Willcox, in his excellent communications, has described the reasons for the outbreak very fully.

The high incidence amongst the native troops at Kut, in comparison with that of the Europeans, is explained on dietary grounds; the latter had fresh meat to supplement their meagre diet, and the former were more susceptible owing to diminished vitality from preventable causes. He states that the knowledge we then possessed for the prevention of scurvy and beri-beri was not fully appreciated and acted upon by those in authority—i.e., that the food must have the necessary accessory factors, as well as sufficient calories as estimated by the protein, carbohydrates, and fat. The fact that over 11,000 cases of scurvy occurred in the last six months of 1916 indicates the importance of the subject. The following table shows the amount of scurvy in the Indian troops and beri-beri in the British.

Year.	Scurvy (Indian).	Beri-beri (British).
1916	11,445	104
1917	2,197	84
1918	825	51

The crews of British men-of-war were also affected to a small extent, and as has been shown repeatedly by the endemic presence in the Persian Gulf of ship beri-beri, this is a question of some importance, more particularly in times of extra strain, as was the case in these years. Much has been done to prevent these outbreaks by increasing and supplementing the diet, but climatic conditions in that area always make the provision of efficient food-supplies difficult.

In other war areas, Serbia and the remainder of the Balkans, famine conditions have necessarily given rise to an enormous number of scurvy cases. In Northern Russia it is very prevalent each winter, particularly in the prisons. Even on the Western Front the cases of incipient scurvy were very numerous, due in great part to the use of tinned provisions. By Japanese investigators the conditions produced in guinea-pigs on a deficiency diet is considered to be similar to that found in ship beri-beri, a disease which undoubtedly links up human scurvy and beri.

The post-war hardships in the civil populations have also induced scurvy, and the gallant fight being made against this and other diseases by individuals of all classes and peoples throws a glimmer of sunshine on what is otherwise a dark and tempestuous horizon. The work in Vienna of Dr. Harriette Chick and others to ameliorate the sufferings of the infantile population is particularly noteworthy. From a scientific point of view such an overwhelming mass of cases has increased our knowledge of the clinical features of scurvy enormously, and many new facts have been ascertained.

Relationship of Scurvy to other Deficiency Diseases.

The ætiology, thanks to the work of British, American, French, and other observers, has now been placed on a sure foundation, following the elaborate investigations that have for years been carried out in establishing the ætiology of beri-beri as a deficiency disease. The close relationship of beri-beri, ship beri-beri, scurvy, and rickets was well shown in a diagram (Fig. 1) by Darling in 1915, which I here reproduce; it shows the gradation from polyneuritis avium at one end to that of rickets at the other. (Table I.)

[1] A paper read before the War Section of the Royal Society of Medicine on May 10th, 1920.

He recognised several varieties of beri-beri—infantile beri-beri, asylum beri-beri, wet-and-dry beri-beri (which Vedder and Clark look upon as distinct), the Brazilian type, endemic dropsy, and endemic peripheral neuritis. Darling, with Surgeon-General Gorgas, spent much time in investi-

FIG. 1.

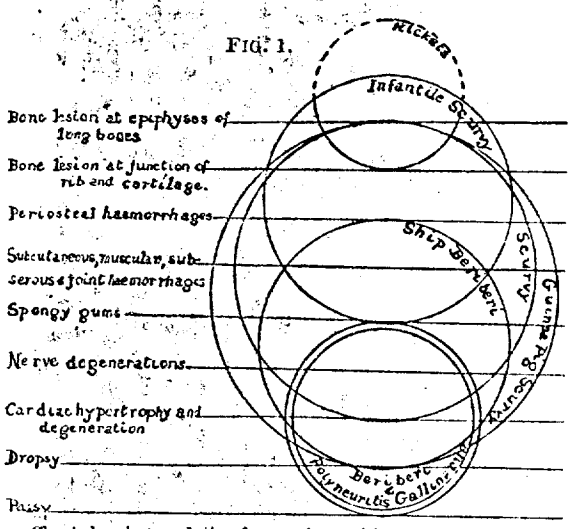


Chart showing gradation from polyneuritis avium to rickets. (Darling.)

TABLE I.—*Showing Affinities and Pathologic Features of Scurvy, Beri-beri, &c.* †

	Rickets.	Infantile scurvy.	Scurvy.	Guinea-pig scurvy.	Ship beri-beri.	Beri-beri.	Polyneuritis gallinarum.
Bone lesions at epiphyses of long bones	+	+	*	+			
Bone lesion at junction of ribs and cartilage	+	+	+	+			
Subperiosteal hæmorrhages		+	+	+			
Joint, subserous, subcutaneous and muscle hæmorrhages		+	+	+	+		
Spongy gums		+	+	+	+		
Nerve degeneration			+	+	+	+	+
Cardiac hypertrophy and degeneration...			+	+	+	+	+
Dropsy...				+	+	+	+
Palsy						+	+

† Trop. Dis. Bull., vol. v., 1915. * Depending on age of patient.

gating the endemic scurvy found among the native miners on the Rand in South Africa. The cardiac condition resembled beri-beri in the concentric hypertrophy of the right heart and degeneration of the vagus nerves. The disease appeared to depend upon the following factors: over-milled corn as a chief article of diet; over-cooked corn; over-boiled meat; insufficient vegetables.

Clinically, cases were seen of every degree of severity, from those who had spongy gums only to those with excessive hæmorrhages. Some cases showed marked rickety symptoms, with extreme destruction of the chondro-costal junctions, others were similar to beri-beri with cardiac degeneration and changes in the vagus nerves. Personal and racial factors had an unknown influence on the resultant symptoms. A deficiency diet in a tropical African negro miner caused severe scurvy symptoms, and in a Cape Colony African labourer mild scurvy, a diet that in some African negroes caused scurvy, in others produced neuritis. In all cases the knee-jerks were exaggerated. (Those who have experience in ship beri-beri have often been puzzled by the persistence of knee-jerks in these cases also.) The scurvy on the Rand, besides showing cardiac changes, differs from the ordinary forms found in time of poverty and famine in that it does not so readily yield to treatment.

Darling sums up in the following words: "The striking excentric hypertrophy and dilatation of the right heart with extensive fatty degeneration of the muscle, and the severe degeneration of the vagus nerves, in cases of scurvy seen on the Rand, furnish new and additional facts which show the intimate relationship between scurvy and beri-beri as to etiology. The affinities between these two diseases and certain other cachexias lend emphasis to the opinion that they are all the result of the continued use of a one-sided and deficient diet."

An Efficient, Portable, and Palatable Antiscorbutic.

Recognising the antiscorbutic that cannot be supplied by the preserved lime juice from West Indian sources as at present made, it has been my object to provide an efficient, portable, and palatable substitute for the use of ships as occasion requires, and for such expeditions as may be undertaken in polar exploration, &c. For the last six months I have been experimenting at the R.N. College, Greenwich, with guinea pigs, which are extremely susceptible to scurvy, to obtain such an antiscorbutic preparation to replace or add to the lime juice ration. The value of these experiments with guinea-pigs in relation to human scurvy has received abundant proof by experiments on monkeys carried out by Barnes and Hume, Chick, Hume and Skelton, and others.

Much of the work done at Greenwich has not been original, but was nevertheless necessary before passing on to the methods of prevention and cure. The use of dried orange juice has been largely employed by Givens and McClugage, who found that in the fresh state it was equal to that of lemons, and that it retained its antiscurvy properties for a considerable time; but in their preparation the employment of a certain amount of heat diminishes the efficiency of the product. In the investigation carried out at Greenwich various methods of preparing the juice were tried, evaporation at 60° C., de-acidification, impregnation of filter paper, rapid drying, &c.

Method of Preparation.

The final laboratory method evolved, which has been continuously in use, is as follows:—

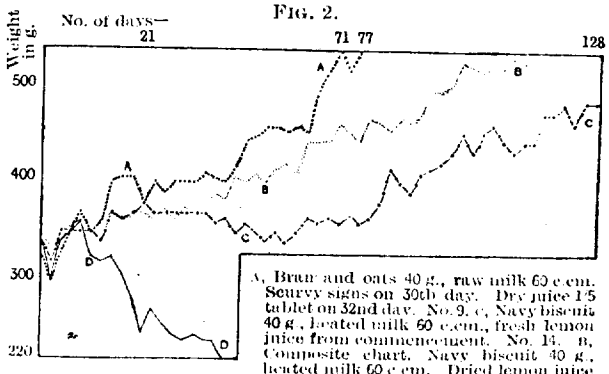
The juice of fresh lemons is roughly filtered through muslin and then through filter paper under reduced pressure by means of a water pump. The filtered juice is evaporated, in vacuo over sulphuric acid at ordinary temperature (13·5°–15·5° C.). The residue of non-crystallisable syrup is worked up into as stiff a paste as possible with a mixture of anhydrous lactose 97 per cent., gum tragacanth 3 per cent. The paste is cut up into sections, each containing the juice of half a lemon. These are rolled, faced with the mixture, and pressed to assume the lozenge form. These tablets have been stored at 15°, 30°, and 37° C. for months, and have been employed in the experiments. The average yield of juice from one lemon is about 48 c.cm., and each tablet contains the equivalent of 24 c.cm. of unfiltered juice. The whole process takes about five days; no heat whatever is used.

Experimental Results.

Two series of experiments were carried out. (Fig. 2.)

1. Using a basal diet of bran and oats, 60 c.cm. of milk heated in steam steriliser for 1¼ hours, and water ad lib.

FIG. 2.



A, Bran and oats 40 g., raw milk 60 c.cm. Scurvy signs on 30th day. Dry juice 1·5 tablet on 32nd day. No. 9. c, Navy biscuit 40 g., heated milk 60 c.cm., fresh lemon juice from commencement. No. 14. B, Composite chart. Navy biscuit 40 g., heated milk 60 c.cm. Dried lemon juice 1·5 tablet from commencement. Nos. 17, 18, 19, 20. D, Composite chart. Bran and oats, heated milk, 60 c.cm. Nos. 1, 2, 4, 7, 8.

In this there was abundant water-soluble anti-beri-beri, and fat soluble growth factor. The control animals died between the thirtieth and fortieth day with all the usual signs of guinea pig scurvy and great loss of weight. The addition of navy lime juice did not give any protection.

2. In the second series navy biscuit was used as the basal diet to which was added steamed milk 60 c.cm. and lime water; on one day per week the biscuit was substituted by oats to supply the anti-beri-beri vitamine. Growth was well maintained for over a fortnight, then the weight rapidly decreased, scurvy signs were manifested, and death followed. Control animals with 20 g. of fresh cabbage or 10 c.cm. fresh de-acidified lime juice grew normally. Animals were given 1/5 tablet (= 4·8 c.cm. fresh lemon juice), in solid form, by hand feeding. There was generally a loss of weight at first, but when they became accustomed to the diet the animals grew and remained healthy for over three months.

A further series was carried out to find the minimum amount of tablet which would act as a preventive dose. The daily amount was reduced to 1/10th (= 2·4 c.cm. fresh lemon juice). In two animals this was sufficient.

FIG 3.

Suprarenal gland in scurvy showing fatty degeneration. × 120.

but in two others signs of paralysis appeared, and they had to be placed on normal diet again; this was followed by recovery.

Keeping properties.—Animals were also given tablets which had been stored for three months and more, and no loss of protective power was noticed. Tablets kept at 37° C. became dark in colour and softer, due to caramelisation of the lactose, and this appears to effect to a slight extent the vitamine properties. In no instance were the tablets found to have become mouldy, whether kept at room temperature or blood heat.

TABLE II.—*Comparative Results.*

(A) Original weight. (B) Weight at death. (C) Weight (mgm.). (D) Mgm. per 100 g. of body-weight at death.

	Scurvy animals.	Animal. (A)	Animal. (B)	Suprarenals. (C)	Suprarenals. (D)
No. 1.	Died on 48th day. Hæmorrhages and joint signs. Suprarenals—local and diffused hæmorrhages.	340	235	270 225	115 96
No. 2.	Died on 44th day. Hæmorrhages and joint signs. Suprarenals: Hæmorrhages and degeneration of cells in cortex.	360	235	237 200	101 85
No. 3.	Died on 53rd day. Mucous and muscular hæmorrhages. Suprarenals, diffuse hæmorrhages, and slight fatty changes.	340	270	255 225	94 83
No. 4.	Died on 44th day. Joint signs. Suprarenals, intense hæmorrhagic condition whole gland. Fatty changes in cortex.	320	240	185 175	77 73
No. 5.	Killed 27th day. Hæmorrhages and joint signs. Ophthalmia. Suprarenals—extreme fatty degeneration of cortical cells, slight hæmorrhages	370	260	220 182	85 70

Five Scurvy Animals—Suprarenal Bodies.

Average weight per 100 g. body-weight at time of death ... 87·9 mgm.

Six Normal Animals—Suprarenal Bodies.

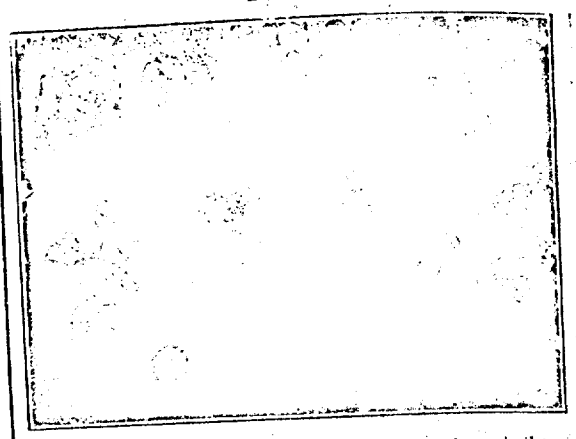
Average weight per 100 g. body-weight at time of death ... 25·8 mgm.

Therapeutic action.—If administered to an animal following scurvy signs, which were not too marked, a cure was effected, but if diarrhœa had set in, or a great loss of weight had occurred, the animals died.

As they have been so fully reported elsewhere, it is unnecessary to describe the scurvy symptoms in the animals or the post-mortem findings, but the condition of the suprarenal bodies, as emphasised by McCarrison, deserves further mention.

He has shown that in guinea-pigs these organs are enlarged, hæmorrhagic, and have a reduced adrenalin content, and with this there is a tendency to secondary bacterial blood infections (found once in my cases). In my experiments the changes in the suprarenals of the scorbutic animals were very marked. (Fig. 3 and 4.) There was

FIG. 4.



Another section showing circumscribed hæmorrhages in the cortical area. × 350.

marked congestion of the whole gland, with diffused or circumscribed hæmorrhages and fatty degeneration of the cells chiefly affecting the cortical areas. The weight of the glands was roughly three times that found in healthy guinea-pigs. The reduction of the normal secretion is, according to McCarrison, an early factor in the scurvy syndrome, and influences very markedly the metabolic changes in the animal, and he concluded that the evidence, so far available, points to the dependence of the functional perfection of the adrenal glands upon the adequate supply, in the food, of the accessory food factors of the various classes.

Conclusions.

Many observations have shown that the onset of scurvy is very gradual. Dr. J. R. Comrie, in an account of scurvy in Russian prisons, stated that it might be 4½ months, but generally it is as long as 6 months, before symptoms are manifested. In the pre-scurvy stage, owing to the deprivation of antiscurvy factors, the metabolism of food is incomplete and a positive agent is added, which acts like a toxin, causing changes in the adrenals and their secretions, favouring the hæmorrhagic conditions which are such marked features of the declared disease.

The use of this concentrated preparation has shown that it has effective antiscurvy properties for animals. We may therefore, with confidence, apply its use to man under conditions of service which preclude the regular provision of fresh fruit and other known preventive substances. It could also be employed in the mercantile marine. Though it is not yet possible to say how long the activity of the retained antiscurvy vitamine will remain effective, it is probable that this will be of extended duration if the preparation is kept dry and cool. Dr. Chick, in Vienna, has found that 5 c.cm. of lemon juice is a prophylactic dose for infants, therefore 2½ c.cm. would probably be ample for an adult (half a lemon). The tablets dissolve fairly readily if added to water containing a small quantity of bicarbonate of soda, and it is suggested that these dissolved tablets, containing antiscurvy accessory factor, should be added to the ordinary non-active pleasant-tasting lime juice at present issued.

For general service, when large quantities are required, the non-heated concentrated syrup, as

obtained before being mixed with lactose, might be kept in bottles and the equivalent of 24 c.cm. of fresh lemon juice be given per man, after solution in water to which sugar has been added at the time of issue. It is probable that this syrup would keep without mould formation and this aspect is at present under consideration.

In this investigation I am greatly indebted to Mr. R. C. Frederick for his able assistance in preparing the tablets used, and to Sick-berth Steward A. Coules for his careful attention in feeding the animals.

A SHORT ACCOUNT OF AN OUTBREAK OF SMALL-POX IN OCCUPIED GERMANY.

BY G. G. JOHNSTONE, M.A., M.D., D.P.H. CAMB.,
CAPTAIN, R.A.M.C. (T.F.).

DURING the month of December, 1918, it will be recalled that the British Army of Occupation was moving through the Ardennes and the bleak and mountainous Eifel district on the way to take up its position along the Rhine. The Germans had, on the whole, been very successful in their rapid retreat, and beyond broken-down motor lorries and cars, and the frequent spectacle of a zone or border of discarded shrapnel helmets along each side of the roads at a distance of about 15 yards, there were few signs to show that the war had so recently ended. Inside some of the hospitals, however, there certainly were signs of blockage and overloading, and in the rush backwards in the case of the retreating army, and forwards in the case of the liberated prisoners of war, the German medical work must have been sorely taxed.

It happened, then, that in the Hospital of St. Vith—a little market town in the province (late German and now Belgian) of Malmedy—there was a case of small-pox in a Polish soldier, who had recently been demobilised from the German army. In the rush and pressure of the constant movement the diagnosis was not made until the infection had spread to other patients in the hospital. The Polish soldier himself was sent on an ambulance train across the Rhine undiagnosed. On the arrival of the first British troops at St. Vith the senior civilian local doctor reported that there was small-pox in the town. There were three cases in the local hospital and one in a house in the centre of the town. This latter case was being removed at once to the hospital. A car was immediately sent to Cologne—about 70 miles away across the mountains—to procure a supply of fresh lymph for use amongst the civilian population. The town was put out of bounds as a billeting area, and the column of troops—a brigade of cavalry and three whole infantry divisions—marched right through the town.

On investigation at the hospital it was found that the Polish soldier had been nursed in the same ward as a man who was suffering from pulmonary tuberculosis. The latter was frequently visited by a cousin—a midwife in practice in the town. The midwife was taken ill with small-pox in her own home on 30/11/18, a day or two after she had attended two confinements. On 15/12/18 her husband was removed to the hospital also suffering from small-pox. On each occasion after the removal of a case to the hospital the house was disinfected by the local authorities. The aged mother of the midwife (81 years) was removed to hospital at the same time as her daughter for the purpose of isolation. On 4/1/19 she was allowed to return to her home, which had been disinfected three weeks before, and on 18/1/19 she also contracted small-pox and was removed to the hospital, where she died. These were the only three cases occurring in the town of St. Vith, and all of them came from the same house.

Hospital Conditions.

A brief sketch of the local hospital will help to explain the cause of the incidence of other cases in this epidemic. On the outskirts of the town there was a building, or rather a composite group of buildings all within the same grounds. In these buildings were housed (1) the local hospital for general diseases; (2) a chapel; (3) an almshouse for aged people; (4) a small convent, the inmates of which were the nurses in the hospital; (5) a large orphanage and school of domestic economy. Here, then, we have a very suitable medium for the growth of a small-pox epidemic, and it seems almost surprising that the number of cases was not greater than actually did occur.

It has been already noted that a supply of lymph had been sent for from Cologne, and on 6/12/18 the inhabitants of the hospital and other buildings, and, as far as possible, of the town, were vaccinated by an organisation of the two local doctors and the Kreisarzt, or medical officer of health for the Kreis of Malmedy. In spite of vaccination, three nurses and one attendant were taken ill on 21/12/18. Two of the nurses, including the matron, died. Altogether five of the nursing staff of a total number of about 20 took the disease, and, in spite of vaccination both recent and remote, the type of the illness was grave. There was no proper isolation block in the hospital, and there was no small-pox hospital, or even proper infectious disease hospital for the district, so that the cases were isolated in a part of the building which was occupied by the aged pensioners, and below them by the nursing sisters.

It was not altogether surprising, then, that the incidence of cases amongst these aged people was very high. Eight (about two-thirds) of them were taken ill, and fortunately there was only one death. Their ages were respectively 40, 63, 75, 70, 72, 81, 52, and 70. At the time of the vaccination the authorities were rather of opinion that it was not essential to insist upon the vaccination of these aged folk, and thus five out of the eight had not been vaccinated since their childhood. Two of them were ill with bronchitis or influenza at the time, and it happened that the only fatal case was that of a woman who refused to be vaccinated in spite of advice.

In the same ward as that in which lay the original Polish soldier it has been mentioned that there was another patient—also a demobilised soldier of the German army. He took the disease, and before the diagnosis had been made he was frequently visited by: (1) his cousin, the midwife, already referred to; and (2) his father (aged 60), who lived in a village about 4½ miles away across the hills. This man was taken ill on 17/12/18 in his house, and as soon as the case had been diagnosed the whole of the population was vaccinated and the village was put out of bounds to British troops. The patient was removed to the St. Vith Hospital on 26/12/18. On 30/12/18 the mother and three children were all taken ill with the disease in a mild form in the hospital where they had been removed for isolation. They were vaccinated on 22/12/18. Fortunately, no other cases occurred in this village.

Vaccination Orders.

It was considered wise, however, by the British authorities that vaccination should be undertaken more extensively in the neighbouring villages. Many points had to be taken into consideration. St. Vith was the market town for that part of the country. Farmers, and others, were constantly coming in and going back to their isolated villages amongst the hills. No check had been made in the early days of visitors to the hospital. Owing to scarcity of civilian doctors and the long distances to be travelled, the poorer people did not seek medical advice unless an illness was of a severe or painful nature. Cases, then, of slight small-pox might lie ill in these villages for days and even weeks without isolation or any other precautions. Although the great majority of these villages were not in use at the moment as billeting areas for British troops, it was not known when the occasion for such use might suddenly arise. St. Vith was also a railway centre of some temporary importance, for it was here that many of the railway wagons and engines were collected from all parts of Germany and repaired previous to being sent over the border to France and Belgium under the terms of the armistice.

An order was then issued by the British military authorities that there should be a compulsory vaccination

Internat. Ztschr. Vitaminforsch. 24: 38-40
1952

Vitamin C and Carbohydrate Metabolism

By N. K. Basu

Received for publication march 20, 1952

Two previous papers (1, 2) have been concerned with a part of the vitamin B complex that has been designated the «anti-diabetic factor». In a later communication (3) the name «adiabetin» was suggested for it. Work by Biskind and Schreier (4) appears to have supported the conclusions drawn from the earlier work.

These authors mention vitamin C in passing, but the almost invariable occurrence of low ascorbic acid levels in the urine of diabetics seems first to have been recorded in 1945 (5). Subsequent papers by Biskind have dealt further with these matters (6, 7).

A private communication from Banerjee in 1950 (8) recorded failure to establish vitamin B deficiency in diabetics or any rise in blood-sugar levels after the administration of nicotinic acid (niacin) or nicotinamide (niacinamide). There was, however, always some degree of hypovitaminosis C, and glucose tolerance improved with increased ascorbic acid intake. Some cases, indeed, then ceased to give sugar in their urines. Banerjee and his colleagues argue from these results that the secretion of insulin depends on the presence of ascorbic acid in the body and that an inadequate level in the diet will cause a deficient production of the hormone. Vitamin C may, they argue, thus be involved in the etiology of diabetes mellitus.

Diabetic patients, I have found, as did Biskind improve on treatment with the vitamin B complex, thus differing from Banerjee, who, however, it must be remembered, only used a single member of the complex (nicotinic acid) on his cases. Biskind recorded consistently satisfactory results from giving the vitamin B complex to diabetics: we have found improvement with a constituent of this complex, but one that appears to be different from any of the recognised members.

There is no doubt that diabetics suffer from hypovitaminosis C, and on this question my own results, both experimental and clinical, agree with those of Banerjee. In none of my cases, however, could urinary sugar be completely abolished by use of ascorbic acid alone. From the experiments summarised below, however, it appears that vitamin C can improve the carbohydrate metabolism of diabetics, and can therefore be a valuable adjunct to insu-

lin and the vitamin B complex, though it has little effect when given by itself.

In all the cases studied the blood-sugar level was found to lie between 160 and 220 mg per 100 ml: this was reduced to normal by restricting dietary carbohydrate to between 150 and 200 g and by injecting 40 to 80 units of insulin daily.

Group I. Ascorbic acid, 25 mg, was injected alternately with the insulin, the same amount being used irrespective of the blood-sugar levels found. After 10 such injections it was found possible to maintain the required blood-sugar levels with half the amount of insulin previously used, but the carbohydrate in the diet could not be increased.

Group II. Ascorbic acid, 25 mg, was injected daily, along with the insulin. Again it was found possible to reduce by half the amount of insulin, but now the dietary carbohydrate could also be increased by 100 g per day while still maintaining the desired blood-sugar levels.

It would thus seem that the effect of ascorbic acid in carbohydrate metabolism is greater when it is injected simultaneously with insulin than when it is given alternately. It is possible that the vitamin has a similar physiological action to insulin, but more probable that it helps to prolong considerably the effect due to insulin given alone.

There is some fairly clear evidence against the first explanation, though it is admittedly based on too few cases. During the last 8 years 250 cases of diabetes have been temporarily deprived of insulin and given massive doses of ascorbic acid. In every one urinary sugar rose and the patient's condition deteriorated. Yet the same doses made it possible to reduce the effective doses of insulin when both were injected simultaneously.

Ascorbic acid is now being given along with the vitamin B complex as a routine to all diabetic patients, and the results are consistently satisfactory.

ZUSAMMENFASSUNG

Vitamin C und Kohlehydratstoffwechsel. Von N. K. Basu.

Es scheint, daß die Wirkung der Askorbinsäure auf den Kohlehydratstoffwechsel größer ist, wenn sie gleichzeitig mit dem Insulin injiziert wird, als wenn sie alternierend gegeben wird. Es ist möglich, daß das Vitamin eine ähnliche physiologische Wirkung wie das Insulin hat, aber es ist wahrscheinlicher, daß es die Wirkung des Insulins allein erheblich zu verlängern vermag.

CONCLUSION

Le métabolisme des hydrates de carbone et la vitamine C. Par N. K. Basu.

L'influence de la vitamine C sur le métabolisme des hydrates de carbone est plus grande, quand elle est administrée ensemble avec l'insuline.

REFERENCES

1. *Basu, N. K.:* Indian Science Congress, Physiology Section, 1938. — 2. *Basu, N. K.:* J. Pediatrics, 1942, 9, 33. — 3. *Basu, N. K.:* J. Council of Scientific and Industrial Research, 1945. — 4. *Biskind, M. S. and Schreier, H.:* Exper. Med. and Surgery, 1945, 3, 299. — 5. *Basu, N. K.:* Indian Medicine, Congress, Physiology Section, 1945. — 6. *Biskind, M. S.:* J. Insurance Medicine, 1950. — 7. *Biskind, M. S.:* J. Insurance Medicine, 1951. — 8. *Banerjee, S.:* Note from Min. of Health, Govt. of India.

Oncology 23: 33-43

0695

Oncology 23: 33-43 (1969)

Laboratory of Biochemistry, National Cancer Institute, Bethesda, Md.

Synergistic Killing of Ehrlich Ascites Carcinoma Cells by Ascorbate and 3-Amino-1,2,4,-triazole

L. BENADE,* T. HOWARD and D. BURK

Introduction

The present study shows that ascorbate (vitamin C) is highly toxic or lethal to Ehrlich ascites carcinoma cells *in vitro*. This toxicity is greatly increased synergistically by concomitant administration of 3-amino-1,2,4,-triazole (ATA). ATA, when given alone, is virtually harmless to the cancer cells, except for specifically inhibiting their catalase (H_2O_2-decomposing) activity. The cytocidal property of the ascorbate is believed to be due in major part to the intracellular generation of toxic hydrogen peroxide produced upon oxidation of the ascorbate by the cells. The synergistic enhancement by ATA is believed to be due to its inhibition of the enzyme catalase, thereby decreasing or destroying the ability of the cancer cells to detoxify the H_2O_2 effectively. Previous studies have established that Ehrlich ascites cells, in common with most other cancer cells, are ordinarily very low in catalase activity [13] in contrast to comparable normal cells, by a factor of 10–100 fold [1]. Thus, a given concentration of ATA can effectively bind all the catalase of a neoplastic cell while providing no such effective impairment of the catalatic activities of comparable normal cells. The great advantage that ascorbate and ATA possess as potential anticancer agents is that they are, like penicillin, remarkably nontoxic to normal body tissues, and they may be administered to animals in extremely large doses (up to 5 or more gm/kg) without notable harmful pharmacological effects.

* Doctoral candidate, Department of Biochemistry, George Washington University, and Guest Worker, National Cancer Institute.

Materials and Methods

Ehrlich ascites carcinoma cells were propagated intraperitoneally in C3H/HeN mice. Upon removal from the mice, the ascitic cells and fluid were taken up in 10-20 volumes of Krebs-Ringer-0.1% $NaHCO_3$ medium, so that the final cell suspensions contained 5-10% ascitic serum. In experiments where 0.2% glucose was added also, the concentration of $NaHCO_3$ was raised to 0.2%, yielding a slightly higher pH. The 'packed volume' of ascites cells per unit volume of suspension was determined by centrifugation of the cell suspension in duplicate hematocrit tubes, and the packed volume was converted to dry weight by a factor of 0.16 on the basis of many previous direct determinations.

Oxygen consumption by cells was determined at 37° in the manometric trough-sidearm vessels of WARBURG and KRIPPAHL [16]. All vessels contained 0.8 cm^3 3M 4½. 1 $KHCO_3$-K_2CO_3 buffer mixture in the trough and sidearm above and separated from the cell suspension on the main floor of the vessel [11]; this buffer mixture provided a gas phase of ca 5% CO_2 in air. The final volume of cell suspension in the main compartment of each vessel was 3.3 cm^3 and contained approximately 40 mm^3 (6.4 mg dry weight) of ascites cells. QO_2 values reported represent mm^3 O_2 gas (STP) consumed/mg dry weight/hour. The pH of the cell suspensions was determined at the end of every experiment by means of a Beckman Model G pH meter.

The proportion of dead cells in an ascites cell population was determined by the widely employed trypan blue staining procedure. Two drops of 0.05% trypan blue in saline solution were added to 0.5 cm^3 of cell suspension in the well of a spot plate (final trypan blue concentration ~ 0.01%). After five minutes, the fraction of distinctly blue-stained (dead) cells was determined under a microscope set at high-dry magnification. The results were frequently checked and confirmed by the same procedure using eosin instead of trypan blue. It is common experience with various killing agents that an Ehrlich carcinoma cell population with 50% or more stained cells is no longer transplantable, even though some of the population may will be otherwise 'viable' by other criteria, including metabolic ones. Even so, many of the unstained cells will show extensive, irreversible blebbing with one to many relatively clear protrusions, as well as internal cellular structural derangements, representing earlier, less drastic stages of death compared to well-stained cells.

The ascorbic acid Na salt (C) and 3-amino-1,2,4,-triazole (ATA) used in these experiments were obtained from Mann Research Laboratories. α-D-glucose was obtained from Allied Chemical and menadione (vitamin K_3-bisulphite) from Sigma. These compounds were dissolved in concentrated stock form in Krebs-Ringer-bicarbonate medium before their addition to the vessels, with the exception of glucose, which was added in solid form directly to the Krebs-Ringer-bicarbonate medium in which the cells were taken up. Crystalline catalase employed was from Nutritional Biochemicals, and bovine plasma albumin from Armour Company.

Results

Table I is a typical set of data from one of the earlier experiments demonstrating the marked inhibition of repiration by, and cytotoxic action of, ascorbate, and, more important, the synergistic enhancement of this phenomenon upon combining ascorbate with ATA. It will be noted that although ascorbate is the prime lethal factor, its combination with ATA provides for a much more rapid and complete realiza-

Table I. Synergistic cytotoxicity of ascorbate and ATA

Addition	—	ATA 3 mg/cm³	C[1] 3 mg/cm³	ATA+C
Q_{O_2} 1st h	7.3	7.3	7.5	4.2
2nd h	7.5	7.4	5.3	2.8
3rd h	7.5	7.4	1.8	0.7
Final pH	7.28	7.30	7.28	7.25
Cell staining	trace (0-2%)	trace	50%	>95%

[1] C = Vitamin C (as Na-ascorbate)

tion of the inhibition effects. The Q_{O_2} values for the control cells and the cells that received only ATA remained relatively constant over the three-hour period, at the end of which no significant cell staining was observed. The cells that were given ascorbate exhibited a normal Q_{O_2} value for the first hour, but showed considerable respiratory decline during the following two hours, and yielded intermediate staining at the end of the experiment, together with marked blebbing of the unstained cells. On the other hand, the cells that received both ATA and ascorbate showed an immediate inhibition of respiration that was almost complete at the end of 3 h. The staining test revealed that at least 95% of the cells had been killed.

Since there was *a priori* reason to believe that the toxicity of the ascorbate was due primarily to intracellularly produced hydrogen peroxide, and that the synergistic effect with ATA was due to inactivation of catalase, experiments were carried out with hope of preventing some or all cell damage by the addition of adequate cata-

Table II. Catalase protection against ascorbate-ATA effect

Catalase added		Q_{O_2}	Staining
None	—	7.6	trace
	ATA (3 mg/cm³)	7.3	trace
	C (3 mg/cm³)	5.8	40%
	ATA + C (3 mg ea/cm³)	3.7	50%
0.3 mg/cm³	—	7.9	trace
	ATA	7.9	trace
	C	7.7	5%
	ATA + C	7.8	5%

Duration of experiment, 1 h.

lase to the vessel suspensions. The results reported in Table II bore out our expectation in considerable measure. In the set of vessels to which no catalase was added, the usual metabolic and staining patterns were observed. However, the cells treated with 0.3 mg catalase/cc showed no metabolic impairment of Q_{O_2}, nor was marked cell staining evident at the conclusion of the experiment. Though not reported in Table II, it was further found in this experiment that if the amount of ATA added was increased by several fold, the effect of the added catalase was eliminated, and if the amount of catalase added was decreased to 0.1 mg/cm³, notable cell death was again observed.

The catalase protection indicated in the bottom half of Table II was only partial, however, since considerable blebbing occurred in the presence of C±ATA, even in the absence of marked staining. The specificity of catalase for this partial protection was indicated by the fact that no such protection was obtained in other experiments when human serum albumin or bovine plasma albumin was added at even 30–100 times the catalase weight concentration. Neither of these proteins at 10 mg/cm³ Krebs-Ringer-bicarbonate medium exhibited appreciable 'catalase activity', i.e., they yielded no O_2 upon addition of H_2O_2; nor, similarly, did they have any observable effect upon the catalase activity of centrifuged (cell-free) ascitic serum when H_2O_2 was added: the rate of O_2 formation and total O_2 produced was unaffected by these albumins.

The catalase protection against intracellularly produced H_2O_2 can scarcely be expected to be more than partial (i.e., not complete), since extracellularly added catalase would appear not to effectively enter the cells but act only on H_2O_2 that comes out of the cells, as described in extensive experimental detail by WARBURG [7, pp. 515–523].

When the experiments of Table I and the upper half of Table II were repeated with cells taken up in centrifuged (cell-free) ascitic serum medium instead of Krebs-Ringer-bicarbonate medium, C±ATA no longer yielded stained cells, even after many hours incubation, evidently because of the demonstrably high catalase content and activity of such serum (half-time for decomposition of added $H_2O_2 < 1$ min]. Nevertheless, although no staining was observed, considerable blebbing was noted, in harmony with expectation and conclusions from Table II. It is certain from other experiments that the partial protection reported in connection with the lower half of Table II would have been more complete if the medium employed had had more added catalase; and it is likely that the inhibitions by C±ATA in the upper half of

Table II would have been greater if the medium had not contained a small amount of ascitic serum, or if washed cells had been employed.

Because many metabolic effects achieved with ascites cells are affected in one way or another by the presence of glucose, glucose was added to the vessel contents for the next series of tests. It was found that glucose at blood-serum levels conferred complete protection against the lethal effects of C±ATA (Tab. III). Those vessels that contained no glucose in the medium exhibited the usual toxicity results, whereas the vessels with 0.2% glucose (and 0.2% bicarbonate) showed no appreciable staining, blebbing or respiratory impairment*.

Table III. Protection against ascorbate-ATA effect by glucose

Glucose added	—	ATA (5 mg/cm³)	C (3 mg/cm³)	ATA+C (5 mg and 3 mg/cm³)
None	Staining trace Final pH 7.35	<5% 7.30	50% 7.35	98% 7.25
0.2%	Staining trace Final pH 7.58	trace 7.42	trace 7.4	trace 7.4

Duration of experiment, 2½ h.

Low oxygen pressure was also found to confer the same overall protection as glucose against C±ATA toxicity (Tab. IV). The 'low' pO_2 vessels were gassed with a mixture of 1.5% O_2/5% CO_2/93.5% N2. The gas phase for the 'high' pO_2 was the usual 5% CO_2 in air. The value of '1%' O_2 in Table IV represents an approximated average of initial 1.5% O_2 and final ca 0.5% O_2.

Table IV. Protection against ascorbate-ATA effect by low pO_2

pO_2	High (21%)[1]			Low (1.0±0.5%)[1]		
	—	C (3 mg/cm³)	ATA+C (3 mg ea/cm³)	—	C	ATA+C
Staining	trace	25%	95%	trace	trace	trace
Final pH	7.32	7.36	7.31	7.30	7.29	7.39

Duration of experiment, 2 h
[1] 5% CO_2 in gas phase.

* Manometric measurements of cellular O_2 consumption, not reported in Tables III to VI, were made in all these experiments, and usually indicated early inhibition effects in advance of the final staining values.

Table V. Prevention of glucose and low pO_2 protection against ascorbate-ATA effect by vitamin K_3-bisulfite

K_3 added	None				1 mg/liter (1 ppm)			
	—	ATA (3 mg/cm³)	C (3 mg/cm³)	ATA + C (3 mg ea/cm³)	—	ATA	C	ATA + C
Staining	trace	trace	trace	trace	trace	10%	90%	98%
Final pH	7.35	7.56	7.56	7.61	7.44	7.58	7.52	7.57

Duration of experiment, 2½ h; all vessels at low pO_2 (1.0 ± 0.5%) and with 0.1% glucose in medium.

Since both low oxygen pressure and some (but low) glucose would be encountered by ascites cells in ascitic serum *in vivo*, a search was undertaken for a third compound that might overcome these protective conditions. One of the most promising compounds found was menadione (vitamin K_3·bisulphite), which acted catalytically: a concentration of only 1 part per million or less was found to overcome glucose and low pO_2 protection, not only *in vitro* (Tab. V and VI) but also *in vivo*, and to yield marked lethality in the presence of C±ATA, at concentrations of menadione too low to be effective by itself.

Table VI. Prevention of glucose and low pO_2 protection against ascorbate-ATA effect by vitamin K_3-bisulfite at 1 ppm

Glucose added	None				0.1%			
	—	ATA (3 mg/cm³)	C (3 mg/cm³)	ATA+C (3 mg ea/cm³)	—	ATA	C	ATA + C
Staining	trace	5%	100%	100%	3%	5%	65%	98%
Final pH	7.6	7.7	7.45	7.5	7.48	7.55	7.5	7.5

Duration of experiment, 2½ h; all vessels at low pO_2, with 1 ppm vitamin K_3-bisulfite in medium.

Menadione represents in reduced form one of the relatively uncommon hydroquinones that is actively autoxidized at oxygen pressure well below 0.01 (even 0.001) atmosphere O_2. Thus, upon reduction by substances such as ascorbic acid in much larger concentration, it can catalytically yield large amounts of H_2O_2 from O_2 during the oxidative phases of its rapid turnover cycles, far more than ascorbate alone can yield at low pO_2.

The important role of ATA in reducing or destroying the catalase activity of Ehrlich ascites carcinoma cells can scarcely be overestimated, as indicated in Table VII, which reports on two cell concentrations and two ATA concentrations, with respect to both initial rate of O_2 evolution from added H_2O_2 and total O_2 evolved before all catalase

Table VII. Inhibition of catalase activity of Ehrlich ascites carcinoma cells by ATA

Cells[1] ($mm^3/3\ cm^3$)	ATA mg/cm^3	Initial rate of O_2 evolution 1-3 minutes after H_2O_2 added ($mm^3\ O_2$/mg dry wt. cells/h)	Total $mm^3\ O_2$ evolved from added H_2O_2 after 20 min
50	0	300	144 (—4%)
	1	22 (--93%)	32 (—79%)
	5	7 (—98%)	10 (—93%)
10	0	310	120 (—20%)
	1		2 (—99%)
	5		0 (100—%)

[1] 5-day Ehrlich ascites cells in final total volume (after tipping 0.5 cm^3 of dilute H_2O_2 equivalent to 150 $mm^3\ O_2$) was 3 cm^3 Krebs-Ringer-0.1% bicarbonate medium. Initial respiratory Q_{O_2} of cells $\sim$3.5 before tipping H_2O_2 at 2 h after incubation of cells with ATA; final respiratory $Q_{O_2} \sim O$ after cessation of O_2 evolution from added H_2O_2 30 min later (complete inhibition of respiration by H_2O_2 added). Manometric measurements taken with Mecrolab automatic recorder printing out point readings every 10 sec. Values in parentheses are calculated percent inhibition.

activity ceased. Under the conditions employed, 1 mg ATA/cm^3 produced virtually total elimination of catalase activity with 10 mm^3 cells/cm^3, and a high degree of elimination with 50 mm^3 cells/3 cm^3. In the absence of added ATA, 50 mm^3 cells/3 cm^3, in other experiments, decomposed added H_2O_2 to the extent of over 1000 $mm^3\ O_2$ produced. ATA also totally eliminated catalase activity found in centrifuged (cell-free) ascitic serum. By contrast, 3.3 mg/cm^3 added human serum albumin or bovine plasma albumin had no observable effect on ascitic serum catalase activity, as already indicated.

Discussion

It should be emphasized that the various effects reported in this paper (e.g., percentage staining, extent of blebbing, protections) will obviously vary from experiment to experiment as a function of agents

added, their concentrations, duration of the experiment, and status of the ascites cells (age, dilution, medium, etc.). Thus, concentrations of ascorbate well below 1–3 mg/cm³ can alone, without ATA, eventually yield, with sufficient time, high percentages of cell staining. $K_3 \pm$ ATA can produce considerable staining even in the absence of ascorbate, or in the presence of glucose that otherwise always protected against harm from C$\pm$ATA, at K_3 concentrations above 1 ppm.; VON ARDENNE and collaborators have reported extensively along such lines [1–6] as well as host pharmacology with K_3, and have demonstrated H_2O_2 formation by ascites cells in the presence of adequate K_3 [4], and notably so under hyperthermic conditions (e.g., 42°C).

It is indicated in the literature that ascorbate can generate H_2O_2 upon oxidation by O_2 gas in biological systems, as early adduced by SZENT-GYÖRGYI [12]. Various types of new evidence presented in this paper now extend this view to Ehrlich ascites carcinoma cells in particular. Thus, catalase, which rapidly breaks H_2O_2 down to O_2 and H_2O, specifically ameliorated cell damage attributable to undetoxified H_2O_2 following incubation of the cells with either ascorbate alone or ascorbate and ATA together. If the concentration of ATA—a compound that specifically and irreversibly combines with the protein moiety of catalase and thereby prevents catalatic activity [9, 10]—is sufficiently increased, or if the amount of catalase is sufficiently decreased, cytocidal results are observed. It is thus reasonable to assume that the cytotoxin primarily involved is hydrogen peroxide produced intracellularly by the action of ascorbate. Extracellular H_2O_2 production does not appear to be involved, even though it has long been known that ascorbate readily undergoes oxidation by O_2 in the absence of cells as a result of catalysis by copper impurity in the ascorbate, since many specific copper inhibitors can prevent such oxidation, even when the rate of oxidation is increased by adding copper. However, in our studies of this noncellular oxidation of ascorbate by O_2, we have never been able to detect the formation of H_2O_2 as any measurable fraction of the O_2 consumed.

On the other hand, we have recently observed that this noncellular ascorbate oxidation can be *completely inhibited by catalase*, at concentrations as low as 10^{-8} M, with our Na-ascorbate containing an estimated 1 part per million of copper impurity [8]. Nevertheless, the action of catalase here is not catalatic, nor is it specific, since we have observed that many proteins, including human serum albumin, bovine plasma albumin, bovine serum albumin, and casein (all copper binders)

0695

likewise inhibit noncellular ascorbate oxidation, although not quite so effectively as catalase on a weight concentration basis. At low concentrations of copper and catalase (*ca* 10^{8-} M) the copper-catalase ratio for inhibition of noncellular oxidation is of the order of unity, but at higher concentrations ($> 10^{-6}$ M) the ratio may attain 5–10. Catalase and cupric ion may be observed to form a spectrophotometrically demonstrable complex as a function of time and concentration of reactants. We have observed no effect of added cupric ion on the rate of catalase decomposition of H_2O_2 to O_2 and H_2O under our experimental conditions, in inorganic buffer medium. We do have evidence, however, that ascorbate has an inhibitory effect on the rate of decomposition of H_2O_2 to O_2 and H_2O by crystalline catalase in inorganic buffer media, but negligible such effect on cellular catalase activity. The rate of O_2 production by ascites cells from added H_2O_2, and also the total amount of O_2 produced, was little affected by the presence of 3 mg Na-ascorbate/cc of Krebs-Ringer-bicarbonate medium, and nonenzymic interaction between ascorbate and H_2O_2 negligible.

Further studies are now in progress on the mechanisms of effects described in the preceding paragraph and earlier ones, including the mechanism of 'glucose protection'. Needless to say, we are also extending our *in vitro* findings regarding ascorbate lethality to *in vivo* conditions in animals, with adjuvants such as aminotriazole and other highly nontoxic compounds, including nordihydroguaiaretic acid [7], various dopa derivatives, amygdalin, glucosamine, stilbestrol, and [14, 15] certain B vitamins and delta-aminolevulinate.

In our view, the future of effective cancer chemotherapy will not rest on the use of host-toxic compounds now so widely employed, but upon virtually host-nontoxic compounds that are lethal to cancer cells, of which ascorbate (± aminotriazole) represents an excellent prototype example of a number of potentially effective combinations now under investigation. Such highly nontoxic compounds have been largely if not totally excluded from consideration in the screening program of the Cancer Chemotherapy National Service Center, in view of the general practice of this Center not to examine compounds at dosages greater than 500 mg/kg body weight, even though the $L.D.^{10}$ may be 10–20 times such dosages and is clearly a far more important criterion for determination of dosages yielding maximally effective chemotherapeutic indices; and, still more important, is the bypassing of testing combinations of highly nontoxic, cancer-lethal compounds in the screening program of this Center.

0695

Summary

Ascorbate is shown to be extremely lethal to Ehrlich ascites carcinoma cells *in vitro*. This lethal effect of ascorbate is synergistically enhanced by 3-amino-1,2,4,-triazole. Catalase, low oxygen tension, and glucose protect the cells from the lethal effects of ascorbate and aminotriazole. Vitamin K_3 (as bisulfite = menadione), at a concentration of no more than 1 $\mu g/cm^3$ (1 ppm), is capable of overcoming such protection. It is concluded that the cytotoxic effect of ascorbate is due to the intracellular production of hydrogen peroxide, and that the enhancement of the ascorbate effect by ATA is due to its inhibition of cellular catalase, thereby inhibiting the ability of the cells to detoxify hydrogen peroxide.

Acknowledgement

We wish to thank Dr. MARK W. WOODS for his interest in and advice concerning these studies.

References

1. ARDENNE, M. VON and REITNAUER, P.G.: Selektive *in-vivo*-Thermosensibilisierung und Therapie des Ehrlich-Mäuse-Aszites-Karzinoms durch Vitamin K_3-Natrium-Bisulfit. Dtsch. Gesundhwes. *40:* 1879–1885 (1967).
2. ARDENNE, M. VON and REITNAUER, P.G.: Überadditive Verstärkung der selektiven Thermosensibilierung von Krebszellen mit Vitamin K_3 durch Methylenblau. Z. Krebsforsch. *70:* 165–171 (1967).
3. ARDENNE, M. VON and REITNAUER, P.G.: Versuche zum Einfluss einer Diät mit erhöhtem Gehalt an mehrfach ungesättigten Fettsäuren auf die Thermosensibilisierung von Krebszellen mit Vitamin K_3. Arch. Geschwulstforsch. *31:* 252–259 (1968).
4. ARDENNE, M. VON and REITNAUER, P.G.: Zum Mechanismus der Thermosensibilisierung von Krebszellen mit Vitamin K_3 durch intrazelluläre Bildung von H_2O_2 und Lipidperoxyden. In press.
5. ARDENNE, M. VON and REITNAUER, P.G.: Zur Toxikologie der Attackenkombination Tumorübersäuerung, Tween 80, Äthylalkohol, Diäthylstilbestrol, Vitamin K_3 und 40°C-Hyperthermia bei der Krebs-Mehrschritt-Therapie. Arzneimittelforschung *18.* In press (1968).
6. ARDENNE, M. VON; REITNAUER, P.G. and SCHMIDT, D.: Zur Toxikologie des Vitamins K_3-Natriumbisulfits und seiner Kombination mit Methylenblau. Arzneimittelforsch. *17:* 1339–1346 (1957).
7. BURKE, D. and WOODS, M.: Hydrogen peroxide, catalase, glutathione peroxidase, quinones, nordihydroguaiaretic acid, and phosphopyridine nucleotides in relation to X-ray action on cancer cells. Radiat. Res. Suppl. *3:* 212–246 (1963).
8. HOWARD, T. and BURK, D. (In preparation).

0695

9. MARGOLIASH, E. and NOVOGRODSKY, A.: A study of the inhibition of catalase by 3-amino-1:2:4-triazole. Biochem. J. *68:* 468–475 (1958).
10. MARGOLIASH, E.; NOVOGRODSKY, A. and SCHEJTER, A.: Irreversible reaction of 3-amino-1:2:4-triazole and related inhibitors with the protein of catalase. Biochem. J. *72:* 339–350 (1960).
11. STAMBUK, B. and BURK, D.: Cytotoxic action *in vitro* of human serum on the metabolism of mouse ascites tumor cells and counteraction by endotoxin and other polysaccharides. J. nat. Cancer Inst. *26:* 681–689 (1961).
12. SZENT-GYÖRGYI, A.: Studies on biological oxidation and some of its catalysts. P.76 (Barth Verlagsbuchhandlung, Leipzig 1937).
13. WARBURG, O.: New Methods of Cell Physiology. Pp. 501, 511, 515, 524, 585 (Interscience Publishers, New York 1962).
14. WARBUNG, O.: The Prime Cause and Prevention of Cancer (English edition by DEAN BURK). P. 16 (Triltsch, Publisher, Würzburg 1967).
15. WARBURG, O.; GEISSLER, A.W. and LORENZ, S.: Wirking von Riboflavin und von δ-Amino-Lävulinsäure auf wachsende Krebszellen *in vitro*. Z. Physiol. Chem *348:* 1683-1685 (1967).
16. WARBURG, O. and KRIPPAHL, G.: Further development of manometric methods. J. nat. Cancer Inst. *24:* 51–55 (1960).

0704

AMER. JOUR. PHYSIOL. 192(3): 511-513, 1958

Effects of Niacin and Vitamin C on Blood Sugar

G. N. BERA AND J. CHOWDHURY

From the Department of Physiology, Presidency College, Calcutta, India

ABSTRACT

BERA, G. N. AND J. CHOWDHURY. *Effects of niacin and vitamin C on blood sugar.* Am. J. Physiol. 192(3): 511-513. 1958.—Intramuscular injection of niacin in normal rats induces hyperglycemia which lasts approximately 1 hour. In adrenalectomized rats niacin produces a hypoglycemia. Subcutaneous injection of vitamin C lowers blood sugar in normal and diabetic rabbits, the effect being pronounced when the blood level of vitamin C is high. The simultaneous administration of niacin and vitamin C produced a pronounced hypoglycemia. The rise in blood sugar normally produced by niacin is nullified if vitamin C is injected simultaneously or shortly thereafter. In both normal and diabetic animals, vitamin C injections produce an increase in liver glycogen. The vitamin C content of liver is lower in normal than in alloxan diabetic animals.

CONFLICTING results regarding the effects of niacin (1-5) and vitamin C (6-9) on blood sugar have been reported. Recent studies have revealed that niacin on intramuscular (i.m.) injection produces hyperglycemia in rabbits (10, 11). Bera showed (10) that the rise in blood sugar after niacin injection is due to liberation of adrenaline from the adrenal glands. For confirmation of this, experiments were repeated in adrenalectomized animals. The effects of niacin and vitamin C on blood sugar level have been considered in establishing their antagonistic roles.

EXPERIMENTAL

In *series I* the effects of niacin injection on blood sugar was determined in fasting male albino rats weighing 100-200 gm before and after adrenalectomy. The operation was performed through the back and control rats were subjected to sham operation. After the experiment was over, a post-mortem examination was made to ensure that the removal of the adrenals was complete. In *series II* blood sugar tolerance after oral administration of glucose was tested in rabbits, with or without the administration of niacin and ascorbic acid. In *series III* changes in sugar and vitamin C concentrations in blood of rabbits was determined after injection of vitamin C with or without oral administration of glucose. Blood sugar was determined by Hagedorn and Jensen's method (12). Vitamin C content of liver was determined by the method of Farmer and Abot (13) and glycogen content by that of Grattan and Jensen (14).

Received for publication March 13, 1957.

RESULTS AND DISCUSSION

In *series I* niacin injection (2.5 mg) in rats caused a rise in blood sugar level during the 1st hour which came down to normal in the 2nd hour (fig. 1). In adrenalectomized rats niacin injection caused no rise but a fall of blood sugar, which might be due to lack of gluconeogenesis in absence of the adrenal cortex. This has to be verified by adrenocortical administration.

In *series II* after oral administration of 1 gm glucose/kg body weight in rabbits, blood sugar reached maximum level between 60 80 minutes in both controls and in those injected with 25 mg niacin intramuscularly, simultaneously, or 20 minutes after the administration of glucose. These results were affected by vitamin C which reduced the peak of blood sugar rise. Injection of niacin and vitamin C in fasting rabbits lowered the blood sugar level (table 1) probably by rendering niacin inactive in its effect on adrenals liberating adrenaline.

TABLE 1. AVERAGE MAXIMUM RISE AND RANGE OF VARIATION IN RISES IN BLOOD SUGAR OF RABBITS*

Exp.		Av. Max. Rise in Blood Sugar and Range of Variation, mg
Ia	Ingestion of 1 gm/kg of sugar	85 (69–101)
Ib	Same + inj. after 20 min. of 25 mg niacin	115 (102–123)
Ic	Same but simultaneous inj. of 25 mg niacin	110 (99–118)
Id	Inj. of 25 mg niacin only	31 (22–40)
IIb	Inj. of 300 mg vit. C and ingestion after 20 min. of sugar as before	63 (36–90)
IIc	Same but simultaneous ingestion of sugar as before	56 (31–75)
IId	Same but without ingestion of sugar	−3.2 (0 to −7)
IIIb	Inj. of 500 mg vit. C and ingestion after 20 min. of sugar and inj. after 20 min. of niacin as before	55 (42–67)
IIIc	Same but simultaneous ingestion of sugar and inj. of niacin as before	59 (42–84)
IIId	Inj. of 500 mg vit. C and 25 mg niacin only	20 (9–35)

* Average increase in blood sugar 1 hr. after the injection of niacin in normal rats is 15.7 mg/100 cc and the variance is 8.8. On the other hand, the blood sugar decreases if niacin is injected after adrenalectomy. In every case the average reductions 1 hr. after the injection being 7.8 mg % and the variance is 12.7. There is some evidence of a negative correlation between the increase in blood sugar due to niacin before adrenalectomy and the reduction in blood sugar due to injection of niacin after adrenalectomy. Glucose and niacin increases the blood sugar by a considerably higher amount than does glucose and vitamin C in the administered dose. Glucose, vitamin C and niacin applied in succession causes an average maximum rise of 54.4 mg % in the blood sugar while for glucose alone the figure is 92.8 mg %. Thus vitamin C lowers blood sugar considerably.

Study of the mechanism of action of vitamin C on blood sugar level: in *series III* vitamin C lowered the blood sugar level in both fasting rabbits and in those after oral administration of glucose (fig. 2). Whether vitamin C stimulated insulin secretion, was determined by injection of it in alloxan[1] diabetic animals (fig. 3). Vitamin C level rises and sugar falls in blood and therefore Vitamin C must be acting independent of insulin, accelerating glycogenesis and/or glucose oxidation directly. Glycogen and vitamin C content of the liver of normal rabbits and those with alloxan diabetes with or without injection of glucose were estimated (table 2). Vitamin C content of liver of control rabbits was lower than in the diabetic which might be due to the using up of vitamin C for glycogenesis, which is retarded in the diabetic livers. The role of vitamin C on glucose oxidation has not been studied in these experiments.

Experiments to investigate the role of vitamin C on neoglucogenesis: vitamin C was injected alone or combined with sugar orally and nitrogen and vitamin C content of urine were studied. While in normal animals urine contained 0.349 gm % nitrogen and 1.55 mg % vitamin C, an injection of 300 mg vitamin C changed the values to 0.279 gm and 12.85 mg % and after simultaneous administration of glucose and vitamin C the values were 0.278 gm and 8.86 mg %, respectively. These results indicate that vitamin C prevents neoglucogenesis and increases excretion of vitamin C.

Thanks are due to Professor N. M. Basu, formerly of Presidency College, Calcutta, for his guidance; to —

[1] Alloxan (Roche) was generously supplied by Voltas Ltd., Bombay.

TABLE 2. EFFECTS OF VITAMIN C ON GLYCOGEN AND VITAMIN C OF NORMAL AND ALLOXAN DIABETIC ANIMALS

Animals	Glycogen and Vitamin C/gm of Liver Substance of Fasted Animals		Same After Simultaneous Injection of 300 mg Vitamin C and Ingestion of Sugar 1 gm/kg 3 Hr. Before Animals are Killed		Same After Injection of 300 mg Vitamin C 3 Hr. Before Animals are Killed	
	Glycogen	Vit. C	Glycogen	Vit. C	Glycogen	Vit. C
	mg	*mg*	*mg*	*mg*	*mg*	*mg*
Normal	9.4 (8.5–10.7)	0.183 (0.15–0.25)	12.3 (11.1–14.4)	0.39 (0.35–0.46)	10.94 (10.7–11.1)	0.383 (0.36–0.4)
Alloxan diabetic	4.5 (1.0–4.8)	0.31 (0.3–0.34)	7.6 (7.4–7.8)	0.75 (0.72–0.78)	5.25 (5–5.5)	0.625 (0.58–0.67)

Average figures of 6 normal and 6 diabetic animals with range of variation.

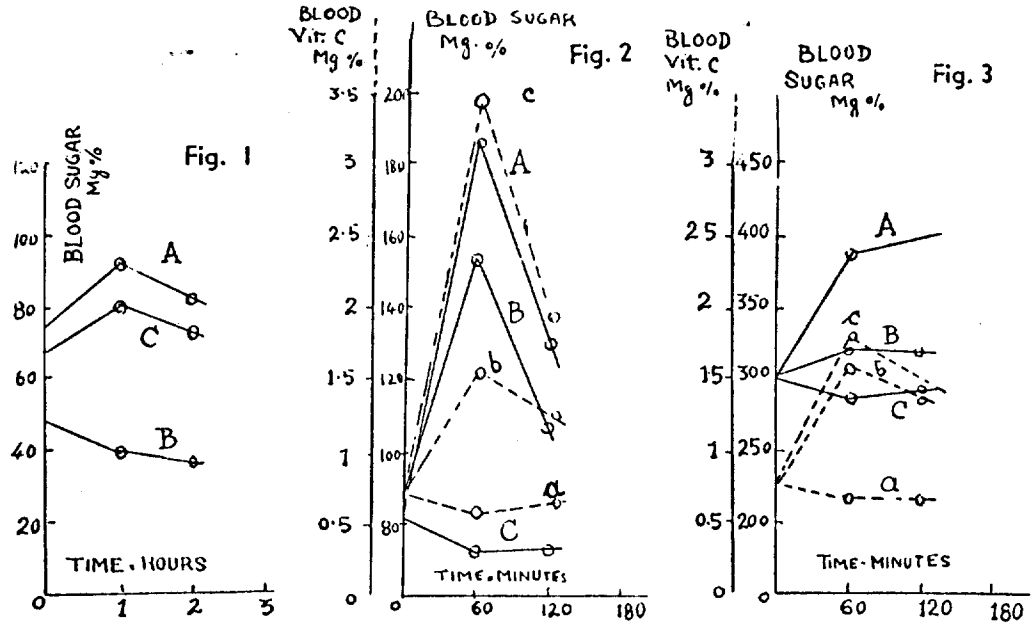


FIG. 1. *A*, effect of injection of 2.5 mg niacin on normal rats; *B*, effect of the same on adrenalectomized rats; *C*, effect of the same on sham-adrenalectomized rats.

FIG. 2. Normal animals. Effect of ingestion of glucose on *A*, blood sugar, *a*, blood vitamin C; effect of simultaneous injection of 300 mg vitamin C and ingestion of glucose on *B*, blood sugar, *b*, blood vitamin C; effect of injection of 300 mg vitamin C on *C*, blood sugar, *c*, blood vitamin C.

FIG. 3. Alloxan diabetic animals. Effect of ingestion of glucose on *A*, blood sugar, *a*, blood vitamin C; effect of simultaneous injection of 300 mg vitamin C and ingestion of sugar on *B*, blood sugar, *b*, blood vitamin C; effect of injection of 300 mg vitamin C on *C*, blood sugar, *c*, blood vitamin C.

Dr. D. K. Sen for technical help; to Mr. J. Roy, statistician, Indian Statistical Institute, for evaluation of results; and to the Government of West Bengal, Education Directorate, for a grant-in-aid for this work.

REFERENCES

1. ESER, S. *Bull. fac. méd. Istanbul* 2: 82, 1948.
2. PAPPALARDO, P. *Acta Neurol. (Naples)* 1: 102, 1948.
3. ARTUNKAL, S. *Bull. fac. Méd. Istanbul* 7: 4024, 1944.
4. FICHERA, G. AND S. VASTA. *Biochim. e. terap. sperr.* 28: 97, 1941.
5. FICHERA, G. AND S. VASTA. *Chem. Zentr.* 2: 2221, 1941.
6. KIVERIN, M. D. *Biokhimiya* 16: 222, 1951.
7. LOREOGINI, G. *Arch. ist. Biochem. Ital.* 12: 135, 1940.
8. LOREOGINI, G. *Chem. Zentr.* 2: 2966, 1941.
9. BASU, N. AND G. N. BERA. *Science and Culture (India)* 10: 123, 1944 45.
10. BERA, G. N. *Am. J. Physiol.* 175: 206, 1953.
11. SEN, S. C. AND S. SEN. *Indian J. M. Res.* 42: 605, 1954.
12. HAGEDORN, H. C. AND B. N. JENSEN. *Biochem. Ztschr.* 135: 46, 1923; 137: 92, 1923.
13. FARMER, C. J. AND A. F. ABOT. *Proc. Soc. Exper. Biol. & Med.* 32: 1025, 1935 and 34: 146, 1936.
14. GRATTAN, J. F. AND H. JENSEN. *J. Biol. Chem.* 135: 511, 1940.

0736

Biochem Pharmacol 7(1): 59-64. Illus 1961.

THE EFFECT OF CARBON TETRACHLORIDE, ETHIONINE AND CHLORETONE ON THE ASCORBIC ACID CONTENT OF RAT LIVER

MARY L. C. BERNHEIM

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina

(*Received* 23 *November* 1960)

Abstract—In rats with acute CCl_4 poisoning, the ascorbic acid content of the liver is very low. Chloretone (chlorobutanol) given to normal rats much increases the ascorbic acid content of the liver, but when given to rats poisoned with CCl_4, it does not. In rats which have received injections of ethionine, the ascorbic acid of the liver is within the normal range. Chloretone, given to such rats, lowers the ascorbic acid value of the liver. This apparent reversal of the action of Chloretone, which occurs when the synthetic mechanism for ascorbic acid is not disturbed, is at present not explained.

INTRODUCTION

THERE are several studies of the effect of CCl_4 on the vitamin content of the liver. Shils *et al.*[1] found a decrease of vitamin A and of certain of the vitamin B group in the livers of CCl_4-poisoned rats. Using liver slices and mitochondrial preparations, Christie and Judah[2] concluded that CCl_4 damages the mitochondria. Vitamin E has also been studied in relation to CCl_4 poisoning.[3] Ascorbic acid, however, has not been studied, possibly because it is not a vitamin for the rat.

Homogenates of most normal tissues form fatty acid peroxides during incubation and these react with 2-thiobarbituric acid to give a red color. Ascorbic acid is necessary for the oxidation,[5] and the amount of color formed in homogenates can usually be increased by the addition of ascorbic acid to the incubating mixture. It was noted that the color formed in liver homogenates of rats with acute CCl_4 poisoning was much reduced, and that the addition of ascorbic acid to the mixture before incubation resulted in a marked increase of color. This suggested a low ascorbic acid content in the poisoned tissues. Therefore, the ascorbic acid concentration in rats livers after CCl_4 administration was determined and compared to that of the livers of rats given ethionine. The effect of Chloretone (chlorobutanol), which is known to stimulate the synthesis of ascorbic acid, was also investigated.

EXPERIMENTAL

Five groups of rats were used. Groups 1, 2 and 3 consisted of a mixed lot of Wistar rats of different ages and sexes. The animals of group 1 were injected intraperitoneally with a single dose of CCl_4, 1 mg/g or sometimes less; they were killed by a blow on the head, usually 17 hr later. Those of group 2 were injected with Chloretone, 30 mg/rat, dissolved in 0·1 ml ethyl alcohol, and the rats were killed at 17 hr. The animals

0736

of group 3 received both substances and were similarly treated. Groups 4 and 5 consisted of female rats which were fasted for 12 hr and then were given ethionine, 75 mg/100 g, divided into three doses at intervals of 2½ hr as described by Koch-Weser *et al.*;[6] they were killed 48 hr later. The animals of group 5 were injected with Chloretone 17 hr before death.

The livers were removed and iced, and 1 g was weighed and homogenized with 9 ml of water, or, for ascorbic acid determinations, with 5% metaphosphoric acid.

The ascorbic acid in the liver was determined by the method of Roe[7]. The metaphosphoric acid homogenate was centrifuged and 1 ml of the supernatant fraction, which was clear or slightly opalescent, was treated with 9 ml of a solution of 2:6-dichlorophenolindophenol in sodium acetate solution and the color was read at once in the Evelyn colorimeter at 520 mμ. The dye was then decolorized completely by the addition of excess ascorbic acid and the value given by the blank was subtracted from the experimental values. The amount of ascorbic acid corresponding to the amount of reduced dye was read from a standard curve.

In order to estimate the total ascorbic acid (the reduced and the dehydroform), the method described by Roe[8] was used. Ascorbic acid was oxidized by treatment with activated Norit, and the color formed with dinitrophenylhydrazine-thiourea and sulfuric acid was estimated in the Evelyn colorimeter at 540 mμ. For this determination the liver homogenate was diluted 1 : 25 with 6% trichloracetic acid, centrifuged and Norit was added. After filtration, 4 ml were used for the determination.

The total nitrogen values were determined on the liver water homogenates, using 2 ml of a 1 : 1000 dilution. The usual Kjeldahl procedure was employed, followed by Nesslerization, and the color was read at 420 mμ. For dry weights, a 3 ml sample of the 1 : 10 water homogenate was dried and weighed.

TABLE 1. THE EFFECT OF CCl_4 ON THE ASCORBIC ACID CONCENTRATION IN RAT LIVER

	Dry weights g/100 g liver	Nitrogen g/100 g liver	Ascorbic acid mg/100 g liver	mg ascorbic acid / g nitrogen
Normal male rat	27·6	2·7	23·0	8·5
Normal female rat	30·7	3·5	27·0	7·8
Male rats after CCl_4	27·4	3·9	11·5	2·9
	26·9	3·3	14·0	4·2
Female rats after CCl_4	20·6	2·15	7·8	3·6
	20·0	2·15	7·0	3·3

RESULTS

Table 1 shows the results of ascorbic acid determinations on the livers of rats with acute CCl_4 intoxication. A definite reduction was found in the amount of ascorbic acid in these livers.

Under the conditions of these experiments, the livers of female rats treated with CCl_4 were grossly larger and appeared more fatty than the livers of males. This impression was confirmed by the dry weights and total nitrogen content of the livers, as can be seen in Table 1. In the table, the data for two individual female and two male

REFERENCES

1. M. E. SHILS, M. SASS, M. WOLKF, G. MARKS, L. J. GOLDWATER and A. BERG, *Brit. J. Ind. Med.* **8**, 284 (1951).
2. G. S. CHRISTIE and J. D. JUDAH, *Proc. Roy. Soc.* B **142**, 241 (1954).
3. E. L. HOVE and J. O. HARDIN, *Proc. Soc. Exp. Biol., N.Y.* **77**, 502 (1951).
4. F. BERNHEIM, M. L. C. BERNHEIM and K. M. WILBUR, *J. Biol. Chem.* **174**, 257 (1948).
5. A. OTTOLENGHI, *Arch. Biochem. Biophys.* **79**, 355 (1959).
6. D. KOCH-WESER, E. FARBER and H. POPPER, *A.M.A. Arch. Path.* **51**, 498 (1951).
7. J. H. ROE, *Meth. Biochem. Anal.* **1**, 121 (1954).
8. J. H. ROE, *Meth. Biochem. Anal.* **1**, 127 (1954).
9. H. E. LONGENECKER, H. H. FRICKE and C. G. KING, *J. Biol. Chem.* **135**, 497 (1940).
10. E. FARBER, M. V. SIMPSON and H. TARVER, *J. Biol. Chem.* **182**, 91 (1950).
11. J. J. BURNS, C. EVANS and N. TROUSOF, *J. Biol. Chem.* **227**, 785 (1957).
12. O. TOUSTER, R. W. HESTER and R. A. SILER, *Biochem. Biophys. Res. Com.* **3**, 248 (1960).

0736

effect of injections of CCl_4 on the concentration of the vitamin in the liver, nor of attempts to alleviate the damage by giving it.

The results shown in this paper indicate that there is a rapid lowering of the vitamin C concentration in the CCl_4-poisoned liver of rats. A few hours after a single intraperitoneal injection of CCl_4 the concentration of ascorbic acid in the liver is reduced; the reduction is maximal between 12 and 17 hr; the values then begin to rise again, but recovery is not complete in 48 hr.

These results are in contrast to those obtained when ascorbic acid was measured in the livers of ethionine-treated rats. In these fatty livers, the vitamin C values per 100 g wet weight were slightly lower than normal, but the ratio of ascorbic acid per gram of nitrogen remained within normal limits. The fatty infiltration without necrosis, typical of ethionine poisoning, does not affect the synthesis of vitamin C by the liver.

Ethionine has been shown to be more toxic for the livers of female rats than for those of males.[10] There is a tendency for the same sex difference under the conditions of these experiments with CCl_4, but the effect is not so marked.

Of the many drugs which increase the urinary output of vitamin C, Chloretone was chosen because it increases the daily vitamin C content of rat urine, up to 100 times the normal range. In guinea pigs, which cannot synthesize vitamin C, the D-glucuronic acid content of the urine rises after barbital,[11] suggesting that there is an increased synthesis of substances which, in most animals, yield ascorbic acid. There is no obvious reason for the increased ascorbic acid formation. Increased formation of glucuronic acid from glucose does not have any known relationship to the metabolism of the group of drugs, which, indeed, have no common chemical structure. Some of them are metabolized, but some are excreted unchanged. Moreover, an increased utilization of glucuronic acid, such as occurs when α-naphthol or borneol are conjugated, does not alter the output of vitamin C. It has been suggested that the effect of the drugs is a hormonal one, for it does not occur in hypophysectomized animals.

In these experiments, Chloretone increased the concentration of vitamin C in the livers of normal rats. However, when given to animals which had received CCl_4, no increase was observed. Even one-quarter the dose usually given was enough to prevent the Chloretone effect.

The effect of Chloretone given to ethionine-treated rats is quite different. In these livers, in which, judging from their ascorbic acid content, the mechanism for the production of ascorbic acid is still intact, Chloretone causes a reduction, rather than an increase, in the amount present. Koch-Weser *et al.*[6] discuss in detail the differences between the lesions of CCl_4- and ethionine-treated animals. Their description shows clearly that the fatty accumulations following the injection of ethionine are not associated with necrosis, nor did they find changes in the activity of certain enzymes. After CCl_4, however, necrotic and regenerative changes were also present, and enzyme activities were altered. These include increased alkaline phosphatase and decreased esterase activity. However, the experiments reported in this paper show that, even in the ethionine-fatty liver, the process by which ascorbic acid is synthesized is not normal. This is also indicated in a recent paper[12] which shows that barbital, which, like Chloretone, increases the excretion of ascorbic acid, does not do so in ethionine-treated rats. The mechanism of the changes cannot be explained.

0736

rats are given, and experimental values were obtained for many others. For instance, in a group of five normal rats (three males and two females) the mean total nitrogen content of the livers was 3·2 g per 100 g wet weight, and ascorbic acid value was 23·6 mg, with a ratio of mg of ascorbic acid/g of total N of 7·4. The corresponding figures for six male rats treated with CCl_4 were, nitrogen, 3·3 g, ascorbic acid 14·1 mg, and a ratio of 4·4. For a group of eight similarly treated females, the nitrogen was 2·15 g, the ascorbic acid 7·8 mg, and the ratio 3·6. It can be seen that the ascorbic acid content of the CCl_4-treated animals is low, expressed either in terms of wet weight or nitrogen content. This is true for both males and females.

Dehydroascorbic acid is not estimated under the conditions employed for ascorbic acid, so it was possible that, in CCl_4-poisoned livers, the low values for ascorbic acid might be the result of a change of equilibrium between the two forms, and the oxidation of a greater part than normal, without change in the total amount. In order to test this, the total ascorbic acid was estimated after the oxidation of the ascorbic acid fraction with activated Norit. The values for two normal rats were, ascorbic acid, 18·6 and 22·0 and the total ascorbic acid 20·0 and 25·5 mg/100 g of liver, and for two rats which had been injected with CCl_4 the values were 4·0 and 3·7, and 4·2 and 4·0, respectively. Therefore, the low amounts of ascorbic acid found in the CCl_4-livers are not the result of oxidation of part of the acid, but represent a real lowering of the total amount present.

The reduction of the ascorbic acid content of the livers of rats treated with CCl_4 is rapid. A few experiments showed that the process is already beginning after 5 hr, and is maximal at from 12 to 15 hr after the CCl_4 injection. Recovery follows relatively slowly, and at 48 hr the ascorbic acid values were still lower than normal.

It seemed possible that fasting might contribute to changes in the concentration of ascorbic acid in the liver. Animals poisoned with CCl_4 eat little if any food, so some experiments were carried out to determine the ascorbic acid values on fasting rats. Food was removed for 15 hr during the night before they were killed. The values for ascorbic acid were as high as or higher than normal, and a group of five rats averaged 27·4 mg/100 g.

A remarkable list of drugs, chemically unrelated to one another, cause an increase in the urinary output of ascorbic acid in rats and other animals.[9] Among these Chloretone is especially effective and increases the excretion in rats to amounts up to 100 times the normal range. Therefore, the effect of this drug on the ascorbic acid content of CCl_4 livers was investigated.

Single doses of Chloretone (30 mg) were given by intraperitoneal injection to rats, which were killed 17 hr later. Table 2 shows that this drug increased the ascorbic acid content of the normal liver, a finding which was to be expected in view of its known effect on the urinary excretion of the acid. However, in rats receiving Chloretone with CCl_4 (1 mg/g) the stimulation of the production of ascorbic acid was prevented, and the values resembled those obtained after CCl_4 alone; smaller doses of CCl_4 caused this effect. For instance, in a series of twenty rats, four normal ones gave an average value for the ascorbic acid of the liver of 23·0 mg/100 g. Six rats were given 30 mg of Chloretone alone and the mean value found was 36·6 mg. For four rats which received 30 mg of Chloretone, and 1·0 mg of CCl_4 per g, the value was 7·5 mg; four receiving 0·5 mg of CCl_4 per g gave 10·4 mg/100 g, and two receiving 0·25 mg of CCl_4 per g gave 8·4 mg of ascorbic acid per 100 g. Even increasing the dose of

0736

Chloretone up to 100 mg in three rats did not increase the low ascorbic acid values caused by 0·25 mg of CCl_4 per g.

For comparison, the ascorbic acid content of fatty livers produced by ethionine was determined, using female rats only. Forty hours after the injection, when maximum damage should have occurred,[6] the animals were killed and the livers homogenized. The mean value for the ascorbic acid for four rats was 18·4 mg/100 g of liver (compared to 23·6 for untreated rats and 7·8 for female rats after CCl_4), while the ratio

TABLE 2. THE EFFECT OF CHLORETONE ON THE ASCORBIC ACID CONTENT OF THE LIVER OF RATS TREATED WITH ETHIONINE OR CCl_4

	Nitrogen g/100 g liver	Ascorbic acid mg/100 g liver	mg ascorbic acid / g nitrogen
Chloretone	3·3	36·0	10·9
	3·1	32·5	10·5
	3·1	28·5	9·1
CCl_4	2·15	7·8	3·6
	2·15	7·0	3·3
	3·3	14·0	4·2
CCl_4 and Chloretone	3·1	17·0	5·5
	3·1	14·0	4·5
	3·1	10·5	3·4
Ethionine	2·67	19·3	7·2
	2·70	23·5	8·7
	2·36	15·7	6·6
	2·56	15·0	5·8
Ethionine and Chloretone	2·90	9·5	3·3
	2·67	12·0	4·5
	1·95	12·5	6·4
	2·59	10·0	3·9

ascorbic acid : nitrogen remained within the normal range (Table 2). The effect of ethionine is thus in marked contrast with that of CCl_4.

Chloretone was given to rats which had received ethionine 24 hr previously. The animals were killed 17 hr after the Chloretone injection. The results show that Chloretone, in contrast to its effect on normal or CCl_4 livers, definitely lowers the ascorbic acid content of the livers of the ethionine-treated animals (Table 2).

DISCUSSION

Christie and Judah[2] were able to show that the changes in the vitamin B complex reported by Shils *et al.*[1] in CCl_4-treated rats were related to the leakage of pyridine nucleotides from the mitochondria, and consequent disorganization of the enzymes of the Krebs cycle. The activity of these enzymes could be completely or partially restored by the addition of the coenzymes *in vitro*. Attempts have been made to protect animals by the addition of vitamins to the diet. Hove and Hardin[3], after finding evidence that vitamin E was deficient in CCl_4-poisoned animals, were able to increase the survival rate of rats receiving one intraperitoneal dose of CCl_4 by injecting them with vitamins E and B_{12}. Nowhere, however, is there reference to vitamin C and the

J. Amer. Med Assoc. 111: 1290-1298

075

Jour. A. M. A.
Oct. 1, 1938

Special Article

VITAMIN C

METHODS OF ASSAY AND DIETARY SOURCES

OTTO A. BESSEY, Ph.D.
BOSTON

This article and others recently published or to be published comprise a new series on the present status of our knowledge of the vitamins. They have been prepared under the general auspices of the Council on Pharmacy and Chemistry and the Council on Foods. The opinions expressed are those of the authors and not necessarily the opinions of either council. Reprints are not available but the articles will be published later in book form.—Ed.

The isolation, synthesis and development of practical methods of assay have given a great stimulus to the study of vitamin C.[1] More papers have appeared on methods of assay and distribution for vitamin C than for all the other vitamins combined. Therefore, no attempt is made here to present a systematic review of the literature. This has been done amply in the monograph by Sherman and Smith,[2] the yearly volumes of the *Annual Review of Biochemistry*,[3] the bulletin of the United States Department of Agriculture on "Vitamin Content of Foods"[4] and numerous reviews.[5] The facts and comments presented in this review may serve to give a general idea of the present state of the subject.

BIO-ASSAY

Chemical methods have rapidly replaced vitamin C determinations by bio-assay for many types of investigations. However, the more specific animal tests continue to be necessary in order to avoid the risk of misinterpretation of the chemical tests.

Guinea pigs kept on certain purified diets fail to gain weight, and the specific pathologic changes of scurvy develop. The degree of protection or cure of the deficiency bears a quantitative relation to the amount of the vitamin administered. This principle forms the basis for biologic methods of vitamin C analyses.

Preventive Method.[6]—The technic of Sherman and his associates,[2] built on the experience of earlier investigators as well as their own studies, has continued to be the most generally used for animal assay purposes. Briefly, the method is as follows: Young guinea pigs are fed ad libitum a basal vitamin C-free diet which theoretically supplies everything necessary for their normal nutrition except vitamin C. Negative control animals are kept on the basal diet only, while other animals are fed graduated amounts of the material to be tested. After from six to ten weeks the degree of protection is estimated by a standard postmortem appraisal of the extent of hemorrhage, the beading and enlargement of the costochondral junctions, the softness of the mandibles, scapulas and ribs, and the looseness of the incisors. An amount of test material just

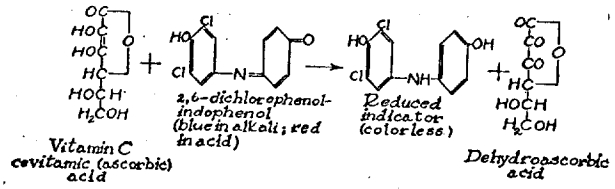
From Harvard Dental School and the Department of Pathology, Harvard Medical School.

1. Now named ascorbic acid or cevitamic acid.
2. Sherman, H. C., and Smith, S. L.: The Vitamins, American Chemical Society Monograph, New York, Chemical Catalog Company, Inc., 1931.
3. (a) Harris, L. J.: Vitamins, in Annual Review of Biochemistry, Stanford University, Calif., Stanford University Press **1**: 357, 1932; **2**: 263, 1933; **3**: 264, 1934; **4**: 347, 1935. (b) von Euler, Hans: The Water Soluble Vitamins, ibid. **5**: 365, 1936. (c) Sherman, Caroline C., and Sherman, H. C.: The Vitamins, ibid. **6**: 346, 1937.
4. Daniel, Esther P., and Munsell, Hazel E.: Vitamin Content of Foods, Miscellaneous Circular 275, U. S. Department of Agriculture, Bureau of Home Economics, June 1937.
5. King.[23] Fellers.[6b]
6. Sherman and Smith.[2] Coward.[14] Coward and Kassner.[16]

The identification of vitamin C as a substance with striking reducing properties made it apparent that it had been studied previously as an unknown reducing substance in plant and animal tissues.[17] Tillmans[18] in studies of the freshness of natural foodstuffs had pointed out that a reducing substance is present in fresh fruits and vegetables which can be estimated by titration against an indicator, 2,6,dichlorophenolindophenol, and which appears to run parallel with their reported vitamin C content. The establishment of the identity of vitamin C by Waugh and King[19] stimulated experiments by Tillmans and his associates,[20] Harris and his associates[21] and Bessey and King[22] which showed the close correlation between the amounts of vitamin C as determined by biological assay and by titration with 2,6,dichlorophenolindophenol in many plant and animal tissues. Additional evidence has supported the view that the titration test when properly used is reasonably specific for vitamin C in natural products.[23]

The essential features of the test are an extraction of the material under examination with an acid-precipitating agent to remove certain interfering substances and to stabilize and bring into solution the vitamin C and subsequent titration of the vitamin with a standardized indophenol solution.

The use of 2,6,dichlorophenolindophenol[22] to determine vitamin C is based on the fact that the colored



Reduction of indicator solution by vitamin C in acid solution.

indicator solution is quantitatively and rapidly reduced by vitamin C in acid solution to a colorless compound, as represented in the equation.

Indophenol Method.[24]—The indicator solution is prepared by dissolving the required amount of dry dye in hot water (50 mg. for food and 10 mg. for blood or urine per hundred cubic centimeters). Although the solid dye is stable, the aqueous solution slowly changes even when kept in the refrigerator and for this reason should be restandardized frequently. It is standardized against a carefully prepared solution of ascorbic acid containing 20 ± 0.1 mg. per hundred cubic centimeters of 3 per cent metaphosphoric acid.[25] A good commercial ascorbic acid is usually sufficiently pure for this purpose. However, the purity should be checked frequently by titration with iodine.[22] One should dilute 5 cc. of the aforementioned solution to 100 cc. with 3 per cent metaphosphoric acid for standardizing the more dilute dye solution. The strength of the reagent is expressed in milligrams of ascorbic acid per cubic centimeter of indophenol solution.

Extracts of plant and animal tissues for vitamin C determinations are best obtained by thoroughly rupturing the cellular structures in metaphosphoric acid. The acid prevents the rapid oxidation of vitamin C due to enzymes[26] liberated by the macerated cells or by the presence of traces of certain metals.[27] Metaphosphoric acid[28] has replaced the use of other acid-extracting reagents because of its marked protective properties and its inertness toward the indicator. Trichloroacetic acid causes fading of the indicator, which may be very rapid with some batches.[29] Sulfuric acid reacts with the dye and should never be used.[15] Tungstic acid[30] has been used in blood determinations and, although it is a more complete protein precipitant, lacks the protective characteristics of metaphosphoric acid.

An extract of a representative portion of the plant or animal tissue under examination (from 5 to 10 Gm.) is prepared by grinding in a small mortar with sand and fresh 3 per cent metaphosphoric acid until a thin paste is formed. The solids are thrown down in a centrifuge and the clear extract is decanted. Another portion of extracting liquid is used to wash the mortar and is stirred into the solids, which are again centrifuged. The rinsing and washing process is repeated, and the decanted aliquots of the extracts are diluted to 50 cc. Aliquots (10 cc.) of the extracts are titrated with the standard 2,6,dichlorophenolindophenol solution until a faint pink end point is reached. A 10 cc. microburet graduated in 0.01 cc. is convenient.

The indophenol reacts rapidly with vitamin C and should be dropped in at a brisk rate. The end point for most plant tissues is stable for twenty seconds or more, while animal tissues usually fade after from ten to fifteen seconds because of a slow reaction by other substances. Such extracts should be titrated only to an end point at which rapid fading ceases. A correction for the amount of dye required to bring 10 cc. of the extracting acid to the same end point should be applied to all determinations, including the standardizations. This quantity is greater than the usual indicator corrections and should never be neglected. The results are most conveniently expressed in terms of milligrams of ascorbic acid per hundred grams of fresh weight, but in studying vitamin C in tissues which vary considerably in water content, one may get a clearer picture by basing the analysis on dry weight.

Comment.—Since the indophenol method depends on the reduction of the reagent by vitamin C, many substances having a reducing potential lower than the dye are possible sources of interference. However, most such substances found in natural systems react with the

17. Szent-Györgi, Albert: Observations on the Function of the Peroxadase Systems and the Chemistry of the Adrenal Cortex: Description of a New Carbohydrate System, Biochem. J. **22**: 1387 (July) 1928. Harris, L. J.: Vitamins, in Annual Review of Biochemistry, Stanford University, Calif., Stanford University Press **4**: 347, 1935. Tillmans, Hirsch and Hirsch.[16]

18. Tillmans, J.; Hirsch, P., and Hirsch W.: The Reducing Property of Plant Foods and Its Relation to Vitamin C, Ztschr. f. Untersuch. d. Lebensmitt. **63**: 1 (Jan.) 1932.

19. Waugh, W. A., and King, C. G.: The Chemical Nature of Vitamin C, Science **75**: 357 (April 1) 1932; The Isolation and Identification of Vitamin C, J. Biol. Chem. **97**: 325 (April) 1932. Harris, L. J.: Vitamins, in Annual Review of Biochemistry, Stanford University, Calif., Stanford University Press **1**: 357, 1932; **2**: 263, 1933.

20. Tillmans, J.; Hirsch, P., and Vanbel, R.: The Reducing Value of Plant Tissues and Its Indication of Vitamin C, Ztschr. f. Untersuch. d. Lebensmitt. **65**: 145 (Feb.) 1933.

21. Harris, L. J., and Ray, S. N.: Specificity of Hexuronic (Ascorbic) Acid as Antiscorbutic Factor, Biochem. J. **27**: 580 (Feb.) 1933.

22. 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 (Dec.) 1933.

23. King, C. G.: Vitamin C, Ascorbic Acid, Physiol. Rev. **16**: 238 (April) 1936.

24. Harris, L. J.: Vitamins, in Annual Review of Biochemistry, Stanford University, Calif., Stanford University Press **3**: 264, 1934. Harris and Ray,[21] Bessey and King,[22] Musulin and King.[29]

25. Metaphosphoric acid solution is slowly converted to orthophosphoric acid. It should be prepared fresh frequently and should be kept in the refrigerator.

26. Kirtesz, Z. I.; Dearborn, R. B., and Mack, G. L.: Vitamin C in Vegetables: IV. Ascorbic Acid Oxidase, J. Biol. Chem. **116**: 717 (Dec.) 1936. Johnson, S. W., and Zilva, S. S.: The Oxidation of l-Ascorbic Acid by Plant Enzymes, Biochem. J. **31**: 438 (March) 1937.

27. Barron, E. S. G.; DeMelo, R. H., and Klemperer, Friedrich: Biological Oxidation: V. Copper and Hemachromagens as Catalyst for the Oxidation of Ascorbic Acid, J. Biol. Chem. **112**: 625 (Jan.) 1936.

28. Fujita, Akiji, and Iwatake, Danzo: The Determination of Vitamin C with 2,6-Dichlorophenolindophenol, Biochem. Ztschr. **277**: 293, 1935.

29. Musulin, P. R., and King, C. G.: Metaphosphoric Acid in the Extraction and Titration of Vitamin C, J. Biol. Chem. **116**: 409 (Nov.) 1936.

30. Taylor, F. H. L.; Chase, D., and Faulkner, J. M.: Ascorbic Acid in Serum and Plasma, Biochem. J. **30**: 1119 (July) 1936.

sufficient to afford complete protection from macroscopic scurvy is selected as a unit, and the results of all analyses are expressed in this unit.[7] With experience and by the use of the survival period, weight and degree of scurvy, one can estimate the degree of protection afforded by the lower test levels, thus bringing into a quantitative rating those animals which are not completely protected. The general uniformity of the test can now be improved by comparing the rate of growth and the intensity of the macroscopic lesions in the test animals with those in a similar group of animals which received graded levels of pure ascorbic acid.[8]

Curative Method.—Harris[9] and his associates have introduced a recovery method which is very useful. Less material is required and there is considerable saving in time and expense. Guinea pigs are kept on a basal diet as previously described, and after from two to three weeks animals are selected which show symptoms of scurvy and have begun to lose weight evenly. Graduated daily doses of the test material are then fed. After from ten to fourteen days the vitamin C value of the unknown is estimated by comparing the growth response with that obtained by feeding a like series of animals with graded amounts of pure ascorbic acid. Thus, with a continuous supply of guinea pigs acquiring scurvy, a test is completed in about two weeks. Care must be exercised in selecting animals for this test, because those which are "too sick" or which show signs of infection will not respond uniformly.

Tooth Method.—Höjer[10] has proposed a method based on the observation that the degree of scurvy can be estimated by histologic examination of the teeth. A cross section of the root of a normal guinea pig incisor shows a row of long parallel odontoblasts surrounding the pulp and active in the formation of dentin. Höjer has described ten stages in the development of scurvy based on the amount of disorganization of the odontoblasts, the width, irregularity and structure of the dentin and the degree of calcification of the predentin. Key and Elphick[11] have described a modification in which, by the same criteria, they distinguish four degrees of scurvy. Using a large series of animals receiving graded doses of ascorbic acid, they plot the average degree of protection against the size of the dose, thus deriving a curve which shows the ratio of protection to dose at any level. When once established, this curve of reference may be used for calculations in successive assays, although it may be revised as additional data accumulate. An example will best show how this curve is used for a determination: A group of healthy growing guinea pigs (from 250 to 300 Gm.) were kept for a few days on a scorbutic diet plus greens. The test was started by withholding the greens. After three days one group of six animals received 0.9 mg. daily of ascorbic acid (International standard). Another group of six animals received 1 cc. of test material (orange juice) daily. Two guinea pigs were kept on the basal diet only (negative control animals). After fourteen days all the animals were killed and the lower jaws fixed and decalcified in 5 per cent trichloroacetic acid, embedded in celloidin and sectioned just anterior and parallel to the first molar at right angles to the longitudinal axis of the mandible. This method gives a cross section of the incisor root, which runs parallel with the jaw at this point.[12] Sections were stained with hematoxylin and eosin and with connective tissue stains. The degree of protection was scored from 1 to 4 for each tooth and the average obtained for all animals on a like level. The guinea pigs receiving the orange juice showed an average protection of 1.3, while those receiving the ascorbic acid averaged 1.8. On the basis of a curve previously prepared as described by Key,[13] the ratio of the protection in terms of ascorbic acid was found to be 0.62 divided by 0.90, which equals 0.69. Since, by definition, 1 mg. of ascorbic acid equals 20 international units, the potency of the orange juice equaled 13.8 international units per cubic centimeter. The curve of response is a device for calculating the relative potency of two materials which do not give an identical response.[14] The feeding of a standard with each assay makes possible a correction for differences in sensitivity of different stocks.[4]

Comparison of Methods.—The chief source of uncertainty in such quantitative methods lies in the unavoidable variation of individual experimental animals and in the indefinite nature of the end point. Complete protection from scurvy or a definite degree of partial protection are conditions difficult to judge. The answer to the problem of which is the best method of bio-assay for vitamin C depends on the experience of the investigator, equipment available, material to be tested, time available, purpose of the assay and many other factors. In addition to the economy of time, the tooth method or the curative method is most useful if the quantity of test material is limited or subject to change by storage. The specificity of the tooth method and the Sherman method is an important feature lacking from the growth response method of Harris. On the other hand, the two former methods are liable to cause subjective errors in judging the degree of scurvy. The need of histologic experience and special equipment for the tooth method are objections to its general use, while the other methods are adaptable to the usual laboratory equipment. It is my experience[15] and that of others[16] that a longer test period than that recommended by Harris (e.g., four weeks) is necessary to obtain quantitative results comparable with those of other methods.

CHEMICAL METHODS

In general, chemical methods are more rapid and accurate than biologic methods but may lack specificity. However, when available and used with precaution they are of great help in many problems not subject to study by bio-assay.

7. A Sherman unit, or a minimum protective dose, is equivalent to 0.5 to 0.6 mg. of ascorbic acid.

8. International standards for assay purposes are obtained by application to the Protein and Nutrition Division, Bureau of Chemistry and Soils, U. S. Department of Agriculture, or to E. Fullerton Cook, Forty-Third Street and Woodland Avenue, Philadelphia.

9. Harris, L. J.; Mills, J. I., and Innes, J. R. M.: The Chemical Identification of Vitamin C, Lancet **2**: 235 (July 30) 1932. Harris, L. J., and Ray, S. R.: Vitamin C in the Suprarenal Medulla, Biochem. J. **27**: 2006 (June) 1933.

10. Höjer, J. A.: Method for Determining the Antiscorbutic Value of Foodstuffs by Means of Histologic Examination of the Teeth of Young Guinea Pigs, Brit. J. Exper. Path. **7**: 356 (Dec.) 1926. Sherman and Smith.[2]

11. Key, Kathleen, M., and Elphick, G. K.: A Quantitative Method for the Determination of Vitamin C, Biochem. J. **25**: 888 (March) 1931. Key and Morgan.[13]

12. It is important that the section be made at a consistent level of the tooth, otherwise the degenerating odontoblasts which normally occur in the incisal part of the tooth may be confused with the scorbutic process. In a longitudinal section of a guinea pig tooth may be observed dental tissue in all stages of development. In the root the odontoblasts are young, regularly arranged and active in the formation of dentin; as they proceed up the persistently growing tooth, a normal sequence of senility and degeneration occurs. Near the biting end of the tooth the odontoblasts normally have much the same appearance as those which are present in the root in scurvy.

13. Key, Kathleen M., and Morgan, Barbara G. E.: The Determination of the Vitamin C Value of Ascorbic Acid, Biochem. J. **27**: 1030 (April) 1933. Coward and Kassner.[16]

14. Coward, Katherine H.: The Biological Standardization of Vitamins, Nutrition Abstr. & Rev. **4**: 24 (April) 1935. Daniel and Munsell.[4]

15. Bessey, O. A.: Unpublished data.

16. Coward, Katherine, and Kassner, Elsie W.: The Determination of Vitamin C by Means of Its Influence on the Body Weight of Guinea Pigs, Biochem. J. **30**: 1719 (Sept.) 1936.

indicator at a perceptibly slower rate than ascorbic acid under the conditions of the titration. This is a requirement for the specificity and accuracy of the method. The end point with plant tissues is stable for some time, but many animal tissue extracts and urines contain sufficient amounts of slow-reducing substances to make the end point less distinct. This is especially true when small quantities of ascorbic acid are being titrated. In such cases the accuracy of the titration depends on the skill of the observer in detecting the point at which rapid reduction is replaced by a slow fading of the indicator. It is true that this feature limits the accuracy of the method, but the precision in most cases is still within the range of biologic variation. The errors are of about the same order as those for blood sugar tests. Unknown reducing materials are reported to interfere with the test in nerve tissue,[31] eye lens and aqueous humor,[32] the liver of some species[33] and tumor tissues.[34] Reduction by phenols, tannins, glutathione and many other substances is eliminated by titration in acid solutions below p_H 3. Cysteine, sometimes present in autolyzed tissue, must be estimated separately or removed from the system. Ergothionine, thiosulfate, cysteine and other organic sulfur compounds may be removed by a rather unpractical mercuric acetate treatment suggested by Emmerie.[35] Reductone (glucic acid),[36] reductic acid[37] and other derivatives formed in hot alkaline sugar solutions reduce the dye in a manner indistinguishable from that of vitamin C. Similar compounds are found in yeast, mold, malt extract and beer.[38] These examples illustrate the point that when one is dealing with a type of material not previously checked it is best to establish the validity of the test by other means.

Recent evidence[38a] suggests that there may exist in certain plant tissues significant quantities of physiologically available combined ascorbic acid which is not extracted by the usual acid extracting reagents and therefore is not included in the titration value. Unless the extracts are carefully prepared and titrated without too much delay, oxidation of vitamin C by liberated enzymes or traces of metals may cause a low titration figure. If the oxidation has proceeded beyond the first step as previously indicated,[39] both the chemical and the biologic value will be low. In general, reversibly oxidized ascorbic acid (dehydroascorbic acid) represents an insignificant portion of the total biologic value of fresh or storage products.

The titration value of some tissue extracts may be increased by preliminary treatment with hydrogen sulfide.[35] Some investigators have interpreted this increase as due to reduction of dehydroascorbic acid and have modified the method to include both forms of ascorbic acid. There is some doubt whether it is due to the reduction of dehydroascorbic acid or to the reduction of other substances which are then included in the titration. The uncertainty of the procedure is also increased by the necessity of completely removing the gas because hydrogen sulfide also reduces the indicator. Emmerie and Van Eekelen recommended the use of mercuric acetate to remove cysteine ergothionine and of thiosulfate followed by treatment with hydrogen sulfide. The extra manipulation involved, with uncertainty at every step, makes the method at present of doubtful value.

Microtitration with indophenol for special purposes has been described by Birch, Harris and Ray,[40] Farmer and Abt[41] and Glick.[42] The last named has applied the Linderstrom technic to microtome-cut sections of glandular tissues with some success.

Other Chemical Methods.—Alternative methods based on the reduction of ferrocyanide,[43] phosphotungstic acid,[44] methylene blue[45] phosphomolybdic acid[46] and other color reagents have been proposed. From a theoretical standpoint it would seem that these reagents might be as specific and useful as indophenol, but for one reason or another they have not been widely adopted. In some cases they react too slowly in acid solutions (p_H from 2 to 3 is a necessary condition for specificity). The color changes often vary with time. Many of these reagents react quantitatively with pure ascorbic acid solution but are inhibited or reduced by other materials in urine and tissue extracts. Ascorbic acid in some cases shows side reactions with the reagent, thus interfering with quantitative reduction. A spectroscopic method[47] in which the intensity of absorption (265 millimicrons) is measured before and after the destruction of the vitamin has been of value in checking the accuracy of the indophenol method. Extracts require special attention in removing irrelevant absorbing materials. Tauber and Kleiner[48] advocated the determination of reducing capacity before and after the destruction of the vitamin by an oxidase as a means of increasing the specificity of the test. This method is also not entirely specific, because of the nonspecificity of the

31. Young, F. G., and Mitolo, M.: A Reducing Substance in Brain Tissue, Nature **133**: 572 (April) 1934. Plant, F.; Bülow, M., and Pruckner, F.: Chemical and Spectroscopic Comparison of the Vitamin C Content of Urine, Spinal Fluid and Serum, Ztschr. f. physiol. Chem. **234**: 131 (April) 1935.

32. Birch, T. W., and Dann, W. J.: Ascorbic Acid in Eye Lens and Aqueous Humor of the Ox, Biochem. J. **28**: 638, 1934. von Euler, Hans, and Malmberg, May: Antiscorbutic Action of the Eye Lens and Its Contents of Reductone and Sulphydryl Compounds, Ztschr. f. physiol. Chem. **230**: 225, 1934.

33. Borsook, Henry; Davenport, H. W.; Jeffreys, C. E. P., and Warner, R. C.: The Oxidation of Ascorbic Acid and Its Reduction in Vitro and in Vivo, J. Biol. Chem. **117**: 237 (Jan.) 1937.

34. Kellie, A. E., and Zilva, S. S.: The Identity of the Indophenol Reducing Substance in the Jansen Sarcoma, Biochem. J. **30**: 1216 (July) 1936. Harris, L. J.: A Reducing Substance in Tumor, Nature **132**: 605 (Oct.) 1933. Harris.[34]

35. Emmerie, Adrianus: Separation of Cysteine from Ascorbic Acid by Mercuric Acetate, Biochem. J. **28**: 268, 1934. Emmerie, Adrianus, and Van Eekelen, Marie: The Chemical Determination of Vitamin C with Removal of Interfering Reducing and Colored Substance, ibid. **28**: 1153, 1934; Some Critical Remarks on the Determination of Ascorbic Acid, ibid. **30**: 25 (Jan.) 1936.

36. Nelson, E. K., and Browne, C. A.: The Properties and Chemical Constitution of Glucic Acid, J. Am. Chem. Soc. **51**: 830 (March) 1929. von Euler, Hans, and Martius, Carl: Reductone and Ascorbic Acid, Ann. d. Chem. **505**: 73, 1933. King.[23] Nelson and Mottern.[19]

37. Reichstein, T., and Oppenhauer, R.: Reductic Acid: A Strong Reducing Split Product of Carbohydrates, Helvet. chim. Acta. **17**: 390, 1934.

38. Harris, L. J.: Chemical Tests for Vitamin C and the Reducing Substances Present in Urine and Other Tissues, Nature **132**: 27 (July) 1933. von Euler.[3] Fox and Stone.[49]

38a. Reedman, E. J., and McHenry, E. W.: Combined Ascorbic Acid in Plant Tissues, Biochem. J. **32**: 85 (Jan.) 1938.

39. Borsook and his co-workers.[33] King.[50]

40. Birch, T. W.; Harris, L. J., and Ray, S. N.: A Microchemical Method for Determining the Hexuronic Acid (Vitamin C) Content of Foodstuffs, Biochem. J. **27**: 590, 1933.

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

42. Glick, David, and Biskind, G. R.: The Histochemistry of the Adrenal Gland: I. The Quantitative Distribution of Vitamin C, J. Biol. Chem. **110**: 1 (June) 1935; The Histochemistry of the Hypophysis Cerebri: The Quantitative Distribution of Vitamin C, ibid. **110**: 583 (Aug.) 1935.

43. Tauber, Henry, and Kleiner, T. S.: A Method for the Quantitative Determination of Ascorbic Acid (Vitamin C), J. Biol. Chem. **108**: 563 (Feb.) 1935.

44. Medes, Grace: The Determination of Ascorbic Acid in Urine with Phospho-18-Tungstic Acid, J. Biochem. **29**: 2251 (Oct.) 1935.

45. Cál, Imre: Estimation of Ascorbic Acid by Titration, Nature **138**: 799 (Nov.) 1936.

46. Bezssonoff, N.: Sur l'identification de la vitamine C, Bull. Soc. chim. Biol. **16**: 1107 (July, Aug.) 1934. Bezssonoff, N.; DeLire, A., and Van Wien, H.: L'activité réductrice de la vitamine C et d'autres réducteurs biologiques exprimée en fonction de leur concentration, ibid., p. 1133. Bezssonoff, N., and Van Wien, H.: Sur l'identification et le dosage de la vitamine C dans les milieux biologiques, ibid., p. 1160. von Euler, Hans, and Burstrom, Dagmar: Ascorbic Acid Determinations in Urine by Titration, Biochem. Ztschr. **283**: 153, 1936.

47. Robertson, E. B.: Spectroscopic Estimation of Ascorbic Acid, J. Chem. Industry **53**: 277 (March) 1934. Chevallier, André, and Choron, Yvonne: Spectrophotometric Method for Estimating Vitamin C in Tissues, Compt. rend. Soc. de biol. **125**: 65, 1937.

48. Tauber, Henry, and Kleiner, I. S.: An Enzymic Method for the Estimation of True Vitamin C, J. Biol. Chem. **110**: 559 (Aug.) 1935.

enzyme.[49] Roe[50] has described a method based on another principle, the color reaction between aniline acetate and furfural, a decomposition product of ascorbic acid.

In general the special features of these procedures are outweighed by features which make them less straightforward and less specific than the indophenol method. A discussion of the weaknesses and merits of all the proposed chemical methods is not within the realm of this paper.

Urine Technic.[51]—The excretion of vitamin C in the urine depends primarily on the relative degree of saturation of the tissues and the immediate intake of ascorbic acid. As a state of tissue saturation is approached, the quantity of ascorbic acid excreted in the urine gradually increases until at saturation a marked abrupt elevation occurs. The quantity of ascorbic acid required to induce saturation appears to be a measure of the state of vitamin C nutrition of the patient.[3e]

The analytic procedure for urine is essentially the same as that previously described for tissues. Each specimen of voided urine is measured and a 50 cc. volume containing 10 cc. of 20 per cent metaphosphoric acid is filled to the mark and stoppered. Aliquots should be stored in the refrigerator and titrated within eight to ten hours, during which time relatively little loss occurs. For analysis, the specimen is centrifuged if cloudy, and from 2 to 5 cc., depending on the concentration of ascorbic acid, is diluted to 10 cc. with 3 per cent metaphosphoric acid. The mixture is quickly titrated to a definite pink end point against a standard indophenol solution, a microburet being used. After correction for the titration blank and the added metaphosphoric acid, the total excretion of the specimen is estimated and the twenty-four hour output calculated. The reducing capacity of the urine collected during a preliminary period before the test establishes the excretion level of the patient before treatment with ascorbic acid. This also places the interpretation of the test on a comparative basis, thus correcting for the small constant effect of reducing substances other than ascorbic acid usually present in the urine.[52] Heinemann[53] has reported a significant interference by thiosulfate, especially when the diet is high in protein. However, the virtual disappearance of the reducing value in cases of scurvy and extreme deficiency and its restoration at a predicted rate when ascorbic acid is fed contribute strong evidence for the accuracy of the test. Provided manipulations are carried out properly and interpretations of too fine a character are not attempted, the method is of real value.

The estimation of the excretion of ascorbic acid before and after a single dose is also of value in demonstrating the presence of subnutrition of vitamin C. This procedure undoubtedly shows the absence of saturation, but sufficient data have not been collected to allow for an estimation of the degree of deficiency such as is possible by the repeated dose method.

Blood Technic.[54]—The vitamin C level of normal blood plasma as estimated by titration shows a close correlation with the level of intake, thus providing means for evaluating the reserve vitamin C by a single determination for persons in that wide zone between scurvy and saturation (from 0.3 to 1.8 mg. per hundred cubic centimeters). This rapid direct application of the test will undoubtedly replace the less practical urine technic for many purposes.

With slight variations, the procedure now most widely used for blood titrations is as follows: Five or more cubic centimeters of blood is oxalated in a tube which also contains 2 mg. of potassium cyanide, and 2 cc. of plasma is pipeted into 8 cc. of 3 per cent metaphosphoric acid solution. The mixture is stirred, stoppered and centrifuged. A 5 cc. aliquot (equal to 1 cc. of plasma) of the clear centrifugate is immediately titrated, as previously described, with dilute 2,6,dichlorophenolindophenol. After a correction for the blank, which always should be performed parallel with a determination, the ascorbic acid is calculated and expressed as milligrams per hundred cubic centimeters of plasma. All sources of error being taken into consideration, the titration is probably within 0.1 to 0.2 mg. of the true ascorbic acid content of the blood for the range from 0.3 to 1.8 mg. per hundred cubic centimeters. With plasma of lower value, the errors are greater. Cyanide is added to inhibit the slow inactivation of ascorbic acid which occurs in both plasma and filtrate. This is a good precautionary measure against oxidation due to traces of copper and other catalysts, although I have never observed such high rates of destruction in the absence of cyanide as have been reported.[55] With carefully prepared reagents and water redistilled from glass, the filtrates remain stable for several hours. Metaphosphoric acid is preferred as a deproteinizing agent for reasons previously stated. Plasma is preferred to whole blood for vitamin C determinations because of the greater stability of plasma filtrates.[56] Substances liberated from red cells by deproteinizing reagents catalyze the inactivation of ascorbic acid even in the presence of cyanide. For this reason plasma which shows evidence of hemolysis is unsuitable for ascorbic acid determinations. Another objection to using whole blood filtrates for vitamin C titration arises from liberation of substances that also reduce the dye. This becomes especially significant when the filtrate is treated with hydrogen sulfide before titration, as recommended by some investigators.

A satisfactory method requiring less blood would be of distinct value in preventing the necessity of venous puncture. Micromethods so far published have not been a solution to this problem. The chief difficulty of titrating such small quantities lies with the inaccuracies of the end point determinations. Mindlin and Butler,[57] in an unpublished manuscript, propose a method which yields results satisfactory for clinical purposes by the use of from 0.2 to 0.3 cc. of capillary blood. The decrease in concentration of colored indicator produced by the addition of an amount of blood plasma filtrate insufficient to cause complete reduction of the dye is

49. Fox, F. W., and Stone, William: Specificity of Indophenol in the Estimation of Ascorbic Acid in Fermented Products, Nature **140**: 234 (Aug.) 1937. Johnson and Zilva.[24] King.[30]

50. Roe, J. H.: The Determination of Ascorbic Acid as Furfural and a Comparison of Results Obtained by This Method and by Indophenol Titration, J. Biol. Chem. **116**: 609 (Dec.) 1936.

51. Sendroy, Julius, Jr., and Schultz, M. P.: Studies of Ascorbic Acid and Rheumatic Fever: I. Quantitative Index of Ascorbic Acid Utilization in Human Beings and Its Application to the Study of Rheumatic Fever, J. Clin. Investigation **15**: 3691 (July) 1936. Harris, L. J., and Ray, S. N.: Diagnosis of Vitamin C Subnutrition by Urine Analysis, Lancet **1**: 71 (Jan. 12) 1935. Albasy, M. A.; Harris, L. J.; Ray, S. N., and Marrack, J. R.: Diagnosis of Vitamin C Subnutrition by Urine Analysis, Lancet **2**: 1399 (Dec. 21) 1936. von Euler.[3] Sherman and Sherman.[8]

52. Borsook and his co-workers.[33] Heinemann.[53]

53. Heinemann, M.: Dietary Influence on the Amount of Ascorbic Acid and Other Reducing Substances in Urine, Acta brev. Neerland. **6**: 67 (Aug.) 1936.

54. Abt, A. F.; Farmer, C. J., and Epstein, I. M.: Normal Cevitamic (Ascorbic Acid) Determinations in Blood Plasma and Their Relationship to Capillary Resistance, J. Pediat. **8**: 1 (Jan.) 1936. Ingalls, T. H.: Studies on the Urinary Excretion and Blood Concentration of Ascorbic Acid in Infantile Scurvy, J. Pediat. **10**: 577 (May) 1937. Sherman and Sherman.[3] Bessey.[15] Farmer and Abt.[41] Pijoan and Klemperer.[55]

55. Pijoan, Michel, and Klemperer, Friedrich: Determination of Blood Ascorbic Acid, J. Clin. Investigation **14**: 443 (May) 1937.

56. Borsook and his co-workers.[33] Farmer and Abt.[41]

57. Mindlin, R. L., and Butler, A. M.: The Determination of Ascorbic Acid in Plasma: A Macro and Micro Method, J. Biol. Chem. **122**: 6732 (Feb.) 1938.

measured by means of a photo-electric colorimeter. This eliminates the subjective reading of the end point. An accurately standardized dye is not needed. Observation of the rate of fading of the color with time permits the detection and study of reducing substances reacting at a slower rate than ascorbic acid.

It is important to emphasize the fact that titrations of blood and many other tissues which are extremely low in ascorbic acid have a limited accuracy even when done with the greatest care. A disregard for this fact is apparent from published investigations. Conclusions based on differences far smaller than the errors of the method used are obviously of doubtful value. Such misinterpretation can be avoided only by a thorough understanding and proper application of the technic.

DIETARY SOURCES

This discussion is concerned with the distribution of vitamin C in food and necessarily lays emphasis on this subject. It should be remembered that adequate nutrition is a problem of supplying many factors in proper proportions and can be met only by use of a variety of foods. Condemnation of a food product because it possesses a low vitamin C value, unless this is a consequence of improper processing or handling, is unjustified. Some foods with many excellent nutritional properties are naturally of low antiscorbutic value.

Ascorbic acid value as far as nutrition is concerned is dependent primarily on two factors: (1) the natural potency of the particular plant or animal tissue and (2) the consequence of aging and processing which occurs before its use as food.

Among the richest sources of vitamin C are oranges, lemons, grapefruit (raw or canned), tangerines, tomatoes (raw or canned) fresh strawberries, green peppers and raw cabbage.[58]

Properly prepared green leafy vegetables have good vitamin C value in spite of the relatively large losses from cooking or canning. In this group may be classed cabbage, spinach, brussels sprouts, kale, broccoli and vegetable greens, such as turnip, beet and dandelion.

Apples, bananas, pineapples (canned), potatoes, green beans and green peas, although containing less antiscorbutic activity, are important sources because of the quantities included in the diet.

The lettuce group, including escarole, have a lower vitamin C content than is generally realized but, since they are usually consumed raw, compare favorably with cooked vegetables. Other important vitamin C-containing foods are kohlrabi, onions, turnips, parsnips and cauliflower.

Dry cereals and legumes of all varieties are devoid of vitamin C, but almost any seed soaked in water for twenty-four hours and kept moist for a few days until it sprouts becomes an effective antiscorbutic substance even when cooked. The British committee on accessory food factors has recommended the use of sprouting seeds as antiscorbutic foods in times of famine.

Cooked muscle meat [2] is practically free from ascorbic acid, although experience seems to show that if consumed rare or raw in large quantities it prevents scurvy. Liver is greatly superior to muscle in this respect. The distribution of vitamin C in other animal tissues (not important as food) has been discussed in a previous paper of this series.[59]

Butter, eggs and cheese contain no vitamin C. The vitamin C value of commercial pasteurized milk is very small.

58. Sherman and Smith.[3] Daniel and Munsell.[4] Fellers.[68] Sherman.[52]
59. King, C. G.: Vitamin C Chemistry, J. A. M. A., to be published.

Variety.[60]—Before the introduction of the chemical method, it was not practical to undertake any comprehensive study of the influence of various factors such as variety, type of soil, season and maturity on the vitamin C potency of fruits and vegetables. Enough information has been obtained now to indicate clearly the need of attention to these points in attempting to obtain food of the highest antiscorbutic value.

Certain varieties of tomatoes [61] contain double the quantity of vitamin C of others grown on the same soil under the same conditions. Canners of tomato juice should give as much attention to this fact as to methods of processing in order to provide the most potent product. Some varieties of apples [62] are definitely higher in antiscorbutic value than other varieties. Similar consistent differences according to variety have been reported for spinach,[63] cabbage,[64] beans and peas [65] and probably exist for most fruits and vegetables.

Soil conditions affect the vitamin C content of spinach. Several varieties grown on upland soil were found to average 50 per cent richer than the same varieties grown on muck soil when compared under otherwise similar conditions.[66] Fall spinach when compared with the same varieties grown in the spring was found to be appreciably higher in ascorbic acid content. It therefore seems likely that crops from year to year may vary in ascorbic acid value because of conditions such as moisture and temperature.

Maturity and Origin.—Tomatoes increase somewhat in vitamin C content as the fruit ripens.[67] However, mature green tomatoes may have nearly the same value as the red ripe fruit.[68] The ascorbic acid content of green peppers increases considerably on reddening. A single fruit which is green on one side and red on the other may show a difference of as much as 75 per cent.[69] A lower vitamin C content in oranges sprayed with lead arsenate has been reported.[70] Immature Bramley seedling apples are as rich in vitamin C as mature fruit.[68] Most green leafy and root vegetables contain the greatest concentration of vitamin C during the period of most rapid growth. Sweet corn [71] and green peas [72] show a maximum vitamin C content during the tender stage, and the content decreases as the seed matures. This is consistent with the lack of vitamin C in nonrespiring mature seeds. Ethylene ripening itself has no effect but tends to encourage early picking and may lead to a slightly lower vitamin C value due to immaturity.[73]

How and from what most plants and some animals form ascorbic acid is not clear, but the distribution in animal tissues shows it to occur in the greatest concentration in the most actively functioning tissues, in the

60. Daniel and Munsell.[4] Fellers.[68] Tressler, Mack and King.[75]
61. Tripp, Francis; Satterfield, G. H., and Holmes, A. D.: Varietal Differences in the Vitamin C (Ascorbic Acid) Content of Tomatoes, J. Home Econ. **29**: 258 (April) 1937. Machinn, Fellers and Buck.[77]
62. Smith, G. G., and Fellers, C. R.: Vitamin C Content of Twenty-One Massachusetts Grown Varieties of Apples, Proc. Am. Soc. Horticultural Sc. **31**: 89, 1934. Batchelder.[76]
63. Tressler, D. K.; Mack, G. L., and King, C. G.: Vitamin C Content of Vegetables: I. Spinach, Food Research **1**: 3 (Feb.) 1936.
64. Gould, Stella; Tressler, D. K., and King, C. G.: Vitamin C Content of Vegetables: V. Cabbage, Food Research **1**: 427 (Oct.) 1936.
65. Mack, Tressler and King, footnotes 72 and 75.
66. von Hahn, F. V., and Gorbling, J.: Influence of Fertilizer on the Vitamin C Content of Spinach, Ztschr. f. Untersuch. Lebensmitt. **65**: 601 (April-June) 1933. Tressler, Mack and King.[62]
67. Sherman and Smith.[2] Tripp, Satterfield and Holmes.[61]
68. Fellers, C. R.: The Effect of Processing in Fruit and Vegetables: A Review, Bull. 338, Massachusetts State College Agricultural Experiment Station, Amherst, December 1936.
69. Bessey and King.[22]
70. Nelson, E. M., and Mottern, H. H.: Effect of Lead Arsenate Spray on the Composition and Vitamin Content of Oranges, Am. J. Pub. Health **22**: 587 (June) 1932.
71. Dunker, C. F.; Fellers, C. R., and Fitzgerald, G. A.: Stability of Vitamin C in Sweet Corn, Food Research **2**: 41 (Jan.) 1937.
72. Mack, G. L.; Tressler, D. K., and King, C. G.: Vitamin C Content of Vegetables: II. Peas, Food Research **1**: 231 (May) 1936.
73. Chace, E. M.: Health Problems Connected with Ethylene Treatment of Fruit, Am. J. Pub. Health **24**: 1152 (Nov.) 1934.

adrenal cortex, corpus luteum, thyroid, pituitary body and liver. This distribution proves to be true also in leaves and growing shoots and suggests a fundamental concern with respiratory processes. However, many rapidly growing fruits are low in ascorbic acid, and the respiratory activity of lemons, oranges and tomatoes does not seem to be unusually high, although these are among the richest sources of ascorbic acid. Eggs and seeds are devoid of vitamin C, but the vitamin forms immediately from unknown constituents when a chick embryo develops or the seed sprouts.

Storage.[68]—Time, temperature, care in handling and type of product are important factors in the problem of the stability of vitamin C during storage. Spinach which has stood for a few days at room temperature and has become wilted loses about one half of its ascorbic acid, while the same spinach kept at 37 or 38 F. for the same period retains its original value.[74] This is true in general of green leafy vegetables of this type. Cabbage stored at room temperature loses about 25 per cent of its vitamin C in a month, while at 45 or 50 F. the loss is only 10 per cent.[64] Green beans and peas, like spinach, rapidly lose their ascorbic acid at room temperature but keep well by refrigeration.[75] Sweet corn in the husk retains its vitamin C well for two or three days, after which time a slow loss occurs.[68] Green peppers remain rich sources of ascorbic acid for several weeks if they do not become bruised or wilted.[15] Loss in apples after three months at 45 F. approximates 15 per cent, at six months 25 per cent and at one year 50 per cent.[76] Lemons and oranges stored at 45 or 50 F. for ten months show losses of from 10 to 30 per cent; no detectable loss occurs in two months.[15] Storage of ripe tomatoes at ordinary temperature for a month causes little change in the vitamin C content provided the fruit remains firm and free from bruises and rot.[77] Potatoes very slowly lose their vitamin C under market conditions; about a 50 per cent loss occurs in one year.[22] With some varieties, little loss is observed after from three to six months' storage.[78] Vitamin C in rutabagas, carrots, turnips and sweet potatoes is slowly lost over a period of several months.[68]

These facts demonstrate the great variation in "storage stability" of vitamin C and indicate the advantage of cold storage as compared with ordinary storage.

Freezing.—Preservation of fruits and vegetables by quick freezing has become an important commercial method.[79] Studies on the stability of vitamin C during freezing and storage in general have shown slight destruction, but often incidental processing operations such as shelling, washing and blanching lead to significant losses. Asparagus, apples, potatoes, strawberries, blackberries and orange juice are unaffected by freezing, while some losses are reported in preparatory operation with peas, beans and spinach. Freezing results in a certain amount of cellular disorganization, with liberation of enzymes from some types of plant tissues. Unless these products are properly blanched (to destroy enzymes) before freezing, considerable losses may occur during storage and thawing. Slow-thawing vegetables rapidly lost their vitamin C. Losses of from 80 to 90 per cent may occur in beans, peas or spinach in a few hours. Frozen foods should not be defrosted until ready to cook or serve raw. Losses are reduced to a minimum by cooking without preliminary defrosting.

Processing.—For several reasons, effort should be made to conserve the vitamin C values of the general food supply rather than to depend on the use of foods of special antiscorbutic properties. A larger percentage of the people than is generally realized, because of economic conditions, lack of knowledge or poor food habits, do not include citrus fruits, tomatoes or other richly antiscorbutic foods in their diet. These people necessarily depend on the general food supply for their source of this essential. Also the loss of important food qualities, such as flavor and the content of other vitamins, usually parallels the destruction of vitamin C. In fact, the preservation of ascorbic acid may well be used as a general index to the care with which certain foods have been processed.

The preservation of vitamin C between harvesting and consumption is essentially a problem of combating oxidation, a process dependent on time and the kind and state of the tissue and influenced by temperature, access to air and presence of metals and enzymes. The destruction of vitamin C when tomato juice is heated can be reduced two thirds by rendering the conditions anaerobic.[80] Spinach and cabbage cooked by methods excluding air are from 50 to 100 per cent richer in vitamin C than when prepared by open kettle cooking.[81] The water in which vegetables are cooked may contain sufficient oxygen to become an important factor of destruction.[82] This fact has led to the practice of starting vegetables in hot water.

Crushing or bruising of many vegetables such as spinach, cabbage, turnips and rutabagas causes the liberation of enzymes (oxidases) which in the presence of air catalyze the oxidation of ascorbic acid.[26] This is a rapid reaction and may lead to complete inactivation of the injured tissue in a few minutes. Starting the cooking in hot water quickly inactivates these enzymes. Shredded carrots, cabbage and other roots and leafy tissues rapidly decrease in vitamin C content and should therefore be prepared fresh before serving.

Vitamin C is more stable in an acid medium than in a neutral or slightly alkaline medium.[83] The addition of soda to cooking vegetables may produce a better color but will lead to increased loss of vitamin C. The vitamin C in tomato juice is naturally stable but becomes rapidly inactivated when neutralized or made alkaline with soda. In general, vitamin C is less subject to oxidation in those fruits and vegetables which are acid.

There is perhaps no single factor which accelerates the destruction of vitamin C so much as copper salts. The presence of air and the merest traces of copper lead to complete inactivation often in a few minutes. Contact with bare copper or utensils made of metal containing copper must be avoided. Quantities of copper so small as to be undetectable by the usual chemical methods will lead to immediate and complete destruction of the ascorbic acid in raw milk or cooking vegetables.[84] The insidious nature of this factor makes it

74. Tressler, Mack and King, footnotes 63 and 75.
75. Tressler, D. K.; Mack, G. I., and King, C. G.: Factors Influencing the Vitamin C Content of Vegetables, Am. J. Pub. Health **26**: 905 (Sept.) 1936.
76. Batchelder, Esther L.: Vitamin C in Apples, J. Nutrition **7**: 647 (June) 1934.
77. Maclinn, W. A.; Fellers, C. R., and Buck, R. E.: Tomato Variety and Strain Differences in Ascorbic Acid (Vitamin C) Contents, Am. Soc. Horticultural Sc. **34**: 543, 1937.
78. Fellers.[65] Wood.[60]
79. Nelson, E. M., and Mottern, H. H.: Vitamin C Content of Frozen Orange Juice, J. Indust. & Engin. Chem. **25**: 216 (Feb.) 1933. Daniel and Munsell.[4] Fellers.[65] Tressler, Mack and King.[75]

80. Kohlman, Eddy and others.[62] Sherman.[63]
81. Sherman and Smith.[2] Bessey.[15]
82. Kohman, E. F.; Eddy, W. H., and Gurin, C. Z.: Vitamins in Canned Foods: XI. A Canned Food Diet, J. Indust. & Engin. Chem. **23**: 1064 (Sept.) 1931; Canning Tomato Juice without Vitamin C Loss, ibid. **25**: 682 (June) 1933.
83. Sherman, H. C.: Chemistry of Food and Nutrition, ed. 5, New York, Macmillan Company, 1937.
84. Whitnah, C. H.; Riddell, W. H., and Caulfield, W. J.: The Influence of Storage, Pasteurization, and Contamination with Metals on the Stability of Vitamin C in Milk, J. Dairy Sc. **19**: 373, 1936. Bessey.[16]

very difficult to control, for, in addition to contamination by contact and from the water supply, many foods naturally contain enough copper to be active.

Drying [85] in itself is not detrimental to vitamin C, but for most foods it is almost impossible to prevent oxidation due to other factors sufficiently to prepare dehydrated products of important and lasting antiscorbutic values. Dehydrated foods have repeatedly failed in this respect when used in times of war. Many experiments have been reported showing that certain of the more acid fruits may be prepared so as to conserve a reasonable degree of their vitamin C activity, but commercial products cannot as yet be considered dependable in this respect.

agreement with the previous emphasis on the unfavorable "influence" of contact with copper utensils, access to air, prolonged heating and alkalinity. Modern commercial canning, with careful plant control, can prepare products with a minimum loss of vitamin C which are comparable to carefully cooked fresh food. However, no rule can cover all cases, for there are badly controlled canneries as well as bad cooks. The variations in processing are so great that the products from each process and perhaps each batch would have to be tested before one could be sure of the vitamin C value. Boiling of cabbage in an open kettle or cooking in a pressure cooker leads to almost complete destruction, while starting the cabbage in boiling water and testing both the

The Vitamin C Content of Various Foods per Hundred Grams *

Food	Mg. of Ascorbic Acid	International Units
Apples		
Delicious	2.5	50
Jonathans	2.5	50
McIntosh	2	40
Winesaps	5	100
Golden delicious	4	80
Yellow Newton	5	100
Dried	0	0
Juice	See type of apple	
Apricots, fresh	1	20
Apricots, dried	8	160
Asparagus	20	400
Bananas	8	160
Beans		
Green	10	200
Green, canned	4	80
Sprouted	10	200
Dried	0	0
Beef muscle, cooked	Trace	Trace
Beer	0	0
Beets	5	100
Beet leaves	35	700
Blackberries, fresh	3	60
Blackberries, frozen	3	60
Blueberries, low bush	4	80
Blueberries, high bush	10	200
Broccoli	50	1,000
Brussels sprouts	50	1,000
Butter	0	0
Cabbage, young, green	40	800
Cabbage, old	20	400
Cantaloup	7	140
Carrots	3	60
Cauliflower	30	600
Celery stalk	5	100
Chicory	10	200
Chard, Swiss, stalks	5	100
Chard, Swiss, leaves	20	400
Cheese, all varieties	0	0
Cherries, sweet	8	160
Cider, fresh	See apples	
Chicken meat, cooked	Trace	
Cod liver oil	0	0
Collard	60	1,200
Corn		
Sweet	10	200
Canned	6	120
Dried	0	0

Food	Mg. of Ascorbic Acid	International Units
Cranberries	10	200
Cucumbers	2	40
Currants, black	100	2,000
Currants, red	15	300
Dandelion greens	40	800
Dates, cured	0	0
Egg plant	5	100
Eggs	0	0
Endive	10	200
Escarole	7	140
Figs, fresh	2	40
Figs, dried	0	0
Fish, fresh cooked	Trace	Trace
Fruit juice	See fruit	
Gooseberries	25	500
Grain, dried, all varieties	0	0
Grass, fresh, green	60	1,200
Grape juice	Trace	Trace
Grapefruit juice, fresh	40	800
Grapefruit juice, canned	30	600
Horseradish	100	2,000
Jelly	0	0
Kale	50	1,000
Kohlrabi	70	1,400
Leek	15	300
Lemon juice		
Fresh	60	1,200
Cold storage	40	800
Canned	50	1,000
Lettuce, green leaf	10	200
Lettuce, head	5	100
Lime juice, fresh	30	600
Liver, beef, cooked	10	200
Malt	0	0
Milk		
Human	6	120
Cow's, raw, fresh	2	40
Cow's, pasteurized	0 to 1	0 to 20
Cow's, dried	5	100
Mustard leaves	60	1,200
Nuts, all kinds	0	0
Okra	10	200
Onion	10	200
Orange juice, fresh	50	1,000
Orange juice, canned	45	900
Orange syrup	0	0

Food	Mg. of Ascorbic Acid	International Units
Papaya	40	800
Parsley	175	3,500
Parsnips	5	100
Peas, green	15	300
Peas, canned	5	100
Peaches, fresh	7	140
Peaches, dried	25	500
Pears, fresh	3	60
Pear juice, canned	Trace	Trace
Peppers, green	180	3,600
Peppers, red, ripe	230	4,600
Pineapple, fresh	25	500
Pineapple, canned	10	200
Plums	2	40
Potatoes, new	15	300
Potatoes, old	7	140
Prunes	2	40
Prunes, dried	0	0
Pumpkins	5	100
Quince	5	100
Radishes	12	240
Raisins	0	0
Raspberries	15	300
Rhubarb	15	300
Rutabaga	20	400
Seeds, mature, dry (all varieties)	0	0
Spinach, fresh	60	1,200
Spinach, canned	5 to 10	100 to 200
Sauerkraut juice	0 to 5	0 to 100
Squash	3	60
Strawberries	25	500
Sweet potatoes	8	160
Swede	See rutabaga	
Tangerines	30	600
Tomatoes		
Green	20	400
Vine ripe	30	600
Artificial ripe	25	500
Tomato juice, fresh	30	600
Tomato juice, canned	25	500
Turnips	30	600
Turnip greens	60	1,200
Vegetable juices canned	0 to 5	0 to 100
Watercress	50	1,000
Watermelon	5	100
Yeast	0	0

* Unless otherwise indicated, these values are on fresh foods.

Pickling, salting, curing or fermenting usually results in complete loss of vitamin C. Fresh cucumbers contain from 5 to 8 mg. per hundred grams; salt pickles contain none. Fermented cider and other fruit juices are free from vitamin C. Sauerkraut and sauerkraut juice are unreliable antiscorbutic foods.[86] Commercial orange drinks which do not contain freshly prepared orange juice are practically free from ascorbic acid.[87] Vegetable juices rapidly lose their vitamin C because of active enzymes and a high p_H and therefore are usually poor antiscorbutic foods.

It is difficult to make general statements concerning the loss of vitamin C during such a variable process as cooking [88] or canning. Recent work is in essential

cooking water and the cabbage produce about 25 per cent destruction and 25 per cent loss in the water.[64] Similar studies on spinach, turnip greens and green peas and beans lead to approximately the same conclusions. Commercial canning destroys from 50 to 85 per cent of the vitamin C in peas, lima beans, spinach and asparagus. Home-canned tomato juice is as satisfactory as commercial canned juice, and both are good antiscorbutic substances.[2] Vitamin C is stabilized by acid and therefore suffers less destruction during cooking in acid fruits and vegetables. Canned grapefruit and orange juice are about 70 to 90 per cent as potent as the fresh juice and, like most canned products, remain stable while canned but slowly lose their vitamin C when left open to the air.[87] Baked and boiled potatoes are reported to show only small losses unless overcooked.[89] Fruit jel-

85. Fellers.[68] Sherman.[53]
86. Sherman and Smith.[2] Fellers.[68]
87. Bessey.[15] Fellers.[68]
88. Sherman and Smith.[2] Gould, Tressler and King.[64] Fellers.[68] Sherman.[53]

89. Wood, E.: The Vitamin C Content of the Russel Burbank Potato of Idaho, Bull. 219, University of Idaho Agricultural Experiment Station, June 1935. Fellers.[68]

lies and jams are usually low in vitamin C. Warmed over foods are practically free from ascorbic acid. Canned or cooked apricots, peaches, plums and cherries are practically free from vitamin C. Freshly prepared apple sauce or apple pies retain from 20 to 30 per cent of the original vitamin C value of the apple.[68]

The use of canned sieved vegetables and fruit for children has increased at a rapid rate in the last few years.[90] In spite of the most careful methods of processing, partial destruction of vitamin C is unavoidable. A great deal of variation in this occurs, depending on the type of food, freshness and method of processing, but in general it has been shown that the loss of vitamin C is slightly greater for purées than for the corresponding canned whole vegetables. As a rule these products cannot be classed as potent antiscorbutic foods, although they may contribute materially to the diet because of the quantity consumed.

Milk is the most nearly perfect and the least dispensable food. Nevertheless, the vitamin C content of commercial milk, raw or pasteurized, is not dependable and should never be relied on to supply the requirements. The use of the titration method has led to a better understanding of those factors which may influence the vitamin C content of milk, such as aging, pasteurization and handling. Judging from a large number of determinations, both biologic and chemical, one may expect from 2 to 2.5 mg. per hundred cubic centimeters of ascorbic acid in fresh raw milk.[91] Even when the milk is cold this value slowly decreases, until a loss of from 20 to 30 per cent may occur in twenty-four hours. Sunlight or traces of copper accelerate this change.[92] Pasteurization by the widely used holding method, unless unusual precautions are observed, results in a loss of from 30 to 60 per cent. However, pasteurization by the short time high temperature process with absence of copper leads to slight inactivation.[93] Loss of vitamin C activity due to pasteurization, aging, reheating and diluting, such as usually occurs in preparing a formula for babies, results in a formula of very low antiscorbutic value.[15]

Although Hess[94] found that a pint of milk a day prevented infantile scurvy, the commercial milk of today, which contains from 0.3 to 1 mg. per hundred cubic centimeters, is a rather poor source of vitamin C. The 5 mg. of ascorbic acid represented by a pint of average commercial milk is far below that recommended for optimum nutrition. This fact has led to the general use of orange juice, tomato juice or potent antiscorbutic substances as a supplement to the infant diet. A breast-fed infant of a normally nourished mother receives from 20 to 40 mg. of ascorbic acid daily from the day of birth, because human milk is from four to six times richer in vitamin C than cow's or goat's milk.[95] The implication is strong that vitamin C should be a constituent of the infant's diet at a very early age.

The ascorbic acid value of canned or dried milk[2] depends primarily on the method by which the milk is prepared or handled. Commercially evaporated milk which has been sterilized at a high temperature is almost free from vitamin C, while sweetened condensed milk which has been evaporated in vacuum at lower temperatures retains a proportion of vitamin C comparable to that of pasteurized milk. Several investigators have reported good conservation in dried milk.[68] However, most commercial preparations are practically free from vitamin C.[15]

The vitamin C concentrations of individual foods are summarized in the accompanying table. Most of the analyses reported were on fresh material. It is difficult to estimate values for cooked or processed food because of the lack of uniformity of methods and treatment. Lower vitamin C values than those given must be expected because of immaturity, lack of freshness, poor preservation, long storage or results of processing. The figures given are estimates derived after consideration of the available data,[96] including the results of many unpublished analyses from my own laboratory, and represent my judgment of the expected value of the raw edible portions under good market conditions. The value when the food is ready for serving will depend on the method of preparation. A recent United States Department of Agriculture bulletin[4] contains fuller details of the vitamin C contents of foods and also references to the literature.

---

90. Fellers, C. A.: Vitamin Content of Important Food in the Child's Diet, Am. J. Pub. Health **25**: 1340 (Dec.) 1935. Hanning, Flora: Comparison of the Biological and Chemical Method for the Determination of Vitamin C in Canned Strained Vegetables and a Study of Its Variation from Year to Year, J. Nutrition **12**: 405 (Oct.) 1936.

91. Rasmussen, Russel, and Guerrant, N. B.: The Effect of Breed Characteristics and Stage of Lactation on the Vitamin C (Ascorbic Acid) Content of Cow's Milk, J. Nutrition **11**: 425 (May) 1936. Riddell, W. H.; Whitnah, C. H.; Hughes, J. S., and Leinhardt, H. F.: Influence of the Ration on the Vitamin C Content of Milk, J. Nutrition **11**: 47 (Jan.) 1936. King and Waugh.[93]

92. Kon, S. K., and Watson, M. B.: The Effect of Light on the Vitamin C of Milk, Biochem. J. **30**: 2273 (Dec.) 1936. King.[23]

93. King, C. G., and Waugh, W. A.: Effect of Pasteurization on the Vitamin C Content of Milk, J. Dairy Sc. **17**: 489, 1934. Schwartze, E. W.; Murphy, F. J., and Hann, R. M.: Studies on the Destruction of Vitamin C in the Boiling of Milk, J. Nutrition **2**: 325 (March) 1930.

94. Hess, cited by Sherman.[53]

95. Selleg, Ina, and King, C. G.: The Vitamin C Content of Human Milk and Its Variation with Diet, J. Nutrition **11**: 599 (June) 1936.

Arch. Int. Med., 71:315-324

076

PROTECTIVE ACTION OF VITAMIN C AGAINST EXPERIMENTAL HEPATIC DAMAGE

KARL H. BEYER, PH.D.
MADISON, WIS.

An associate and I [1] previously found that if a hepatoxin, carbon tetrachloride, were given to dogs they could be made to excrete all instead of about half of a given dose of the sympathomimetic amine amphetamine. More recently I showed that if dogs were put on a maintenance dose of the amine, saturating the animals with vitamin C (ascorbic acid) would reduce the excretion of amphetamine to about one third of the amount normally excreted.[2] This together with the in vitro deamination of the amine demonstrated the role of ascorbic acid in the detoxication of these compounds. However, to our surprise, if dogs saturated with vitamin C were given carbon tetrachloride orally, the excretion of amphetamine did not rise above normal.

These observations suggested that ascorbic acid might protect the animals against the toxic action of carbon tetrachloride on the liver. It was deemed more suitable to turn to guinea pigs for the pursuit of the problem. The purpose of this study, then, has been to determine whether ascorbic acid administered to a group of guinea pigs maintained on a scorbutogenic diet would protect them against hepatic damage.

PROCEDURE

All the animals were maintained on a diet of Purina mixed rabbit chow checkers,[3] which is quite adequate except for vitamin C. In some instances the pellets of food were ground and mixed with 5 per cent brewers' yeast to supplement the vitamin B complex without influencing the results. Ecker and Pillemer [4] have reported that guinea pigs maintained on a similar diet showed signs of scurvy in twenty-one days and that 10 to 20 mg. of ascorbic acid per day was necessary to maintain maximal plasma concentration of the vitamin. Throughout the experiments the animals receiving ascorbic acid were given 30 mg. daily. This was made up with half its weight of sodium bicarbonate, dissolved in an equivalent of 1 cc. of distilled water and injected subcutaneously immediately.

Hydrazine sulfate was selected for the experimental production of hepatic damage, for Wells [5] has shown that it is a poison with an almost specific effect on the cytoplasm of the parenchymatous cells of the liver. When this chemical is given, the liver alone evidences structural alterations. The substance attacks the cytoplasm primarily, only much later affecting the nucleus, and causes profound fatty degeneration. The urinary products of dogs seem quite normal after a 50 mg. per kilogram dose of hydrazine sulfate, according to Underhill and Kleiner.[6]

From the Department of Physiology, University of Wisconsin Medical School.

1. Beyer, K. H., and Skinner, J. T.: The Detoxication and Excretion of Beta Phenylisopropylamine (Benzedrine), J. Pharmacol. & Exper. Therap. **68**:419 (April) 1940.

2. Beyer, K. H.: The Action of Vitamin C and Phenol Oxidase in the Inactivation of Beta Phenylisopropylamine, J. Pharmacol. & Exper. Therap. **71**:394 (April) 1941.

3. The composition of the diet was as follows: protein, 15.35 per cent; fat, 2.38 per cent; carbohydrates, 64.61 per cent (including 16.86 per cent fiber); calcium carbonate; phosphorus; sodium chloride; potassium iodide; magnesium sulfate, and per pound (0.5 Kg.) approximately 5,000 international units of vitamin A, approximately 400 international units of vitamin B_1, approximately 3,000 micrograms of riboflavin (vitamin B_2) and negligible vitamin C.

4. Ecker, E. E., and Pillemer, L.: Vitamin C Requirement of the Guinea Pig, Proc. Soc. Exper. Biol. & Med. **44**:262 (May) 1940.

5. Wells, H. G.: The Pathological Anatomy of Hydrazine Poisoning, J. Exper. Med. **10**:457 (July) 1908.

6. Underhill, F. P., and Kleiner, J. S.: The Influence of Hydrazine upon Intermediary Metabolism in the Dog, J. Biol. Chem. **4**:165 (Feb.) 1908.

0767

The experiment was set up as follows: For purposes of comparison it was necessary to know the percentage of lipoid in the livers of normal guinea pigs, of ones rendered deficient in vitamin C and of ones given an injection of 30 mg. of ascorbic acid per day. This was to determine whether low or high intake of the vitamin influenced the lipoid content of the liver. To determine whether vitamin C protected the animals against hepatic damage caused by hydrazine other guinea pigs weighing between 250 and 400 Gm. were divided into two groups of 6 or more each. Both groups were placed on the vitamin C-deficient diet, and their gain in weight was followed every other day. One group (A) received 30 mg. of ascorbic acid subcutaneously daily. The other group (B) did not receive injections of the vitamin. At first the gain in weight in both groups was the same, but later the animals in group A gained more rapidly than did the ones in group B. After about sixteen days the guinea pigs in group B ceased to gain weight. Then the guinea pigs in both groups were given injections of 25 mg. of hydrazine sulfate per kilogram of body weight on two successive days. Two days after the second injection of hydrazine the animals were killed and autopsied. The livers were removed from the bodies, examined grossly and in many instances photographed with kodachrome film for future reference. After that, the livers were weighed and a section was preserved for histologic study. A weighed amount of the remainder of each liver was dried in an oven, weighed again and powdered for extraction of the lipoid fraction. The lipoids of a weighed amount of liver from each guinea pig were extracted

TABLE 1.—*Analysis of Livers from Normal Guinea Pigs*

Number of Guinea Pig	Washed Liver, Percentage of Body Weight	Dry Matter, Percentage of Total Weight of Liver	Fat, Percentage of Dry Matter of Liver
N-1	4.2	32.8	9.1
N-2	4.1	36.2	9.7
N-3	3.8	31.2	9.3
N-4	3.8	30.5	8.0
N-5	3.5	30.7	9.3
N-6	3.9	32.3	11.0
N-7	3.7	30.0	13.5
N-8	3.6	28.0	14.0
N-9	3.2	28.3	12.3
N-10	4.3	32.3	11.9
N-11	2.7	45.0	8.8
N-12	4.0	38.8	7.5
N-13	4.7	30.2	9.2
N-14	4.9	29.6	9.1
Average	3.9	32.5	10.2

with chloroform in a modified Soxhlet continuous extractor, the period of extraction being twenty-four hours, and at least two determinations were made on each sample of liver. After extraction the chloroform was allowed to evaporate from the lipoid fraction at 80 to 90 F.; the residue was weighed and calculated as a percentage of the dry weight of liver. Thus anatomic and chemical evidence of any damage done to the livers was obtained.

RESULTS

Analysis of Normal Livers.—The results of analysis of the livers of 10 normal guinea pigs are given in table 1. The washed livers removed from the bodies of normal guinea pigs represented an average of 3.9 per cent of the body weight, the dry matter 32.5 per cent of the total liver weight and the lipoids 10.2 per cent of the dry matter. Control values for livers from 10 guinea pigs receiving 30 mg. of ascorbic acid subcutaneously for sixteen days are given in table 2. Comparing tables 1 and 2, one can see that the data in the latter table agree remarkably closely with those for the normal guinea pigs.

In an effort to help clear up an uncertainty [7] about the effect of avitaminosis C on hepatic fat and for comparison with other data, 18 guinea pigs were maintained

7. Spellberg, M. A., and Keeton, R. W.: Production of Fatty Livers in Guinea Pigs with Scorbutogenic Diets, Proc. Soc. Exper. Biol. & Med. **41**:570 (June) 1939.

on the vitamin C–deficient diet for sixteen days and then killed, and their livers were examined and analyzed. Grossly, these livers were no different from those of normal guinea pigs. If one expresses the relation as percentage of body weight, the livers from deficient and from normal guinea pigs were similar. On the basis of these data the livers of vitamin C–deficient animals tended to have a higher lipoid content than did those of animals on an adequate diet, but the difference was not significant, especially since only 7 of the 18 vitamin C–deficient animals had a

TABLE 2.—*Analysis of Livers from Guinea Pigs Given Injections of Ascorbic Acid*

Number of Guinea Pig	Washed Liver, Percentage of Body Weight	Dry Matter, Percentage of Total Weight of Liver	Fat, Percentage of Dry Matter of Liver
H-1	3.9	28.4	9.8
H-2	5.0	24.3	9.9
H-3	4.0	28.8	11.1
H-4	3.7	28.8	11.1
H-5	5.3	28.3	10.8
H-6	3.9	30.4	10.0
H-7	3.4	30.8	14.0
H-8	3.7	29.5	12.8
H-9	3.7	31.3	11.8
H-10	3.8	25.6	14.0
Average	4.0	28.6	11.5

TABLE 3.—*Analysis of Livers from Vitamin C–Deficient Guinea Pigs*

Number of Guinea Pig	Washed Liver, Percentage of Body Weight	Dry Matter, Percentage of Total Weight of Liver	Fat, Percentage of Dry Matter of Liver
D-1	3.2	24.7	10.7
D-2	3.7	28.4	13.0
D-3	3.5	29.1	12.6
D-4	3.6	32.0	14.4
D-5	4.0	29.0	17.4
D-6	5.4	27.8	12.5
D-7	5.1	21.8	11.7
D-8	3.6	23.1	9.9
D-9	4.0	21.5	9.1
D-10	3.8	21.8	9.7
D-11	4.0	25.0	12.4
D-12	4.0	27.6	15.5
D-13	4.6	22.0	13.9
D-14	3.8	23.7	14.5
D-15	4.2	26.1	12.9
D-16	4.0	23.1	11.2
D-17	3.8	22.8	12.1
D-18	4.3	25.2	12.4
Average	4.0	25.3	12.5

lipoid content above the average of 12.5 per cent for that group (table 3). On the other hand, since vitamin C deficiency does markedly impair hepatic function, one cannot entirely dismiss this slight increase.

Vitamin C and Hepatic Damage.—Having found that there is really little difference histologically or in fat content between the livers of guinea pigs that have been maintained on an adequate diet and those maintained on a vitamin C–deficient diet with or without injections of ascorbic acid, I could then examine my original hypothesis—that vitamin C protects against hepatic damage. The experiments were set up as described in the preceding section. Briefly, two groups of guinea pigs were maintained on a diet deficient in vitamin C, and one group (A)

0767

was given daily injections of ascorbic acid. Guinea pigs in both groups received hydrazine on the same days after those in group B ceased to gain weight.

Table 4 presents data on 19 vitamin C-deficient guinea pigs, each of which was given a total of 50 mg. of hydrazine sulfate per kilogram of body weight

TABLE 4.—*Analysis of Livers from Vitamin C-Deficient Guinea Pigs Given Injections of Hydrazine*

Number of Guinea Pig	Washed Liver, Percentage of Body Weight	Dry Matter, Percentage of Total Weight of Liver	Fat, Percentage of Dry Matter of Liver
DH-1	10.2	14.8	20.0
DH-2	5.0	29.4	10.2
DH-3	5.5	19.3	22.6
DH-4	5.3	26.4	27.5
DH-5	6.4	23.5	19.0
DH-6	5.7	24.0	19.8
DH-7	4.3	26.8	18.9
DH-8	4.4	26.0	20.0
DH-9	4.0	24.5	17.4
DH-10	5.9	26.6	25.8
DH-11	5.2	22.3	29.8
DH-12	7.1	21.8	30.0
DH-13	5.5	25.7	23.2
DH-14	5.1	21.4	20.5
DH-15	4.7	26.6	24.3
DH-16	5.3	23.7	17.0
DH-17	5.3	22.0	24.0
DH-18	4.5	29.6	34.2
DH-19	4.9	29.0	31.8
Average	5.5	24.4	23.3

TABLE 5.—*Analysis of Livers from Guinea Pigs Given Both Vitamin C and Hydrazine*

Number of Guinea Pig	Washed Liver, Percentage of Body Weight	Dry Matter, Percentage of Total Weight of Liver	Fat, Percentage of Dry Matter of Liver
HH-1	5.5	25.0	15.6
HH-2	4.8	24.5	16.7
HH-3	4.8	27.6	18.5
HH-4	4.7	27.2	14.8
HH-5	5.6	22.0	15.8
HH-6	4.0	21.3	14.2
HH-7	4.2	21.5	15.9
HH-8	4.2	21.9	15.2
HH-9	5.0	19.0	12.0
HH-10	4.0	23.3	16.3
HH-11	3.3	22.3	15.2
HH-12	4.5	20.8	12.1
HH-13	3.8	28.2	17.8
HH-14	4.4	23.0	16.2
HH-15	4.7	19.5	14.7
HH-16	5.5	23.0	14.8
HH-17	4.2	22.5	15.4
HH-18	4.1	29.6	14.3
Average	4.5	23.4	15.5

subcutaneously. Compared to the vitamin C-deficient animals in table 3, there was a 37.5 per cent increase in the liver weight as compared to body weight in the case of the animals rendered toxic. The figures for percentage of water and dry matter are almost identical in the two sets of data. One has only to compare the lipoid content of the two groups of animals to visualize the fatty nature of the livers of the animals receiving hydrazine. There was an 86.5 per cent increase

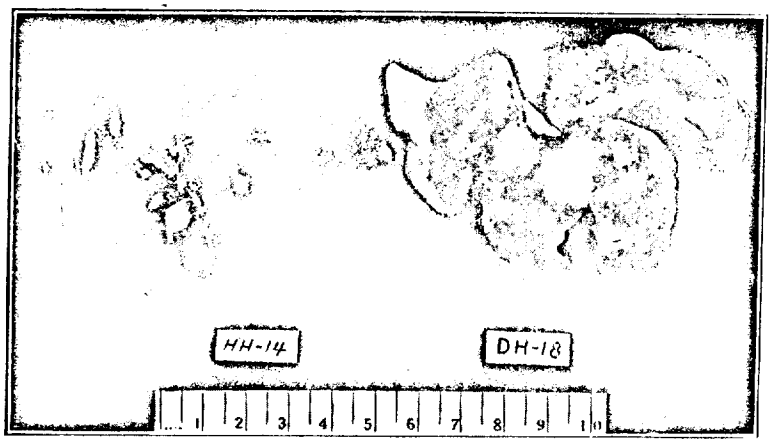


Fig. 1.—The effect of hydrazine on the livers of guinea pigs. Guinea pig HH-14 had received subcutaneous injections of 30 mg. of ascorbic acid per day. Guinea pig DH-18 had not received vitamin C. Both animals were on a scorbutogenic diet for sixteen days before being given hydrazine sulfate, 25 mg. per kilogram on two successive days. Expressed as percentage of total body weight, the liver of guinea pig DH-18 was 68 per cent heavier than that of guinea pig HH-14.

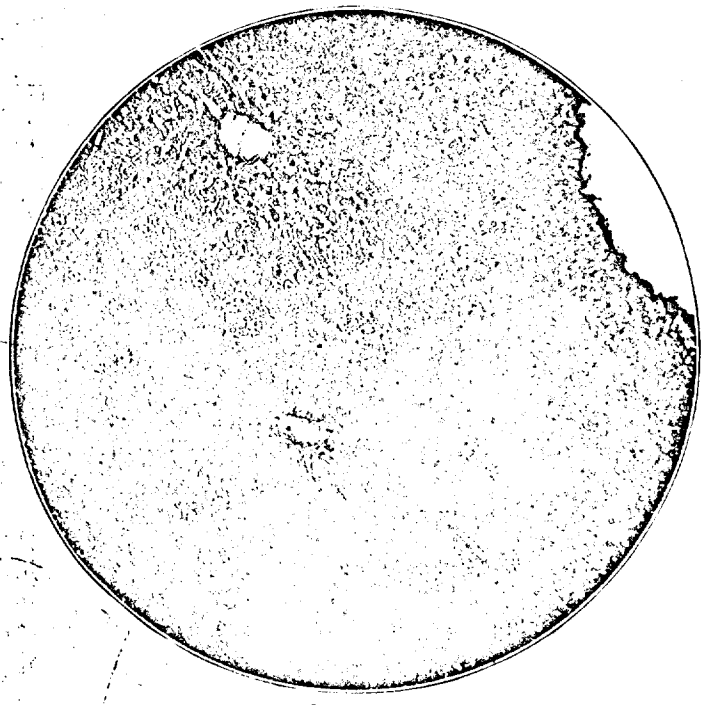


Fig. 2.—A section from the liver of guinea pig HH-14; × 100, stained with hematoxylin and eosin. This section was selected to show one lobule with the central degeneration typical of hydrazine poisoning. The architecture around the smaller central vein is more typical of the animals that have received adequate vitamin C.

0767

in the lipoid content of the livers of vitamin C–deficient animals that received hydrazine over that of the control avitaminotic animals.

Hydrazine did increase the lipoid content of the liver in animals that received an adequate amount of vitamin C (table 5), as compared with the controls given injections of the vitamin (table 2). There was no significant increase in the weight of these livers, though the percentage of dry matter was decreased 18.2, which suggests the hydropic changes verified by histologic examination.

Finally, a comparison of tables 4 and 5 is of interest. As a result of the hydrazine poisoning those animals that did not receive an adequate amount of vitamin C showed a 22.2 per cent greater liver weight and a 50.3 per cent greater lipoid content of their livers than did the guinea pigs that received ascorbic acid.

Fig. 3.—A portion of the section shown in figure 2; × 400. The cellular appearance is essentially normal. There are some hydropic changes in the cytoplasm.

Gross and Microscopic Evidence.—These analytic findings are borne out both by gross and by microscopic evidence.

Grossly, the livers of animals that received vitamin C and hydrazine were almost indistinguishable from the livers of normal guinea pigs. The livers of the guinea pigs deficient in vitamin C and given injections of hydrazine were relatively large, pale to definitely yellow, friable, somewhat hemorrhagic and yellowish on cut surfaces. Figure 1 compares the livers from 2 guinea pigs. Both animals were given injections of hydrazine, but guinea pig HH-14 received vitamin C and guinea pig DH-18 did not. The difference in size is not so apparent unless one appreciates the difference in weight of the 2 animals. Guinea pig HH-14 weighed 430 Gm., and its liver weighed 19.1 Gm. Guinea pig DH-18 weighed 290 Gm., and

its liver weighed 20.6 Gm. The paleness of the liver of guinea pig DH-18 as compared with that of guinea pig HH-14 is evident. Actually, the former liver was quite yellow.

Microscopically, it is in the livers of guinea pigs having received ascorbic acid that the early stages in the pathogenesis of this fatty degeneration due to hydrazine can be traced best. Here one sees that the process begins at the center of the lobule, decreasing in severity as it spreads toward the periphery. The process begins as a cloudy swelling of the cytoplasm. As the degeneration develops, one sees areas of condensation and rarefaction in the cytoplasm, as manifested by staining reactions; cell demarcation becomes indistinct, and the architecture of

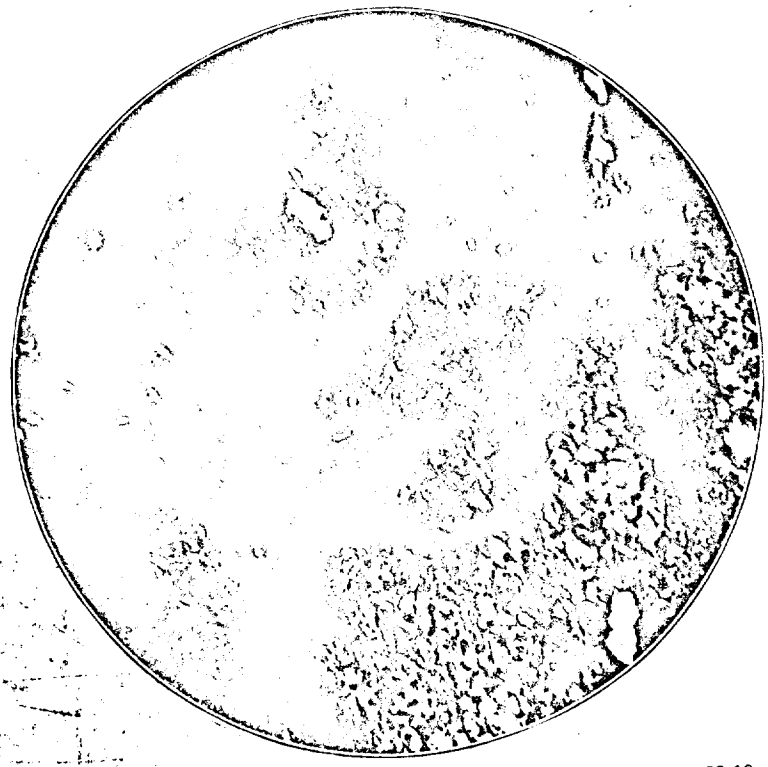


Fig. 4.—A section from the liver of vitamin C-deficient guinea pig DH-19 which had been damaged by hydrazine; × 100, stained with hematoxylin and eosin. Though the magnification is the same as that of figure 2, the increase in the size of cells, the general loss of architecture and the destruction of cells and nuclei involved in the fatty necrosis are obvious. Both this liver and that represented in figures 2 and 3 had lipoid contents approximating the average for the respective experimental groups.

the lobule becomes increasingly distorted. Finally, one may see isolated cells containing vacuoles in the cytoplasm, presumptive evidence of the presence of fat at one time. Manifestation of nuclear involvement is not apparent until relatively late in the process, and then it is one of early karyolysis. This is seldom seen in the livers of animals receiving adequate amounts of vitamin C.

Figures 2 and 3 show a section from the liver of guinea pig HH-14 at two different magnifications. The section under low power (fig. 2) has been deliberately selected to show the central involvement of the lobule typical of the patho-

0767

genesis of this process but infrequently advanced to this degree in the livers of guinea pigs that have received vitamin C. The granular, fairly normal appearance of the cytoplasm is evident at the higher magnification (fig. 3).

Examination of sections from livers of vitamin C-deficient guinea pigs that have received hydrazine reveals the final stages in this pathogenesis. Here one sees relatively little of the early stages, for in almost all the lobules there is widespread vacuolation of the swollen cells, some of them containing nuclei having some semblance of normality and others showing only a hematoxylin-stained shell or nuclear membrane. In some lobules the process has gone on to necrosis. In these the remnants of cells are large, containing little or no cytoplasmic material

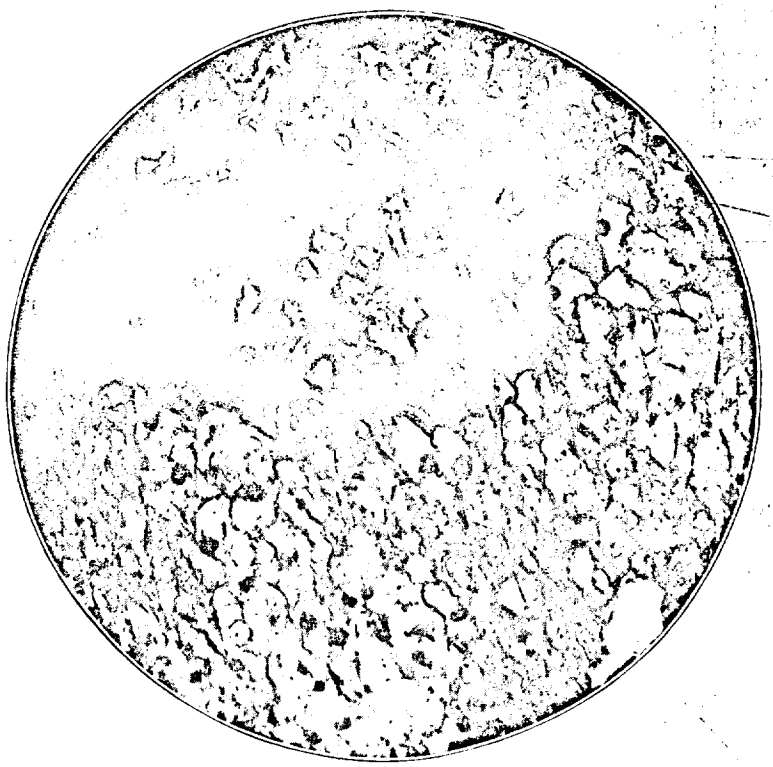


Fig. 5.—A portion of the section shown in figure 4; × 400.

and sometimes no evidence of a nucleus. These later developments are to be seen in figures 4 and 5, the magnifications of which are the same as those of figures 2 and 3, respectively.

COMMENT

It is not to be suggested that ascorbic acid supplant the use of dextrose in the treatment of acute and subacute hepatic damage. Rather, it would seem that the two substances used together would be more efficacious than either alone. There is evidence for such a viewpoint.

Beddard,[8] in 1908, was probably the first to report the use of carbohydrate in the treatment of a hepatic disease, though the work of Rosenfeld[9] also led

8. Beddard, A. P.: A Suggestion for Treatment in Delayed Chloroform Poisoning, Lancet **1**:782 (March 14) 1908.

9. Rosenfeld, G.: Fat Synthesis: Part II, Ergebn. d. Physiol. **2**:50, 1903.

0767

him to suggest that dextrose might be beneficial in the prevention and treatment of hepatic necrosis induced by chloroform anesthesia. While the use of this form of therapy has had wide acceptance, the actual explanation of how it works is not yet clear. Recently it has been suggested that a diet emphasizing protein and carbohydrate may be better than one of carbohydrate alone.[10]

There is increasing evidence that ascorbic acid plays a significant role in the metabolism of proteins and carbohydrates in the liver, as well as in detoxication and the healing of wounds. Miwa[11] showed that in experimental scurvy the Golgi apparatus was decreased, but if ascorbic acid was given both the vitamin C level and the Golgi apparatus increased in the liver and the kidney. Also, the number of Golgi apparatus nearly paralleled the content of vitamin C in the damaged livers of rabbits. Murakami[12] found that feeding guinea pigs on a vitamin C–deficient diet decreased the excretion of bile and of bile pigment together with the detoxicating functions of the liver. If ascorbic acid was given to the animals, the disturbance in these functions was removed. In view of the present understanding of the function of the Golgi apparatus, these observations of Miwa and Murakami tend to substantiate each other.

It has been known since 1903 that leucine, tyrosine and aminoacetic acid[13] are eliminated in considerable quantities in the urine after poisoning with the hepatotoxin phosphorus, but only recently evidence has been found for a direct or an indirect participation of vitamin C in protein metabolism. Romanyuk[14] has shown that cathepsin activity of kidney, liver and muscle was increased during avitaminosis C in guinea pigs. Levine, Marples and Gordon[15] reported that some premature infants excreted parahydroxyphenyllactic acid and parahydroxyphenylpyruvic acid. If phenylalanine or tyrosine was fed to the infants, the excretion of the substances just mentioned increased, but if human milk or ascorbic acid was given in the diet, the excretion of the compounds dropped to zero. Similar results were obtained by Sealock and Silberstein,[16] who found that vitamin C–deficient guinea pigs fed 0.5 Gm. or more of tyrosine per day excreted homogentisic acid, parahydroxyphenylpyruvic acid and parahydroxyphenyllactic acid. If 10 mg. or so of ascorbic acid was fed daily to the animals, the excretion of these metabolites was prevented.

---

10. (*a*) Miller, L. L., and Whipple, G. H.: Chloroform Liver Injury Increases as Protein Stores Decrease: Studies in Nitrogen Metabolism in These Dogs, Am. J. M. Sc. **199**: 204 (Feb.) 1940. (*b*) Messinger, W. J., and Hawkins, W. B.: Arsphenamine Liver Injury Modified by Diet: Protein and Carbohydrate Protective, but Fat Injurious, ibid. **199**:216 (Feb.) 1940. (*c*) Ravdin, I. S.: The Protection of the Liver from Injury, Surgery **8**:204 (Aug.) 1940.

11. Miwa, A.: Golgi's Apparatus and Vitamin C: Experimental Studies, Orient. J. Dis. Infants **26**:3 (July) 1939.

12. Murakami, O.: The Influence of Ascorbic Acid upon the Liver Function and the Mutual Relation Between Vitamins B and C: I. Influence of Ascorbic Acid upon the Pigment Excreting Function of the Liver, Jap. J. Gastroenterol. **11**:1 (July) 1939.

13. Abderhalden, E., and Bergell, P.: Concerning the Elimination of Monoaminoacids in the Urine of Dogs Following Phosphorus Poisoning, Ztschr. f. physiol. Chem. **39**:464, 1903.

14. Romanyuk, N. M.: Cathepsin Activity in the Brain and Muscles During C Avitaminosis, Biochem. J. (Ukraine) **14**:354, 1940.

15. Levine, S. Z.; Marples, E., and Gordon, H. H.: Defect in the Metabolism of Aromatic Amino Acids in Premature Infants: The Role of Vitamin C, Science **90**:620 (Dec. 29) 1939.

16. Sealock, R. R., and Silberstein, H. E.: The Excretion of Homogentisic Acid and Other Tyrosine Metabolites by the Vitamin C Deficient Guinea Pig, J. Biol. Chem. **135**:251 (Aug.) 1940.

0767

Since 1865 it has been repeatedly shown that in hepatic damage by hepatotoxins the glycogen content of the liver is rapidly and almost completely exhausted.[17] An interesting isolated observation was made by Ratsimamanga,[18] namely, that in guinea pigs maintained on a vitamin C-deficient diet liver glycogen and muscle glycogen decreased to one fifth of normal and phosphocreatine fell to one third of normal, while muscle lactate and blood lactate tended to increase. The reports of Watanabe,[19] Morelli and d'Ambrosio[20] and Terada[21] are in agreement that ascorbic acid injected into guinea pigs or rabbits increased the glycogen content of the liver. According to Watanabe, vitamin A and vitamin D had no effect and vitamin B_1 and vitamin B_2 had but slight effect on glycogen production. Hjorth[22] and Dienst[23] found that administration of vitamin C increased the dextrose tolerance of normal and of vitamin C-deficient patients. Fan and Woo[24] and Vasile and Pecorella[25] observed that in children with glycogen disease and creatinuria there was a disappearance of creatine and an increase in the excretion of creatinine in the urine if 200 mg. of ascorbic acid was fed daily. This Vasile and Pecorella attributed to an increased utilization of carbohydrate in the presence of the vitamin, resulting in an increased accumulation of glycogen in the tissues.

Since on the basis of existing evidence vitamin C does play a part in the intermediate metabolism of proteins and is necessary for the presence of Golgi apparatus and production of bile and its pigments and since its absence or presence results in a diminution or increase in the glycogen content of the liver with a decrease or increase in dextrose tolerance, it seems a definite factor in the maintenance and repair of hepatic function and structure. The experiments presented in this communication seem to bear out the theory that vitamin C does protect against hepatic damage.

SUMMARY

Analytic and histologic evidence is presented for the protective action of vitamin C against hepatic damage. Vitamin C-deficient guinea pigs showed an average of 50.3 per cent more fat in their livers than did guinea pigs receiving adequate ascorbic acid when both groups receiving the same diet were given the hepatotoxin hydrazine. Gross and microscopic evidence bore out the severity of the fatty degeneration in the vitamin C-deficient animals and the relatively mild involvement of the livers of normal guinea pigs receiving the compound.

While glycogen replacement therapy has met with the accord of time, carbohydrate utilization and glycogen storage, as well as intermediary metabolism of protein, are at least in part dependent on an adequate body content of ascorbic acid.

426 North Charter Street.

17. Saikowsky: Concerning the Fatty Metamorphosis of the Organs Following the Internal Use of Arsenic, Antimony and Phosphorus, Virchows Arch. f. path. Anat. **34**:73, 1865.

18. Ratsimamanga, A. R.: The Influence of Ascorbic Acid on the Behavior of the Organism at Rest, Travail humain **1**:303, 1939.

19. Watanabe, I.: Effect of Various Vitamins on Glycogen Content of Experimentally Damaged Liver, Tohoku J. Exper. Med. **35**:65 (Jan.) 1939.

20. Morelli, A., and d'Ambrosio, L.: Ascorbic Acid and Liver Glycogen, Arch. di sc. biol. **24**:351 (Aug.) 1938.

21. Terada, K.: Effect of Vitamin C and Related Substances upon Glycogen Metabolism, Tohoku J. Exper. Med. **36**:180 (July) 1939.

22. Hjorth, P.: The Influence of Vitamin C on Carbohydrate Metabolism, Acta med. Scandinav. **105**:67, 1940.

23. Dienst, C.: Action of Vitamins B and C in Diabetes, Deutsche med. Wchnschr. **65**: 710 (May 5) 1939.

24. Fan, C., and Woo, T. T.: Effect of Vitamin C on Creatine and Creatinine Metabolism, Proc. Soc. Exper. Biol. & Med. **45**:90 (Oct.) 1940.

25. Vasile, B., and Pecorella, F.: The Influence of Vitamins on the Metabolism of Creatin Substances in Childhood: I. The Effect of Vitamin C, Pediatria **47**:130 (Feb.) 1939.

Indian J. Biochem. Biophys. 8(1): 57-58 1971

0816

Changes in Rat Ascorbic Acid Status & Adrenocortical Activity during Acute Dieldrin Toxicity

S. C. Bhatia, S. C. Sharma, V. N. Damodaran & T. A. Venkitasubramanian

Vallabhbhai Patel Chest Institute, University of Delhi
Delhi 7

Manuscript received 31 *July* 1970; *revised manuscript received* 23 *January* 1971

Rats fed dieldrin at a dose level of 30 mg/kg body weight show a significant increase in plasma, liver, spleen, kidney, heart and brain ascorbic acid concentrations and a decrease in cholesterol and ascorbic acid contents of adrenals as compared to normal controls. Lowered adrenal cholesterol and ascorbic acid contents suggest increased adrenocortical activity in dieldrin toxicity.

DIELDRIN is a widely used organochloro insecticide. Spraying of crops with dieldrin involves risk of its intake by animals and humans. The reports available in literature on the biochemical lesions caused by dieldrin are very scanty. In order to understand the biochemical mechanism of the insecticide poisoning, we have undertaken a systematic study of the various metabolic derangements caused during acute dieldrin toxicity. In the present communication, we report changes in ascorbic acid content of different tissues and plasma of rats. The effect of dieldrin toxicity on the adrenocortical activity has also been evaluated by determining the cholesterol and ascorbic acid contents of adrenals.

Male albino rats (Wistar strain), weighing 110-125 g, were divided into two groups (control and experimental). Dieldrin*, dissolved in groundnut oil, was fed orally to the experimental rats in a single dose of 30 mg/kg body weight. Control animals received appropriate amounts of vehicle groundnut oil only. Immediately after treatment, food was withdrawn from all cages.

After 24 hr of fasting, the animals were stunned by a blow on the head and blood collected by cutting the jugular vein. The rats were then decapitated and liver, spleen, kidneys, heart, brain and adrenals removed. Tissues were freed from adhering blood by dipping in chilled normal saline and pressing between folds of filter paper and homogenized in cold 10% trichloroacetic acid. Plasma was deproteinized with 6% trichloroacetic acid. Total ascorbic acid was estimated in the protein-free extracts by the method of Roe and Kuether[1]. Adrenals were ground in a mixture of chloroform-methanol (2:1, vol./vol.). The chloroform-methanol extracts were evaporated to dryness and lipids were extracted with chloroform. Suitable aliquots of the chloroform solution were used for cholesterol estimation[2].

In rats, dieldrin at a concentration of 30 mg/kg body weight resulted in a significant increase in the ascorbic acid content of plasma, liver, kidney, spleen, heart and brain and a significant decrease in cholesterol and ascorbic acid contents of adrenals (Tables 1 and 2). However, no significant change was observed in the weight of the adrenals.

Ascorbic acid plays an important role in maintaining the normal intracellular material of cartilage, dentine and bone, collagen synthesis[3], oxidation and reduction systems[4] and resistance to infection. Its concentration has been found to be altered in a variety of conditions which cause stress to the animal[5-7]. The presence of an insecticide in the body, particularly after an acute exposure, constitutes a stress condition, and the common physiological response which follows is an increased stimulation of the pituitary and adrenal glands[8]. Ascorbic acid and cholesterol contents of the adrenals and their weights have been used as a measure to assess the gland's activity[9]. The observed low levels of these two constituents in the adrenals of dieldrin-treated rats may be interpreted as indicative of increased adrenocortical activity. Lahiri and Llyod[10] have demonstrated that corticotrophins bring about an increase in ascorbic acid titre in liver, spleen and kidney, but a decrease is manifested in the adrenals. Yates *et al.*[11,12] have forwarded a concept that stress leads to increased pituitary adrenocortical activity by increasing the requirement of peripheral tissue cells for cortical hormones. The role of cholesterol as a principal precursor of steroidal hormones has been well established[13] and this may explain the lowered adrenal cholesterol levels encountered in

Table 1 — Effect of Feeding Dieldrin on Plasma and Tissue Ascorbic Acid Levels

(Values are expressed as mean ± SEM. Numerals given in parentheses indicate the number of animals)

Tissue	Ascorbic acid content*		p†
	Control	Experimental	
Plasma	2·10 ± 0·14 (4)	2·57 ± 0·12 (4)	0·02
Liver	383 ± 9 (8)	512 ± 14 (8)	0·01
Kidney	207 ± 14 (6)	346 ± 6 (6)	0·01
Spleen	391 ± 18 (5)	589 ± 32 (5)	0·01
Heart	121 ± 3 (5)	156 ± 4 (5)	0·01
Brain	459 ± 2 (6)	492 ± 12 (6)	0·02

*μg/g tissue in all cases except plasma for which it is expressed in mg/100 ml plasma.
†<0·05 is considered significant.

Table 2 — Effect of Feeding Dieldrin on Cholesterol and Ascorbic Acid Levels in Adrenals

(Values are expressed as mean of results from 6 rats in each group ± SEM)

Estimation	Control	Experimental	p*
Adrenal wt, mg	19·83 ± 0·7	21·47 ± 0·8	—
Cholesterol, mg/g tissue	11·86 ± 0·45	6·91 ± 0·36	0·01
Ascorbic acid, mg/g tissue	6·06 ± 0·17	4·22 ± 0·13	0·01

*<0·05 is considered significant.

*Kindly supplied by Shell Chemicals, Rotterdam.

Indian J. Biochem. Biophys. 8(1): 57-58 1971

0816

Changes in Rat Ascorbic Acid Status & Adrenocortical Activity during Acute Dieldrin Toxicity

S. C. Bhatia, S. C. Sharma, V. N. Damodaran & T. A. Venkitasubramanian

Vallabhbhai Patel Chest Institute, University of Delhi Delhi 7

Manuscript received 31 *July* 1970; *revised manuscript received* 23 *January* 1971

Rats fed dieldrin at a dose level of 30 mg/kg body weight show a significant increase in plasma, liver, spleen, kidney, heart and brain ascorbic acid concentrations and a decrease in cholesterol and ascorbic acid contents of adrenals as compared to normal controls. Lowered adrenal cholesterol and ascorbic acid contents suggest increased adrenocortical activity in dieldrin toxicity.

DIELDRIN is a widely used organochloro insecticide. Spraying of crops with dieldrin involves risk of its intake by animals and humans. The reports available in literature on the biochemical lesions caused by dieldrin are very scanty. In order to understand the biochemical mechanism of the insecticide poisoning, we have undertaken a systematic study of the various metabolic derangements caused during acute dieldrin toxicity. In the present communication, we report changes in ascorbic acid content of different tissues and plasma of rats. The effect of dieldrin toxicity on the adrenocortical activity has also been evaluated by determining the cholesterol and ascorbic acid contents of adrenals.

Male albino rats (Wistar strain), weighing 110-125 g, were divided into two groups (control and experimental). Dieldrin*, dissolved in groundnut oil, was fed orally to the experimental rats in a single dose of 30 mg/kg body weight. Control animals received appropriate amounts of vehicle groundnut oil only. Immediately after treatment, food was withdrawn from all cages.

After 24 hr of fasting, the animals were stunned by a blow on the head and blood collected by cutting the jugular vein. The rats were then decapitated and liver, spleen, kidneys, heart, brain and adrenals removed. Tissues were freed from adhering blood by dipping in chilled normal saline and pressing between folds of filter paper and homogenized in cold 10% trichloroacetic acid. Plasma was deproteinized with 6% trichloroacetic acid. Total ascorbic acid was estimated in the protein-free extracts by the method of Roe and Kuether[1]. Adrenals were ground in a mixture of chloroform-methanol (2:1, vol./vol.). The chloroform-methanol extracts were evaporated to dryness and lipids were extracted with chloroform. Suitable aliquots of the chloroform solution were used for cholesterol estimation[2].

In rats, dieldrin at a concentration of 30 mg/kg body weight resulted in a significant increase in the ascorbic acid content of plasma, liver, kidney, spleen, heart and brain and a significant decrease in cholesterol and ascorbic acid contents of adrenals (Tables 1 and 2). However, no significant change was observed in the weight of the adrenals.

Ascorbic acid plays an important role in maintaining the normal intracellular material of cartilage, dentine and bone, collagen synthesis[3], oxidation and reduction systems[4] and resistance to infection. Its concentration has been found to be altered in a variety of conditions which cause stress to the animal[5-7]. The presence of an insecticide in the body, particularly after an acute exposure, constitutes a stress condition, and the common physiological response which follows is an increased stimulation of the pituitary and adrenal glands[8]. Ascorbic acid and cholesterol contents of the adrenals and their weights have been used as a measure to assess the gland's activity[9]. The observed low levels of these two constituents in the adrenals of dieldrin-treated rats may be interpreted as indicative of increased adrenocortical activity. Lahiri and Llyod[10] have demonstrated that corticotrophins bring about an increase in ascorbic acid titre in liver, spleen and kidney, but a decrease is manifested in the adrenals. Yates *et al.*[11,12] have forwarded a concept that stress leads to increased pituitary adrenocortical activity by increasing the requirement of peripheral tissue cells for cortical hormones. The role of cholesterol as a principal precursor of steroidal hormones has been well established[13] and this may explain the lowered adrenal cholesterol levels encountered in

TABLE 1 — EFFECT OF FEEDING DIELDRIN ON PLASMA AND TISSUE ASCORBIC ACID LEVELS

(Values are expressed as mean ± SEM. Numerals given in parentheses indicate the number of animals)

Tissue	Ascorbic acid content*		p†
	Control	Experimental	
Plasma	2·10 ± 0·14 (4)	2·57 ± 0·12 (4)	0·02
Liver	383 ± 9 (8)	512 ± 14 (8)	0·01
Kidney	207 ± 14 (6)	346 ± 6 (6)	0·01
Spleen	391 ± 18 (5)	589 ± 32 (5)	0·01
Heart	121 ± 3 (5)	156 ± 4 (5)	0·01
Brain	459 ± 2 (6)	492 ± 12 (6)	0·02

*μg/g tissue in all cases except plasma for which it is expressed in mg/100 ml plasma.

†<0·05 is considered significant.

TABLE 2 — EFFECT OF FEEDING DIELDRIN ON CHOLESTEROL AND ASCORBIC ACID LEVELS IN ADRENALS

(Values are expressed as mean of results from 6 rats in each group ± SEM)

Estimation	Control	Experimental	p*
Adrenal wt, mg	19·83 ± 0·7	21·47 ± 0·8	—
Cholesterol, mg/g tissue	11·86 ± 0·45	6·91 ± 0·36	0·01
Ascorbic acid, mg/g tissue	6·06 ± 0·17	4·22 ± 0·13	0·01

*<0·05 is considered significant.

*Kindly supplied by Shell Chemicals, Rotterdam.

0868

Anat. Anz. Bd. 123, S. 250–254 (1968)

Department of Physiology, University College of Science Calcutta-9, India

Role of ascorbic acid on the histochemical changes in the prostate of scorbutic Guinea Pigs

By Narendra Mohan Biswas and Chandicharan Deb

With 6 figures

(Received January 15, 1968)

Introduction

Relationship between ascorbic acid and male reproductive organs have been studied by many investigators. Lindsay and Medes (1925, 1926) have reported degeneration of germinal epithelium together with reproductive failure in male guinea pigs during acute scurvy. Testicular degeneration and spermatogenic arrest in the above condition have also been reported (Biswas and Deb 1965). The changes in seminal vesicles during avitaminosis C have previously been studied (Hill and Bourne 1958, Kocen and Cavazos 1958).

The effect of vitamin C on the histochemical changes in scorbutic testis has also been investigated (Biswas and Deb 1967). The purpose of the present investigation was to determine the histochemical changes in the prostate of scorbutic guinea pigs after ascorbic acid administration.

Experimental Procedure

Thirty adult male guinea pigs of average weight 200–225 gms were selected and kept in the laboratory for a few days prior to experimentation. The animals were divided into three groups of equal number. All the animals were fed a scorbutic diet (Banerjee 1945) and also a concentrate of vitamins A, D and K twice a week. The control group received 5 mg of ascorbic acid per animal per day. Ten scorbutic guinea pigs were injected intramascularly with ascorbic acid (Redoxan 100 mg/100 g body weight) from the 16th day on a scorbutic diet and killed on the 21st day. Both the lateral and dorsal prostate were quickly removed and histochemical reactions were carried out on them as follows.

In fresh frozen sections, succinic dehydrogenase was demonstrated by the method previously reported (Biswas and Deb 1967). The tissues were fixed in 4% cold formol and the following reactions were performed on formol fixed frozen sections.

1. Neutral lipid according to Key and Whitehead (1949).
2. Plasmal reaction by the method of Hayes (1949).
3. Acid phosphatase by Gomori's improved histochemical technique (Gomori 1946).

Alkaline phosphatase was localized in cold acetone fixed tissues also according to Gomori (1950).

0868

Fig. 1. Succinic dehydrogenase activity in prostate of control guinea pig. × 96.

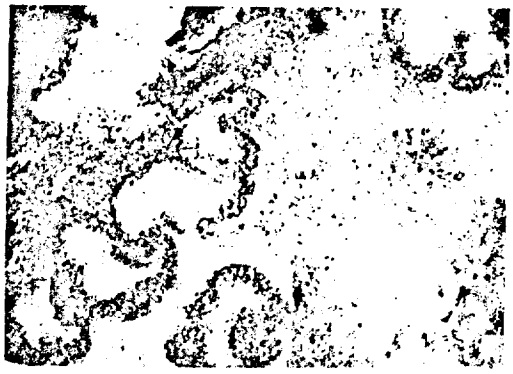


Fig. 2. A fall in succinic dehydrogenase in prostate of scorbutic guinea pig. × 96.

Fig. 3. Distribution of alkaline phosphatase in prostate of control guinea pig. × 96.

0868

Fig. 4. Alkaline phosphatase activity in prostate of scorbutic guinea pig. Same time of incul as fig. (3). A reduction in enzymatic reaction. ×96.

Fig. 5. Prostate from control animal showing acid phosphatase activity. ×96.

Fig. 6. Acid phosphatase activity in prostate of scorbutic animal after similar time of inc batio as fig. (5). A fall in enzymatic reaction observed. ×96.

0868

Results

In ascorbic acid deficient guinea pigs, the size of both lateral and dorsal prostate was found to be smaller than pair fed controls. There was atrophy of this accessory sex organ in severe scurvy, the columnar epithelial cells became flattened. Sudan positive substance was not present either in control or in scorbutic prostate but a fall in plasmal reaction has been noted on the later condition. There was also a fall in acid and alkaline phosphatase as well as succinic dehydrogenase in both lateral and dorsal prostate during avitaminosis C.

On administration of ascorbic acid to scorbutic guinea pigs, all the cytoplasmic constituents returned to control level.

Discussion

ALBERT and LEBLOND (1946) have demonstrated histochemically, a reduction in plasmal content of accessory sexorgans in rats after castration, which restored to normal level on androgen treatment. The fall in plasmal in the prostate of scorbutic guinea pigs, as observed in the present experiment was possibly due to insufficient androgen output from degenerated testes.

Castration in rats has also been observed to cause a reduction in succinic dehydrogenase content of prostate (DAVIS, MEYER and MCSHAN 1949). WILLIAMS-ASHMAN (1954) has noted that most of the enzymes of Krebs Cycle in rat prostate were markedly affected by castration. The reduction in succinic dehydrogenase as observed in ascorbic acid deficiency, might be due to similar reasons.

Role of androgens in regulating alkaline phosphatase (DEMPSEY, GREEP and WENDLER 1949, MELAMPY and CAVAZOS 1953) and acid phosphatase (MELAMPY and CAVAZOS 1953, PORTER and MELAMPY 1952) activity in male accessory sex organs have previously been demonstrated. MCCULLAGH and SCHAFFENBURG (1954) suggested the alkaline phosphatase in ventral prostate to be androgen dependent. Acid phosphatase content of this organ has also been observed to be controlled by male sex hormones. GUTMAN and GUTMAN (1939) have noted maximum amount of this enzyme in the prostate of adult monkey and traces in infants. A fall in both the enzymes content of the accessory sex organ is ascorbic acid deficiency was possibly due to male sex hormone deficiency in the system.

BISWAS and DEB (1967) have recently observed that the histochemical changes in the testes of scorbutic guenea pig return to normal level on ascorbic acid administration. The correction of histochemical abnormalities in the prostate of ascorbic acid deficient guinea pigs in the present investigation indicates the possibility of androgen output by ascorbic acid treatment.

0868

Acknowledgment

Thanks are due to Dr. S. R. MOITRA, Head of the Department of Physiology for laboratory facilities and encouragements. This project is being financed by University Grants Commission, New Delhi.

Summary

Physiology of the prostate has been studied in scorbutic and in ascorbic acid supplemented scorbutic guinea pigs. A fall in plasmal, alkaline and acid phosphatase, and succinic dehydrogenase has been observed in prostate during ascorbic acid deficiency. All these cytoplasmic constituents returned to control level on ascorbic acid administration.

Literature

ALBERT, S., & LEBLOND, C. P., Endocrinology **39**, 386 (1946).
BANERJEE, S., J. Biol. Chem. **159**, 327 (1945).
BISWAS, N. M., & DEB, C., Experimentia **21**, 542 (1965).
— Acta Histochem. **27**, 85 (1967).
DAVIS, J. S., MEYER, R. K., & MCSHAN, W. H., Endocrinology **44**, 1 (1949).
DEMPSEY, E. W., GREEP, R. O., & WENDLER, H., Endocrinology **44**, 88 (1949).
GOMORI, G., Am. J. Clin. Path. **16**, 347 (1946).
Stain Technol. **25**, 81 (1950).
GUTMAN, A. B., & GUTMAN, E. B., Proc. Soc. Exp. Biol. Med. **41**, 277 (1939).
HAYES, E. R., Stain Technol. **24**, 19 (1949).
HILL, C., & BOURNE, G., Brit. J. Nut. **12**, 237 (1958).
KAY, W. W., & WHITEHEAD, R., J. Path. Bact. **55**, 279 (1941).
KOCEN, B., & CAVAZOS, L. F., Proc. Soc. Exp. Biol. Med. **98**, 485 (1958).
LINDSAY, B., & MEDES, G., Proc. Soc. Exp. Biol. Med. **22**, 177 (1925).
— Am. J. Anat. **37**, 213 (1926).
MCCULLAGH, E. P., & SCHAFFENBURG, C. A., Glandular physiology and Therapy. J. B. Lippincott Co., London 1954.
MELAMPY, R. M., & CAVAZOS, L. F., Endocrinology **52**, 173 (1953).
PORTER, J. C., & MELAMPY, R. M., Endocrinology **51**, 412 (1952).
WILLIAMS-ASHMAN, H. G., Endocrinology **54**, 121 (1954).

Author's adress: Dr. N. M. BISWAS and Dr. CH. DEB, Department of Physiology, University College of Science, 92 Acharya Prafulla Chandra Road, Calcutta-9 (India).

Endocrinology 87: 170-3 Jul. '70

0871

Notes and Comments

In Vitro Studies on the Effects of Ascorbic Acid and Dehydroascorbic Acid on Δ^5-3β-Hydroxysteroid Dehydrogenase in Toad Testis

NARENDRA MOHAN BISWAS AND CHANDICHARAN DEB

Department of Physiology, University College of Science and Technology, 92, Acharya Prafulla Chandra Road, Calcutta 9, India

ABSTRACT. The enzyme Δ^5-3β-hydroxysteroid dehydrogenase was demonstrated histochemically in both the tubular and Leydig cells of the toad testes. Addition of 200 μg of dehydroascorbic acid (DHA) to 100 mg of testicular tissue slices increased the activity of Δ^5-3β-hydroxysteroid dehydrogenase, while a similar dose of ascorbic acid (AsA) failed to stimulate the enzyme. The evidence indicates that DHA participates in the activation of Δ^5-3β-hydroxysteroid dehydrogenase. (*Endocrinology* **87**: 170, 1970)

THE ROLE of ascorbic acid (AsA) in the synthesis of steroid hormones has been debated for some time without reaching any definite decision as to its function. AsA has been implicated in the electron transport chain between reduced diphosphopyridine nucleotide (DPNH) and one of the cytochromes (1). The DPN-dependent Δ^5-3β-hydroxysteroid dehydrogenase, which has been demonstrated in the Leydig cells of several mammalian species (2), is concerned with steroid hormone biosynthesis. Administration of AsA to normal toads stimulates testicular Δ^5-3β-hydroxysteroid dehydrogenase (3).

In the present investigation, the effects of AsA and dehydroascorbic acid (DHA) on the testicular Δ^5-3β-hydroxysteroid dehydrogenase have been examined *in vitro*, to elucidate the participation of this vitamin in steroid hormone synthesis.

Materials and Methods

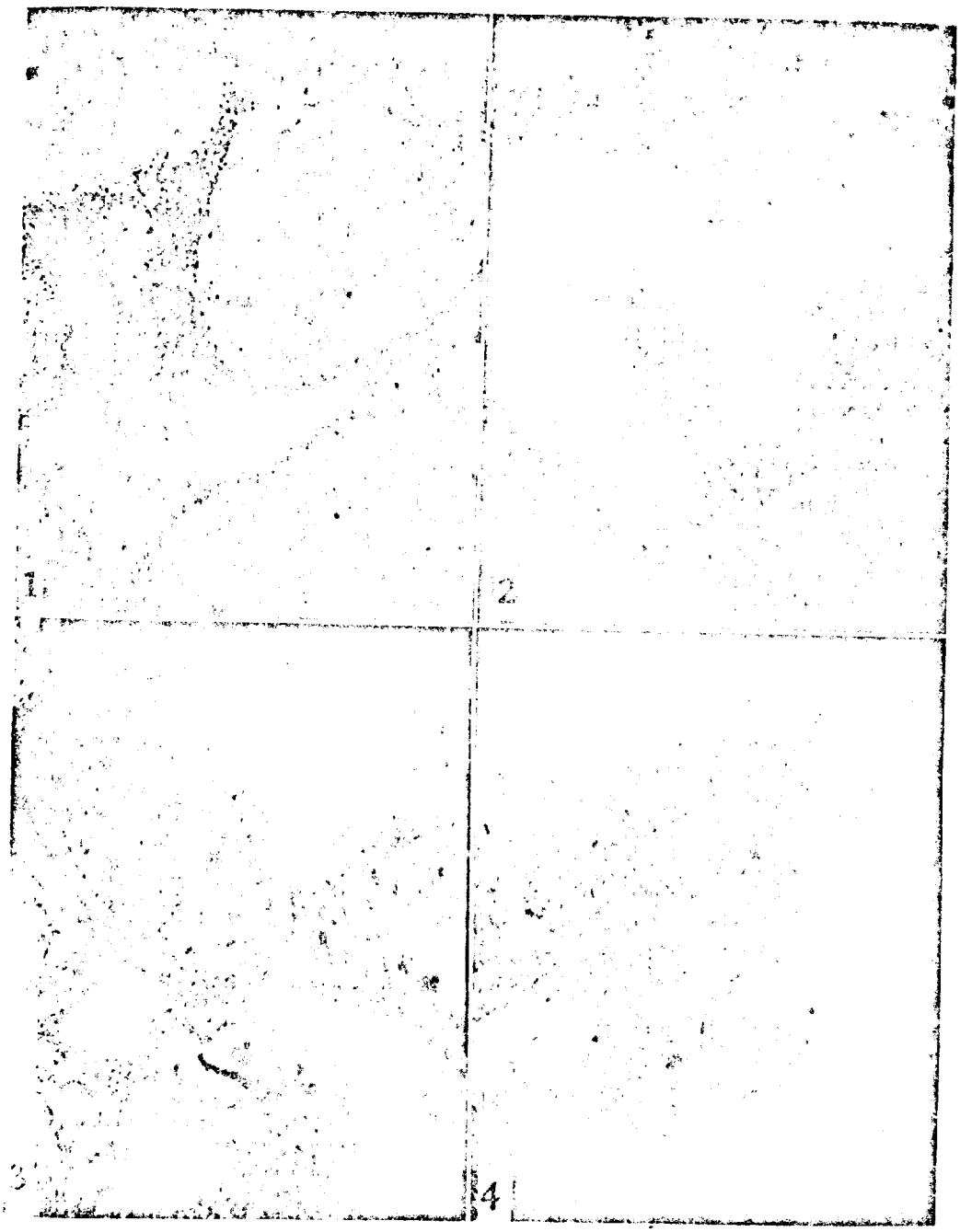
Ten adult male toads (*Bufo melanostictus*) weighing about 50 g were used for each test, during the breeding season. The testes were excised from the pithed animals. About 210 mg of testis taken from all these animals was cut into small slices and distributed equally into 7 vessels, each containing 2 ml of Krebs-Ringer bicarbonate buffer. The vessels were incubated for 2 hr at 37 C in a gas phase of 95% O_2–5% CO_2 (4). Ascorbic acid (AsA) or dehydroascorbic acid (DHA) was dissolved in 0.1M phosphate buffer. The final pH was adjusted to 7.1. Testicular slices in each of 3 vessels received AsA or DHA at dose levels of 100, 200 and 300 μg/100 mg of testis. Slices of testis incubated with an equal amount of phosphate buffer served as control. At the end of incubation, the testicular slices were kept in cold phosphate buffer (pH 7.1). 20 μ frozen sections of the testicular slices and the testis which had been removed from the freshly pithed animals were mounted on cover slips. Sections were first rinsed in cold acetone and then in cold 0.1M phosphate buffer pH 7.1. The Δ^5-3β-hydroxysteroid dehydrogenase activities of different incubated tissues were determined by incubating the sections separately for 60 min, at 37 C, in a substrate (dehydroepiendosterone) medium described by Dean *et al.* (5). As controls, parallel sections were also incubated for 60 min, at 37 C, in a medium containing no substrate. To determine the effect of 2 hr incubation in Krebs-Ringer bicarbonate buffer on the activity of Δ^5-3β-hydroxysteroid dehydrogenase, the activities in sections of fresh tissue incubated for 60 min in substrate medium were compared with the enzyme activities of the incubated tissue slices from the control group.

Results

Histochemically, both the Leydig and tubular cells showed Δ^5-3β-hydroxysteroid dehydrogenase activity in the normal fresh testis (Fig. 1) and in incubated testicular slices (Fig. 2). The Δ^5-3β-hydroxysteroid dehydrogenase activity of the testicular slices incubated in Krebs-Ringer bicarbonate buffer was identical with the enzyme activity of the normal fresh testis. The Leydig cells showed definitely stronger enzyme activity than the tubular cells. The enzyme activity was neither improved nor impaired by 2 hr preincubation with AsA or DHA (100 μg/100 mg of testis) and AsA (200 μg/100 mg of testis). On the other hand, activity of the enzyme in the testis incubated with 200 μg of DHA and 300 μg of AsA or DHA appeared to increase significantly over that seen in controls (Fig. 3). No differences were apparent between the enzyme activity of the testes incubated with 200 μg of DHA and 300 μg of DHA. Sections of the testicular slices incubated in a substrate free medium demonstrated no formazan deposition (Fig. 4). All

Received October 9, 1969.

0871



Figs. 1-4. Fig. 1. Δ^5-3β-Hydroxysteroid dehydrogenase in the unincubated testis of the normal toad. Fig. 2. 3β-Hydroxysteroid dehydrogenase in the incubated testis of the normal toad. Fig. 3. An increased 3β-hydroxysteroid dehydrogenase in the testis incubated with 200 μg of DHA. Fig. 4. Absence of formazan formation in the testis incubated in substrate free medium.

0871

the animals in each group showed similar reaction. After incubation sections were mounted in gum arabic.

Discussion

This investigation reveals that DHA *in vitro*, at a smaller dose level than AsA, stimulates the enzyme Δ^5-3β-hydroxysteroid dehydrogenase in the toad testis. Two hr incubation of testicular slices in Krebs-Ringer bicarbonate buffer does not alter significantly the normal enzyme activity. Presence of Δ^5-3β-hydroxysteroid dehydrogenase in both the seminiferous tubules and Leydig cells indicates that both of them are concerned with steroid hormone synthesis, but the Leydig cells have definitely greater activity. The seminiferous tubules of rat testes are also capable of converting progesterone to androgen *in vitro* (6). In their studies of human testis, Baillie and Mack (7) recorded persistent traces of Δ^5-3β-hydroxysteroid dehydrogenase in the seminiferous tubules, as well as a fairly strong enzyme activity in the Leydig cells. The injection of AsA into normal toads increases the Δ^5-3β-hydroxysteroid dehydrogenase activity (3). Although there is ample evidence regarding the importance of AsA in the maintenance of testicular function of rats (8) and toads (9, 10), the question of whether AsA *in vitro* stimulates the endocrine activity of the toad testes remains uncertain. In the present *in vitro* study, 200 μg of DHA stimulated the activity of Δ^5-3β-hydroxysteroid dehydrogenase, while the same amount of AsA failed to do so. This agrees with the finding of Brady (4) that addition of AsA to the incubating medium exerts little effect on testosterone biosynthesis in mammalian testis.

The role of AsA in steroid hormone synthesis is indeed obscure. Changes in ovarian AsA and cholesterol following injection of luteinizing hormone (11) indicate that AsA is implicated in ovarian steroid hormone synthesis. In the adrenal gland, AsA depletion after corticotropin treatment is concomitant with an increased corticoid production in chicks (12), rats (13) and calf (14). Salomon (16) observed that 20–40% of the fall in adrenal ascorbic acid can be accounted for as dehydroascorbic acid after ACTH administration. On the other hand, monodehydroascorbic acid produced by ascorbic acid-dependent $NADH_2$ oxidase acts as an intermediate electron carrier between NADH and O_2 in pig adrenal (17). This adrenal oxidase enzyme in the presence of AsA releases NADH inhibition of progesterone formation from pregnenolone in the rat (18). Existence of ascorbic acid oxidase has been suggested in other animal tissues (19). Studies are currently under way in our laboratory to localize the ascorbic acid oxidase in toads. Our findings indicate that both the adrenal and the testis of the toad can synthesize DHA from AsA. Administration of DHA to normal toads stimulates Δ^5-3β-hydroxysteroid dehydrogenase of the testes earlier than administration of AsA (unpublished observation). In the previous paper, it was suggested that stimulation of Δ^5-3β-hydroxysteroid dehydrogenase by AsA injection (3) is possibly due to its subsequent conversion to DHA.

In the present *in vitro* studies DHA has been found to simulate Δ^5-3β-hydroxysteroid dehydrogenase activity. Enzymatic stimulation has also been observed with AsA, but the concentration of AsA in the incubating medium needed to obtain an appreciable effect is much higher than with DHA. This can be attributed to the possibility that AsA is first converted to DHA by the testicular slices and thereby stimulates the enzyme activity. The extracellular AsA which has not been converted to DHA has been washed out in cold phosphate buffer before sectioning the testicular slices. On the other hand, DHA crosses the cellular barrier more readily than AsA (20) and this vitamin in its fully oxidized radical form is incapable of reducing the tetrazolium salt as manifested by negative reaction in controls. Moreover, this experimental stimulation of the testicular enzyme by DHA has obvious limitations. DHA at a dose level of 200 μg/100 mg of testis produces the maximum increase in the enzyme activity. Further increasing the dose level of DHA results in no significant further alteration of the enzyme activity.

Thus, it appears that in toad testes responsiveness of steroid hormone synthesis to ascorbic acid is possibly related to and dependent on conversion to dehydroascorbic acid.

Acknowledgments

The authors wish to thank Prof. S. R. Maitra, Head of the Department of Physiology, and Prof. P. B. Sen, Former Head of the Department of Physiology, Calcutta University, for their helpful suggestions and constant encouragement. This work was supported by grants from the University Grants Commission, New Delhi.

References

1. Staudinger, H. J., K. Krisch, and S. Leonauser, *Ann NY Acad Sci* **92:** 195, 1961.
2. Sikita, M., and B.I. Tamaoki, *Endocrinology* **76:** 563, 1965.
3. Biswas, N. M., *Endocrinology*, **85:** 981, 1969.
4. Brady, R. O., *J Biol Chem* **193:** 145, 1951.

5. Deane, H. W., B. L. Rubin, E. C. Driks, B. L. Lobel, and G. Leipsner, *Endocrinology* **70:** 407, 1962.
6. Christensen, A. K., and N. R. Mason, *Endocrinology* **76:** 646, 1965.
7. Baillie, A. H., and W. S. Mack, *J Endocr* **35:** 239, 1966.
8. Biswas, N. M., and C. Deb, *Endocrinology* **79:** 1157, 1966.
9. Biswas, N. M., and M. Mukherji, *Experientia* **23:** 667, 1967.
10. Biswas, N. M., and C. Deb, *Endokrinologie* (in press).
11. Parlow, A. F., *In* Albert, A. (ed), Human Pituitary Gonadotropins, Charles C Thomas, Springfield, 1961.
12. DeRoss, R., *Gen Comp Endocr* **1:** 494, 1961.
13. Slusher, M. A., and S. Roberts, *Endocrinology* **61:** 98, 1957.
14. Lipscomb, H. S., and D. A. Nelson, *Endocrinology* **66:** 144, 1960.
15. Rosenfeld, G., *Endocrinology* **56:** 649, 1955.
16. Salomon, L. L., *Texas Rep Biol Med* **15:** 925, 1951.
17. Kersten, H., W. Kersten, and H. J. Staudinger, *Biochim Biophys Acta* **27:** 598, 1958.
18. Koritz, S. B., *Biochim Biophys Acta* **59:** 326, 1962.
19. Hati, R. N., and A. G. Dutta, *Biochim Biophys Acta* **148:** 310, 1967.
20. Martin, G. R., *Ann NY Acad Sci* **92:** 141, 1961.

Interruption of Pseudopregnancy in Rats by Hemi-ovariectomy

AMAR CHATTERJEE[1] AND M. J. K. HARPER
Worcester Foundation for Experimental Biology, 222 Maple Avenue, Shrewsbury, Massachusetts 01545

ABSTRACT. Hemi-ovariectomy on days 1, 2 and 3 after the appearance of leukocytes in vaginal smears in rats (made pseudopregnant by reserpine injections at estrus) caused a return of proestrus between 2 and 3 days after operation in all animals. There was compensatory ovulation at the first or second postoperative proestrus, as shown by counting healthy implantation sites at laparotomy following a successful mating. Progesterone replacement immediately after hemi-ovariectomy successfully maintained the persistent leukocytic vaginal smears of pseudopregnancy in all animals so treated. Sham operation was without effect. (*Endocrinology* **87:** 173, 1970)

THERE IS good evidence to indicate that the central nervous system chronically inhibits the secretion of prolactin in the rat (1-4). It has been postulated that the factor which stimulates luteotrophin secretion does so by suppressing this neural inhibition, and that, during pseudopregnancy, progesterone may maintain the secretion of luteotrophin by a positive feedback (3). Inactivation of corpora lutea of pseudopregnancy by a pharmacological agent like ergocornine terminates pseudopregnancy. This effect can be delayed, but not reversed by progesterone treatment (5). To study the role of progesterone in the physiology of pseudopregnancy the following experiments were performed.

Materials and Methods

Sixty female rats of the Sprague-Dawley strain, weighing 180-200 g, were used. They were checked for at least 2 regular estrous cycles before use. Pseudopregnancy was induced by a single subcutaneous injection of reserpine (1 mg/kg), which proved to be 100% effective when given between 8:00 and 10:00 AM on the day of estrus.

Between 12:00 noon and 2:00 PM on each of the first 3 days of the leukocytic vaginal smears indicating pseudopregnancy, animals of groups 1, 2 and 3 (Table 1) were subjected to unilateral ovariectomy through a dorsolateral incision without ligation of the blood vessels supplying the ovary and uterus. A sham operation was similarly done on the animals of groups 4, 5 and 6. Vaginal smears were studied daily between 8:00 and 9:00 AM from the day of operation.

On each of the first 3 days of the leukocytic vaginal smear of pseudopregnancy hemi-ovariectomy was performed on the animals of groups 7, 8 and 9 (Table 1) and they were given 2 subcutaneous injections of a long-acting progestin (17α-hydroxyprogesterone caproate, Delalutin—Squibb) at a dose level of 3 mg/day in 0.2 ml sesame oil, the first one immediately following operation and the second 48 hr later. Vaginal smears were studied as described above.

Twelve (50%) of the animals of groups 1, 2 and 3 were mated with fertile males on the first day proestrous smears were obtained, and the remaining 12 were mated on the day of proestrus of the

Received January 15, 1970.

[1] Post-doctoral Fellow, Ford Foundation Training Program in Reproductive Physiology. *Present address:* c/o Professor G. S. Greenwald, Univ. of Kansas Medical Center, Dept. of Obstetrics and Gynecology, Kansas City, Kansas 66103.

Biochim Biophys Acta 78: 795-7, 13 Dec '63

0908

Ascorbic acid protection against toxicity of an azo dye carcinogen for *Neurospora crassa*

While riboflavin has been alleged to overcome azo-dye inhibition of growth of micro-organisms (see BOBB *et al.*[1], for references), to our knowledge a direct effect by ascorbic acid has not previously been reported. The synergistic effect of methylene blue on azo-dye growth inhibition (unpublished observations) led us to test the capacity of an easily oxidizable substance to reverse the inhibition by the carcinogen. This report deals with the ability of ascorbic acid to overcome the growth inhibitory effect of MeMAB on *N. crassa*.

Growth experiments were performed in 125-ml erlenmeyer flasks containing 20 ml of medium N (ref. 2) which contained per l of solution: 2.5 g sodium citrate, 5.0 g KH_2PO_4, 2.0 g NH_4NO_3, 0.2 g $MgSO_4 \cdot 7\ H_2O$, 0.1 g $CaCl_2 \cdot 2\ H_2O$, 5 mg biotin, 20 g sucrose, and trace elements; pH 5.8. For growth of the riboflavin-deficient strain 51602, 5 µg of riboflavin per 20 ml was included. After inoculation with a standardized conidial suspension, duplicate cultures were incubated at 31° without shaking. Growth was determined by drying the mycelial mats to constant weight and averaging the duplicate values.

As reported previously, all Neurospora strains tested were inhibited in their growth by the presence of the azo-dye carcinogen MeMAB, inhibition being manifested primarily by a prolonged lag phase rather than by decreased growth rate or growth capacity[1]. The wild-type strain STA was relatively resistant and the riboflavin-deficient strain 51602 was the most sensitive to azo-dye inhibition.

TABLE I

THE ABILITY OF *l*-ASCORBIC ACID TO REVERSE THE GROWTH INHIBITORY EFFECTS OF MeMAB ON *N. crassa*

Concentrations of medium constituents are given in µg per 20 ml. The figures represent weight of mycelial mats in mg.

Strain	*Days of growth*	*Additions to basal medium*	*Ascorbic acid (m moles)*			
			0	*1*	*2*	*3*
51 602	3	5 B_2	53	56	53	52
		5 B_2 + 50 MeMAB	48	47	50	51
		5 B_2 + 75 MeMAB	28	32	39	36
		5 B_2 + 100 MeMAB	3	10	39	43
			0	*1.5*	*3*	
STA	2	None	61	58	59	
		100 MeMAB	47	63	64	
		125 MeMAB	22	50	58	

As shown in a representative experiment (Table I), *l*-ascorbic acid *per se* did not affect the growth of either strain on a complete medium. Growth of both strains was progressively inhibited by increasing amounts of MeMAB. A definite protection

Abbreviations: MeMAB, 3′-methyl-4-monomethylaminoazobenzene; DAB, 4-dimethylamino-azobenzene.

against inhibition by the dye occurred with both strains if ascorbic acid was also present in the medium. The ascorbic acid effect was most easily seen with the higher concentrations of the dye. At a given dye concentration, increasing amounts of the vitamin produced progressively greater protective effects. There was no superimposed effect of ascorbic acid on the pH of the growing cultures. Beyond a molar ratio of vitamin/dye of about 100:1 no further protective effects were manifested. Growth curves showed that ascorbic acid reduced the lag period incurred with MeMAB. The medium and the mycelial mats retained the bright yellow color of the dye whether or not ascorbic acid was present.

In the above experiments the vitamin was filter sterilized and added aseptically to the medium after autoclaving. The same results were obtained when the vitamin was added to the medium prior to autoclaving for 10 min at 15 lb pressure.

TABLE II

EFFECT OF GSH AND CYSTEINE ON MeMAB INHIBITION

Figures represent 3-day growth in mg of strain 51602 on complete medium (5 μg B_2 per 20 ml).

MeMAB supplement (μg per 20 ml)	*Concentrations (m moles)*							
	GSH				*Cysteine*			
	0	*1.5*	*3*	*5*	*0*	*1*	*3*	*5*
None	48	50	49	46	59	59	57	33
50	49	52	44	38	48	50	41	22
100	10	10	17	22	3	1	2	2

The *l*-ascorbic acid could be replaced by the isomer, *d*-isoascorbic acid, as well as by dehydroascorbic acid. Cysteine was ineffective while GSH was slightly effective at very high concentrations (Table II). These compounds were all filter sterilized.

Furthermore, the ascorbic acid-protective effect was nullified in the presence of either cysteine or GSH (Table III). The growth inhibition of strain 51602 on complete medium by cysteine plus ascorbic acid, but not by either compound alone at these concentrations, cannot be explained. On medium containing 75 μg MeMAB per 20 ml, 1.5 mM cysteine gave added growth inhibition.

The protection offered by ascorbic acid in preventing the toxicity of MeMAB for *N. crassa* would appear to depend on the autoxidation of the vitamin. The protec-

TABLE III

PREVENTION OF ASCORBIC ACID EFFECT BY CYSTEINE AND GSH

Figures represent 3-days growth in mg of strain 51602 on complete medium (5 μg riboflavin per 20 ml).

MeMAB supplement (μg per 20 ml)	*Control*	*Concentrations (m moles)*				
		3.0 l-ascorbic acid	*1.5 cysteine*	*0.75 GSH*	*1.5 cysteine + 3.0 l-ascorbic acid*	*0.75 GSH + 3.0 l-ascorbic acid*
None	52	50	57	52	28	58
50	47	46	—	48	—	48
75	26	45	13	—	6	—
100	4	38	11	7	trace	3

ive action of sulfhydryl compounds such as cysteine and glutathione on the autoxidation of ascorbic acid is well known[3]. A simple lowering by ascorbic acid of the oxidation–reduction potential of the medium enough to initiate growth appears unlikely due to the inability of cysteine to replace ascorbic acid. The greater than catalytic amounts of ascorbic acid required and the activity of the *d*-isomer rules out a growth-factor effect.

The significance of the present findings with respect to the induction of hepatomas in the rat by the feeding of azo-dye carcinogens is uncertain. However, DAB and other carcinogens are known to increase the ascorbic acid level in the liver of rats[4]. Furthermore, rats deficient in riboflavin have a reduced content of ascorbic acid in the tissues[5], and the induction of hepatomas by DAB is favored in riboflavin-deficient rats[6]. Also, it should be noted that certain carcinogenic hydrocarbons are potent in stimulating ascorbic acid biosynthesis as well as inducing the synthesis of several liver-microsomal enzymes (*e.g.*, azo-dye demethylase) which metabolize foreign compounds[7] implying that vitamin-C deficiency may decrease the activity of such enzymes.

This investigation was supported in part by Public Health Service Research Grant C-2869 from the National Cancer Institute to Dr. D. A. SALZBERG and in part by the Max Fleishmann Fund of the Palo Alto Medical Research Foundation. The technical assistance of Mrs. D. LIEBES is gratefully acknowledged.

Palo Alto Medical Research Foundation,
Palo Alto, Calif. (U.S.A.)

DOLORES BOBB

[1] D. BOBB, D. LIEBES AND D. A. SALZBERG, *J. Bacteriol.*, 84 (1962) 242.
[2] H. J. VOGEL, *Microbial Genet. Bull.*, 13 (1956) 42.
[3] W. E. KNOX, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 2, Academic Press, New York, 1960, p. 253.
[4] J. P. GREENSTEIN, *Biochemistry of Cancer*, 2nd edition, Academic Press, New York, 1954, p. 335.
[5] B. SURE, R. M. THEIS AND R. T. HARRELSON, *J. Biol. Chem.*, 129 (1939) 245.
[6] C. J. KENSLEP, K. SUGIURA, N. F. YOUNG, C. R. HALTER AND C. P. RHOADS, *Science*, 93 (1941) 308.
[7] A. H. CONNEY, G. A. BRAY, C. EVANS AND J. J. BURNS, *Ann. N.Y. Acad. Sci.*, 92 (1961) 115.

Received July 29th, 1963

After the six week training period, ten hours exercise did not result in elevated creatinephosphokinase activity in either group of guinea pigs. There was a tendency for training, per se, to elevate creatinephosphokinase activity, but this was not statistically significant. In comparable experimental groups, except for the two showing significant changes, creatinephosphokinase activity in both red and white muscle was always lower in the guinea pigs maintained on low vitamin C levels.

TABLE I

Exercise and Creatinephosphokinase Activity[a] in Red and White Muscle of Trained and Untrained Guinea Pigs: The Influence of Dietary Vitamin C. Mean ± S.E.M. Sample Numbers in Parentheses.

	RED MUSCLE			
	Untrained		Trained	
	High Vitamin C	Low Vitamin C	High Vitamin C	Low Vitamin C
Control	(10) 1598 ± 55	(10) 1213 ± 92	(9) 1795 ± 151	(8) 1452 ± 85
Exercised	(8) 1673 ± 97	(8) 1820 ± 64[b]	(9) 1566 ± 73	(8) 1321 ± 81

	WHITE MUSCLE			
	Untrained		Trained	
	High Vitamin C	Low Vitamin C	High Vitamin C	Low Vitamin C
Control	(10) 4541 ± 299	(10) 3677 ± 302	(9) 5168 ± 381	(8) 4361 ± 515
Exercised	(8) 4552 ± 191	(8) 5719 ± 542[b]	(9) 4545 ± 199	(8) 3796 ± 392

[a] Activity expressed in milliunits (mμM substrate transformed/mg protein/min).

[b] Indicates significant differences at $P < 0.05$, Student t Test.

Muscle Fructose-1,6-Diphosphate Aldolase

Ten hours exercise of untrained guinea pigs maintained on low vitamin C levels resulted in a significant decline of fructose-1,6-diphosphate aldolase

activity (30%) in the red muscle, but did not affect enzyme activity in those maintained on high levels of vitamin C (Table II).

TABLE II

Exercise and Fructose-1,6-Diphosphate Aldolase Activity[a] in Red and White Muscle of Trained and Untrained Guinea Pigs: The Influence of Dietary Vitamin C. Mean ± S.E.M. Sample Numbers in Parentheses.

RED MUSCLE	Untrained		Trained	
	High Vitamin C.	Low Vitamin C	High Vitamin C	Low Vitamin C
Control	(10) 45.3 ± 3.0	(10) 48.1 ± 3.3	(9) 56.3 ± 5.1	(8) 56.3 ± 3.1
Exercised	(8) 39.3 ± 3.7	(8) 34.1 ± 2.2[b]	(9) 60.6 ± 5.2	(8) 63.3 ± 6.9

WHITE MUSCLE	Untrained		Trained	
	High Vitamin C	Low Vitamin C	High Vitamin C	Low Vitamin C
Control	(10) 1409 ± 104	(10) 1628 ± 93	(9) 1729 ± 114	(8) 1499 ± 122
Exercised	(8) 1574 ± 72	(8) 1789 ± 206	(9) 1597 ± 80	(8) 1494 ± 104

[a] Activity expressed in milliunits (mμM substrate transformed/mg protein/min).
[b] Indicates significant difference at $P < 0.05$, Student t Test.

Training prevented the decrease after exercise previously noted in red muscle fructose-1,6-diphosphate aldolase activity. Fructose-1,6-diphosphate aldolase activity tended to increase after training in the red and white muscle of guinea pigs maintained on high levels of vitamin C, but not in those maintained on low levels of vitamin C. There were no other significant changes in the activity of fructose-1,6-diphosphate aldolase in the white muscle.

TABLE III

Exercise and Fructose-1-phosphate Aldolase and Fructose-1,6-diphosphate Aldolase Activity[a] in Liver of Trained and Untrained Guinea Pigs: The Influence of Dietary Vitamin C. Mean ± S.E.M. Sample Numbers in Parentheses.

FRUCTOSE-1-PHOSPHATE ALDOLASE				
	Untrained		Trained	
	High Vitamin C	Low Vitamin C	High Vitamin C	Low Vitamin C
Control	(10) 84.6 ± 4.3	(10) 74.0 ± 3.5	(9) 85.9 ± 11.0	(8) 88.5 ± 4.0
Exercised	(8) 69.2 ± 6.8	(8) 66.4 ± 5.2	(9) 100.7 ± 7.6	(8) 70.5 ± 6.9[b]

FRUCTOSE-1,6-DIPHOSPHATE ALDOLASE				
	Untrained		Trained	
	High Vitamin C	Low Vitamin C	High Vitamin C	Low Vitamin C
Control	(10) 28.6 ± 1.8	(10) 28.4 ± 3.2	(9) 30.9 ± 3.0	(8) 29.0 ± 2.0
Exercised	(8) 24.0 ± 1.4	(8) 27.6 ± 1.7	(9) 33.8 ± 2.0	(8) 26.7 ± 2.0

[a] Activity expressed in milliunits (mμM substrate transformed/mg protein/min).

[b] Indicates significant difference at $P < 0.05$, Student t Test.

Liver Fructose-1-phosphate and Fructose-1,6-diphosphate Aldolase

Liver fructose-1-phosphate aldolase activity tended to decrease after ten hours exercise, but the changes were not statistically significant (Table III). Exercise of trained guinea pigs maintained on low levels of vitamin C resulted in a significant decrease of fructose-1-phosphate aldolase activity (20%); in guinea pigs maintained on high levels of vitamin C, training prevented the decrease after exercise in liver fructose-1-phosphate aldolase activity. There were no significant changes in fructose-1,6-diphosphate aldolase activity.

TABLE IV

Exercise and Plasma Enzyme Activity[a] of Trained and Untrained Guinea Pigs: The Influence of Dietary Vitamin C. Mean ± S.E.M., N = 5.

CREATINEPHOSPHOKINASE				
	Untrained		Trained	
	High Vitamin C	Low Vitamin C	High Vitamin C	Low Vitamin C
Control	327 ± 27	530 ± 65	792 ± 193	729 ± 54
Exercised	982 ± 44[b]	1094 ± 86[b]	625 ± 224	717 ± 238

FRUCTOSE-1,6-DIPHOSPHATE ALDOLASE				
	Untrained		Trained	
	High Vitamin C	Low Vitamin C	High Vitamin C	Low Vitamin C
Control	71 ± 8	72 ± 7	85 ± 5	88 ± 6
Exercised	273 ± 32[b]	382 ± 81[b]	96 ± 12	145 ± 39

FRUCTOSE-1-PHOSPHATE ALDOLASE				
	Untrained		Trained	
	High Vitamin C	Low Vitamin C	High Vitamin C	Low Vitamin C
Control	11.0 ± 1.3	7.5 ± 0.6	10.0 ± 1.6	7.7 ± 1.4
Exercised	11.4 ± 2.0	8.4 ± 0.7	8.0 ± 0.7	8.6 ± 2.1

[a] Activity expressed in units/ml plasma. See Materials and Methods for unit definitions.

[b] Indicates significant differences at $P < 0.01$, Student t Test.

Plasma Enzymes

Prior to exercise creatinephosphokinase activity in guinea pigs maintained on low levels of vitamin C was 62% greater than that in animals maintained on high levels of vitamin C (Table IV). Exercise of either group of animals resulted in a 2-3 fold increase in enzyme activity. The levels of plasma creatinephosphokinase in trained guinea pigs were elevated above those

in untrained ones, but acute exercise of the trained animals did not result in further elevations.

Exercise of untrained guinea pigs maintained on high vitamin C levels resulted in a 285% increase in plasma fructose-1,6-diphosphate aldolase activity. Comparable exercise of the group maintained on low vitamin C levels increased the plasma enzyme activity 431%. Prior training was less effective in preventing the exercise-induced increase of fructose-1,6-diphosphate aldolase activity in guinea pigs maintained on low levels of vitamin C than in those on high levels of vitamin C.

There was no significant change in the plasma fructose-1-phosphate aldolase activity in any of the experimental groups.

Discussion

The low vitamin C diet employed here, as in an earlier study[11], did not induce weight loss or other scorbutic changes, nor was there obvious differences in the exercise performance of the two groups of guinea pigs. Thus, interpretation of the changes in tissue and plasma enzyme activity after exercise were not complicated by the pathological changes associated with scurvy.

A comparison of tissue and plasma enzyme activity after exercise suggests that muscle is the major source of the plasma enzyme elevations, and is in agreement with earlier results[6]. The one change noted in liver fructose-1-phosphate aldolase, an enzyme localized mainly in this organ[7], was not correlated with the activity of this enzyme in plasma. However, the activity of fructose-1,6-diphosphate aldolase, an enzyme found in both liver and muscle[7], showed a significant increase after exercise in the plasma, declined in the muscle, and was unchanged in the liver. Moreover, the activity of creatinephosphokinase, localized chiefly in the muscle[7], increased 2-3 fold in the plasma after exercise.

As previously demonstrated[5], prior training prevented further increases in plasma enzyme activity after exercise, as normally occurs in untrained animals. In one instance, that of plasma fructose-1,6-diphosphate aldolase in guinea pigs maintained on low vitamin C, prior training was less effective in preventing the exercise-induced rise in plasma enzyme activity.

It appeared that red muscle, rather than white muscle, contributed more to the elevations in plasma enzymes after exercise. This might be expected since the vascular supply of red muscle far exceeds that of white muscle[12], affording a greater opportunity for the escape of cellular enzymes to the plasma. After exercise fructose-1,6-diphosphate aldolase activity was significantly decreased in the red muscle, but not in white muscle, while the plasma enzyme activity increased. Note also the relationship between muscle and plasma creatinephosphokinase activity in control animals maintained on different levels of vitamin C. Plasma enzyme activity was higher in guinea pigs maintained on low levels of vitamin C and the decreases in tissue creatinephosphokinase activity in the low vitamin C groups proportionately greater in the red muscle.

It is interesting that each of the significant changes in tissue enzyme activity occurred in the guinea pigs that had been maintained on low levels of vitamin C. These observations, in conjunction with the greater increases in plasma enzyme activity in the hypovitaminotic group, suggest one consequence of vitamin C deprivation may be a generalized increase in membrane permeability, and may in part account for the decreased exercise tolerance previously noted in scorbutic animals[1].

Creatinephosphokinase appeared to be directly influenced by the low vitamin C diet. The activity of this enzyme in control guinea pigs on low vitamin C was 24% less in the red muscle and 19% less in the white muscle in comparison to those maintained on high vitamin C. It is known that reducing agents other than ascorbic acid, such as glutathione or mercaptoethanol, will

enhance creatinephosphokinase activity in certain *in vitro* preparations[13]. This magnitude of inhibition may therefore be of physiological significance since the activity of creatinephosphokinase is closely associated with the contractile function of muscle[14].

Summary

The relationship between tissue and plasma enzyme activity was examined in exercised guinea pigs maintained on high or low levels of vitamin C. The responses of "tissue specific" enzymes, creatinephosphokinase in red and white muscle, fructose-1-phosphate aldolase in liver, and fructose-1,6-diphosphate aldolase in both tissue types, indicated muscle rather than liver contributed to the rise in plasma enzyme activity after exercise. Moreover, the data suggested plasma creatinephosphokinase and fructose-1,6-diphosphate aldolase originated mainly from the red muscle.

Without exception each of the significant tissue enzyme changes occurred in the hypovitaminotic group, implying a causal relationship between vitamin C and muscle metabolism. There was an augmented rise in plasma enzyme activity in the hypovitaminotic animals which was correlated in several instances with a decline in muscle enzyme activity, suggesting that vitamin C deprivation may increase membrane permeability. The consistently low creatinephosphokinase activity in the muscles of hypovitaminotic animals suggests Vitamin C may be required for the optimal *in vivo* activity of this enzyme.

Acknowledgment

The author thanks Mr. Milton G. Parker for determining the plasma enzymes and Mr. Wm. L. Conway for animal maintenance.

References

1. M. CHALOPIN, M. MOUTON, and A. R. RATSIMAMANGA, World Rev. Nutr. Dietet. 6, 165 (1966).
2. A. B. BANERJEE and N. C. GANGULI, J. Biol. Chem. 237, 14 (1962).

3. A. R. RATSIMAMANGA, Thèse Sciences, Paris (1939).

4. P. D. ALTLAND and B. HIGHMAN, Am. J. Physiol. 201, 393 (1962).

5. B. HIGHMAN and P. D. ALTLAND, Am. J. Physiol. 205, 162 (1963).

6. J. GARSUS, B. HIGHMAN, and P. D. ALTLAND, Am. J. Physiol. 207, 467 (1964).

7. J. C. DREYFUS, The Proceedings of the Second Symposium on Current Research in Muscular Dystrophy, p. 127 (1963).

8. M. P. DIETER, Life Sci. 8, 459 (1969).

9. H. U. BERGMEYER, Methods of Enzymatic Analysis, 1064 pp., Academic Press, New York (1963).

10. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL, J. Biol. Chem. 193, 265 (1951).

11. M. P. DIETER, Proc. Soc. Exptl. Biol. Med. 132, 1147 (1969).

12. O. HEROUX and J. ST. PIERRE, Am. J. Physiol. 188, 163 (1957).

13. N. C. KAR and C. M. PEARSON, Proc. Soc. Exptl. Biol. Med. 118, 662 (1965).

14. J. KENDRICK-JONES and S. V. PERRY, Nature 208, 1068 (1965).

PROC. SOC. EXP. BIOL. MED. 1971, 136, 316-322

Further Studies on the Relationship Between Vitamin C and Thymic Humoral Factor (35256)

MICHAEL P. DIETER
(Introduced by P. D. Altland)

Laboratory of Physical Biology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014

The relationship between ascorbic acid and the differentiation, growth, and function of various cellular types have been investigated (1). We demonstrated during age involution of the lymphoid organs that while the total vitamin C concentration decreased, the percentage of dehydroascorbic acid remaining increased, and the percentage of ascorbic acid decreased (2). Treatment with adrenal cortical or androgenic steroid hormones to interrupt differentiation and growth of the lymphoid organs induced similar changes in the concentration and oxidation state of tissue vitamin C (1).

Recent reports have shown that the thymus, in addition to the adrenals and testes, elaborates humoral factors which influence the growth and immunologic function of other peripheral lymphoid organs (3). Subsequently it was demonstrated that cell-free, thymic extracts (thymic humoral factor or THF) were less active when prepared from guinea pigs maintained on lower, than on higher vitamin C supplements (4). The employed bioassay tested the capacity of THF to induce regeneration of weight and hexose monophosphate shunt enzyme activity in lymphoid organs in X-irradiated rats. The efficacy of this bioassay has since been confirmed by other investigators, who showed that the increase in spleen and lymph node weight of THF-treated, irradiated mice was accompanied by increased incorporation of tritiated thymidine or deoxycytidine into DNA (5).

While the initial study pointed out that dietary vitamin C influenced the bioassay for THF, one could not ascertain whether its function extended throughout thymic ontogeny for the production of THF, or whether vitamin C acted directly as a type of cofactor for the expression of THF activity. In an attempt to distinguish between these possibilities THF bioassays were performed using thymic extracts prepared from guinea pigs maintained 6 weeks on four known levels of daily vitamin C intake, or on dialyzed extracts of rat thymus from which the vitamin C had been partially removed. In addition the content of total, reduced, and oxidized vitamin C in the thymic preparations were measured and these parameters correlated with THF activity.

Materials and Methods. Male guinea pigs, 400–500 g, of the Hartley strain from NIH were maintained five to a cage and given a vitamin C deficient diet[1] and water, *ad libitum*. After 1 week they were divided into four groups and orally supplemented every other day with vitamin C equivalent to an intake of 0.25, 0.50, 1.00, or 5.00 mg/24 hr. The vitamin was freshly prepared as an aqueous solution of sodium ascorbate. Body weights were recorded throughout the study. After 6 weeks on the dietary regime, animals were sacrificed with chloroform and the thymus, spleen, liver, and adrenals were removed. Vitamin C from these organs was extracted with metaphosphoric acid and reduced, oxidized, and total vitamin C was determined by the indophenol method (6). Cell-free extracts of the thymus and spleen were prepared in 0.85% sterile saline as before (4). 0.1 ml of the supernate was analyzed for total protein (7), and the preparations were adjusted with sterile saline to a final concentration of 10 mg of protein/ml.

To determine whether ascorbic acid was

[1] Ascorbic acid deficient diet (guinea pig) from Nutritional Biochemicals Corporation, Cleveland, Ohio.

necessary for the expression of THF activity, cell-free saline extracts of rat thymuses were prepared in the same manner as guinea pig tissue extracts, except one portion was subjected to dialysis against 0.85% saline in the cold. Dialysis removed 50% of the ascorbic acid and the activity of this preparation was compared to that of nondialyzed THF.

THF bioassays were performed as outlined previously (4) by measuring regeneration of lymphoid organ weights and hexose monophosphate shunt enzyme activity in 7-week-old male Sprague-Dawley, pathogen-free rats

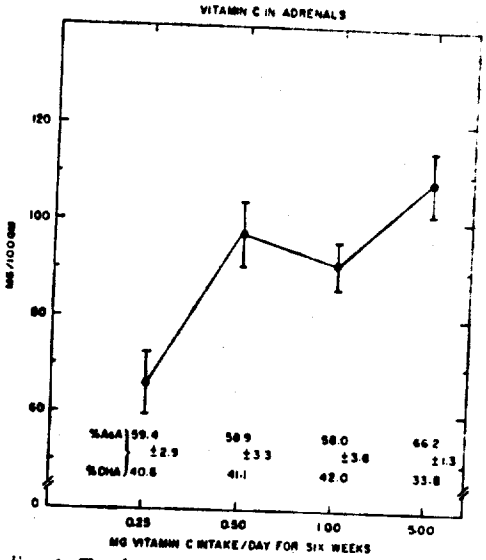


FIG. 1. Total, reduced (ASA or ascorbate), and oxidized (DHA or dehydroascorbate) vitamin C in the adrenals of guinea pigs maintained on four levels of vitamin C: means ± SEM, 10 animals/group.

following 300 R of whole body X-radiation. Starting 6 hr postirradiation the rats were injected ip three times at 24-hr intervals with 0.5 ml of tissue extract, containing 5.0 mg of protein. Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities were determined by previously described methods (8) in the lymph nodes, thymuses, and spleens; the protein content of the supernates was analyzed, and activity was expressed in nanomoles of substrate transformed/milligram of protein/min (mUnits).

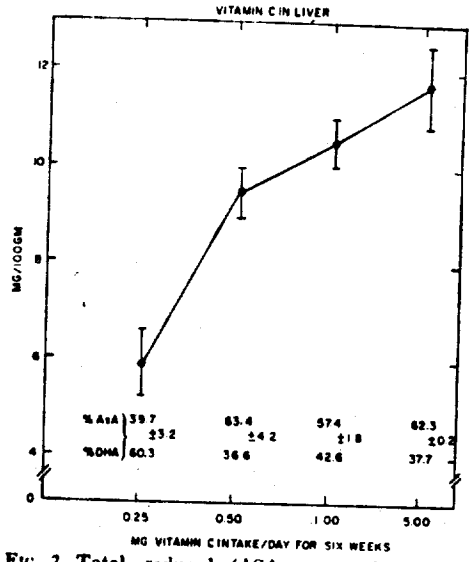


FIG. 2. Total, reduced (ASA or ascorbate), and oxidized (DHA or dehydroascorbate) vitamin C in the liver of guinea pigs maintained on four levels of vitamin C: means ± SEM, 10 animals/group.

Results. The guinea pigs given 0.5, 1.00, or 5.00 mg of vitamin C/day gained an equivalent amount of weight. Those maintained with 0.25 mg/day showed a 17% decrease in final body weight, the thymus and

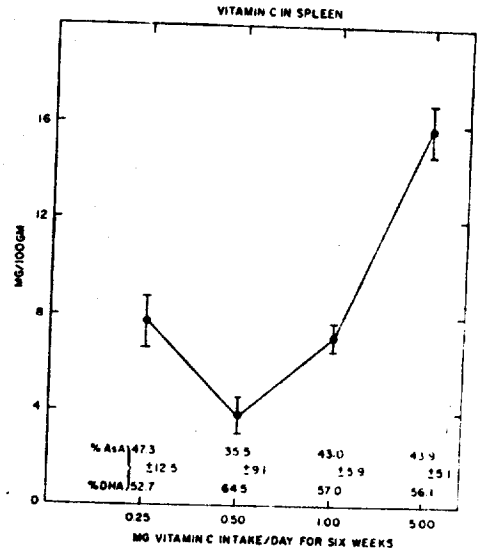


FIG. 3. Total, reduced (ASA or ascorbate), and oxidized (DHA or dehydroascorbate) vitamin C in the spleen of guinea pigs maintained on four levels of vitamin C: means ± SEM, 10 animals/group.

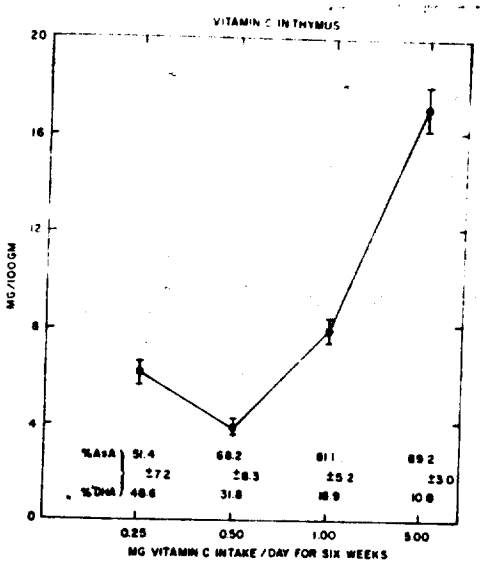


FIG. 4. Total, reduced (ASA or ascorbate), and oxidized (DHA or dehydroascorbate) vitamin C in the thymus of guinea pigs maintained on four levels of vitamin C: means ± SEM, 10 animals/group.

spleen weights did not differ, but the adrenal weights of those on 0.25 mg/day increased 25%.

Maintenance of guinea pigs on decreasing daily intakes of vitamin C resulted in ca. 50% decrease in the concentration of total vitamin C in the adrenals, liver, spleen, and thymus (Figs 1–4). However the only tissue showing a marked change in the oxidation state of vitamin C was the thymus (Fig. 4). There was a linear increase in the percentage of dehydroascorbate (DHA) and decrease in the percentage of ascorbate (ASA), corresponding to the decrease in the daily intake of vitamin C. Expression of total vitamin C, ascorbate, and dehydroascorbate on a total content basis (μg/thymus) did not result in values different from those cited above.

Thymic humoral factor bioassay. Figure 5 illustrates the increases in the lymphoid organ weights of X-irradiated rats following treatment with THF prepared from the thymuses of each of the 4 groups of guinea pigs. Following THF treatment there was a linear increase in the weight of the lymph nodes that was proportional to the level of vitamin C in the various thymic preparations. A somewhat linear relationship was also evidenced by the spleen weights, but the thymus weight was increased the same amount after all of the THF treatments. The increases in organ weight were significantly greater after THF treatment than after

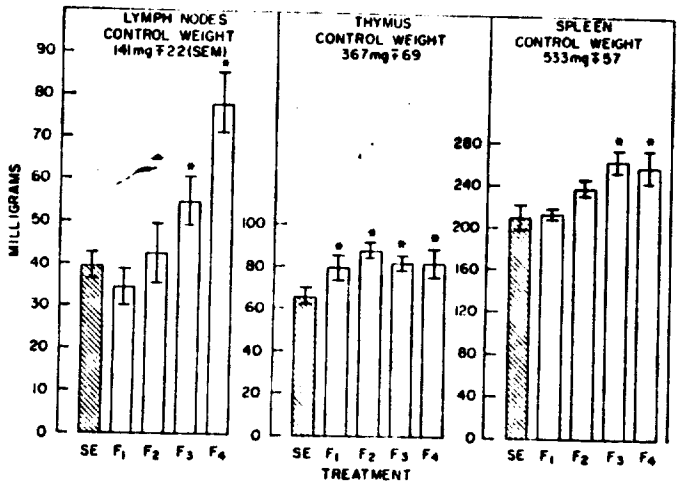


FIG. 5. Lymphoid organ weights of irradiated rats following injections of spleen extract or thymic humoral factor. The spleen extract (SE) was prepared from guinea pigs maintained on 5.0 mg of vitamin C, and the different peparations of thymic humoral factor (designated F_1 through F_4) from those maintained on 0.25, 0.50, 1.00, or 5.00 mg of vitamin C, respectively; mean ± SEM, sample numbers from 4 to 8. *Significantly different from values in irradiated rats injected with spleen extract, $p<0.05$, Student's t test.

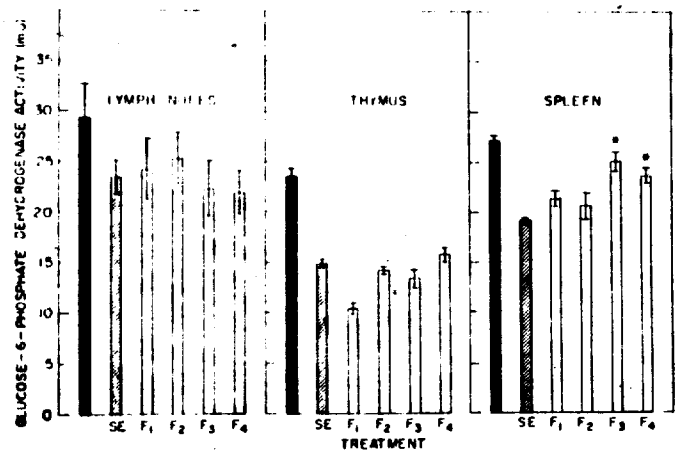


FIG. 6. Glucose-6-phosphate dehydrogenase activity in the lymphoid organs of irradiated rats following injections of spleen extract or thymic humoral factor. The closed bar represents values in nonirradiated rats. Abbreviations and other details explained in Fig. 5; mean ± SEM, sample numbers from 4 to 8. *Significantly different from values in irradiated rats injected with spleen extract, $p<0.05$, Student's t test.

spleen extract treatment in the lymph nodes (rats injected with preparations F_3 and F_4), thymus (all preparations), and in the spleen (preparations F_3 and F_4).

The increases in hexose monophosphate shunt enzyme activities after THF treatment of X-irradiated rats are illustrated in the lymphoid organs in Figs. 6 and 7. Glucose-6-phosphate dehydrogenase activity in the lymph nodes was not significantly greater in the irradiated rats treated with THF than in those treated with spleen extract. The increases in the activity of this enzyme in the thymus were proportional to the level of vitamin C in the various thymic preparations, but did not increase above the levels in rats

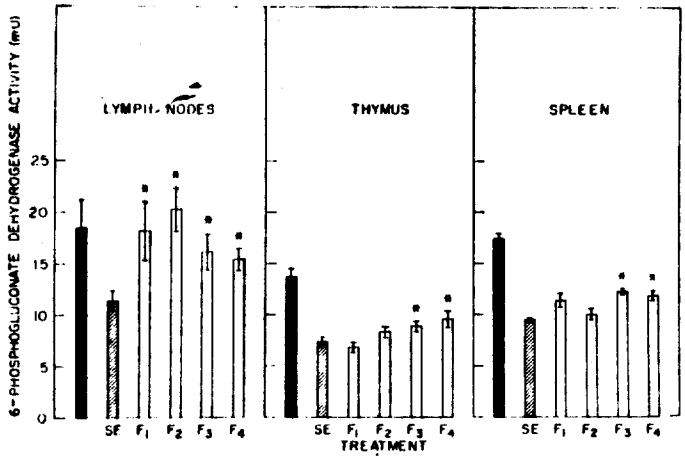


FIG. 7. 6-Phosphogluconate dehydrogenase activity in the lymphoid organs of irradiated rats following injections of spleen extract or thymic humoral factor. The closed bar represents values in nonirraidated rats. Abbreviations and other details explained in Fig. 5; mean ± SEM, sample numbers from 4 to 8. *Significantly different from values in irradiated rats injected with spleen extract, $p<0.05$, Student's t test.

treated with spleen extract. The activity of glucose-6-phosphate dehydrogenase in the spleens of rats treated with THF was somewhat proportional to the level of vitamin C in the thymic extracts, and that in the rats treated with preparations F_3 and F_4 was significantly greater than in rats treated with spleen extract.

The activity of 6-phosphogluconate dehydrogoenase in the rat lymphoid organs was more sensitive to THF treatment than that of glucose-6-phosphate dehydrogenase. In the lymph nodes treatment with any of the THF preparations induced significant increases in 6-phosphogluconate dehydrogenase activity above those in rats treated with spleen extract (Fig. 7). The increases of 6-phosphogluconate dehydrogenase activity in the thymus and spleen were proportional to the level of vitamin C in the thymic extracts; these changes were significant in both organs after injections of either THF preparations F_3 or F_4.

The results of the bioassay for THF activity using nondialyzed and dialyzed rat thymic preparations are presented in Table I. Although about 50% of the ascorbic acid was removed by dialysis, the activity of this preparation was not inhibited, and was in fact, somewhat greater than that of the nondialyzed preparation, even though an equivalent amount of protein was injected. In addition, the partial removal of ascorbic acid by dialysis did not inhibit the stimulation of hexose monophosphate shunt enzyme activity in the lymphoid organs of irradiated, THF-injected rats (not depicted in Table I).

Discussion. Prior to assay the partial removal of the vitamin C from rat thymic preparations did not inhibit THF activity when bioassayed as before (4). It is possible that some of the ascorbic acid remaining in the dialyzed THF fraction (ca. 50%) is bound to protein, since in the determination of thymic ascorbic acid no distinction was made between the bound and unbound vitamin. In other tissues (muscle, liver) bound ascorbic acid accounts for only 10–15% of the total fraction (9, 10), but in the thymus this moiety could be necessary for the expression of THF activity. Nevertheless, the present data suggest that vitamin C is not needed for the expression of THF activity, *per se*, but that it is involved in some manner for the production of THF during thymic ontogeny.

During a period of rapid thymic development guinea pigs were maintained 6 weeks on daily intakes of 0.25–5.0 mg of vitamin C and their thymuses used to prepare THF. The bioassay showed there was a good correlation between the activity of the various

TABLE I. Content of Ascorbic Acid and Activity of Rat Thymic Humoral Factor (THF) in Dialyzed and Nondialyzed Preparations.

Means ± SEM, $n = 5$.

	Ascorbic acid (μg/ml) in THF preparations		
	Before	After 48 hr	After 72 hr
Dialyzed THF:	205	150	110
Nondialyzed THF:	195	200	180
	Activity of THF[a] [organ wt (mg)]		
Treatment	Lymph nodes	Thymus	Spleen
None	137 ± 12	510 ± 45	1188 ± 90
Irradiated			
+ spleen extract	46 ± 3	77 ± 5	451 ± 34
+ dialyzed THF	60 ± 3[b]	111 ± 12[b]	446 ± 22
+ nondialyzed THF	53 ± 4	90 ± 8	422 ± 30

[a] Index for THF activity: increase in organ weight above that in rats treated with spleen extract.

[b] Significantly different from mean immediately above, $p < .05$, Student's t test.

THF preparations and the level of vitamin C in the thymus. In a few instances the lymphoid organs of the irradiated rats injected with THF did not show increases in weight and hexose monophosphate shunt enzyme activity that were strictly proportional to the level of vitamin C in the THF preparations, *e.g.*, increases in hexose monophosphate shunt enzyme activity of the lymph nodes, and increases in the weight of the thymus. These inconsistencies are probably attributable to the heterogeneity of the THF preparations, the nature of the bioassay employed, etc., and are to be expected in biological assays of this type (19).

The guinea pigs serving as a source of THF in this study were maintained on much lower vitamin C levels than those of the preceding study (4), and the vitamin C content in the thymus is correspondingly less. It is pertinent to note that the bioassay responses in the present study were also less marked than in the former one. As before (4), the percentage of dehydroascorbate in the thymus was greater in animals on low than on high vitamin C intakes, and in fact, was inversely proportional to the level of vitamin C in the diet. Judging from the weights of the lymphoid organs and adrenals in the guinea pigs, there was little evidence to indicate that the hypovitaminotic diets elicited a stress response that could result in the release of adrenal cortical hormones, and directly or indirectly affect THF production. It follows then that the level and oxidation state of vitamin C in the thymus are by themselves critical for the optimal production of THF, or else that vitamin C is necessary for the maintenance of particular cells that are concerned with the production of THF.

Changes in the oxidation state of thymic ascorbic acid have been demonstrated during normal age involution (2), after lympholytic steroid hormone treatment (1), or after whole body X-irradiation (unpublished results). In each of these experimental situations, the decrease in lymphoid organ mass has been associated with increased tissue levels of dehydroascorbate and decreased tissue levels of ascorbate. In addition one can demonstrate the opposite shift in the oxidation state of tissue vitamin C (increased ratio of ascorbate to dehydroascorbate) as the thymus increases in mass during normal development (2). An extensive literature exists in both plants and animals that points out the correlation between enhanced cellular growth and the increased cellular concentration of ascorbic acid and decreased concentration of dehydroascorbic acid (11). Thus the association between normal cellular activity and the cellular concentration of ascorbate and dehydroascorbate appears to be universal. Perhaps in the thymus the deficiency of vitamin C augments protein breakdown (THF?) and diminishes protein resynthesis, as it has been shown to do in the liver, brain, and spleen (12). If the vitamin is in fact involved in some facet of protein turnover involving THF, it is apparent that the oxidation state of vitamin C in the thymus assumes particular importance in this regard.

As suggested earlier, vitamin C in the thymus may also function in the maintenance of certain cells that are concerned with the production of THF. In particular the thymic reticular cells, which require vitamin C for their differentiation (13), maintenance (14), and function (15) are likely candidates, and are suspected by some to elaborate THF directly (16), and by others to direct the maturation of lymphocytes (17), or to provide an architectural framework for lymphocyte reconstitution in the thymus (18).

Summary. Cell-free thymic extracts (thymic humoral factor or THF) were prepared from guinea pigs maintained 6 weeks on daily intakes of 0.25, 0.50, 1.00, or 5.00 mg of vitamin C. The concentration of dehydroascorbate in their thymuses increased, and that of ascorbate (and total vitamin C) decreased in direct proportion to the level of vitamin C in the diet. The activity of each preparation was then compared by measuring regeneration of lymphoid organ weight and hexose monophosphate shunt enzyme activity in irradiated, THF-injected rats, and was found to be directly correlated with the level of ascorbate, and inversely correlated with the level of dehydroascorbate in the THF preparations. Thus the data indicate that throughout the ontogeny of the thymus, the level, and particularly the oxidation state of thymic vitamin C is involved in some manner

for the production of THF. No evidence was found that vitamin C was required for the actual expression of THF activity, since prior to bioassay, the partial removal of ascorbic acid from similarly prepared rat THF preparations did not result in diminished reponses.

1. Dieter, M. P., PhD dissertation, University of Missouri, Columbia, Missouri, 1968.

2. Dieter, M. P. and Breitenbach, R. P., Poultry Sci. 47, 1463 (1968).

3. White, A., and Goldstein, A. L., Perspect. Biol. Med. 11, 475 (1968).

4. Dieter, M. P., Proc. Soc. Exp. Biol. Med. 132, 1147 (1969).

5. Goldstein, A. L., Banerjee, S., Schneebeli, G. L., Dougherty, T. F., and White, A., Radiat. Res. 41, 579 (1970).

6. Hughes, R. E., Biochem. J. 64, 203 (1956).

7. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. 193, 265 (1951).

8. Dieter, M. P., Life Sci. 8, 459 (1969).

9. Sumerwell, W. N., and Scalock, R. R., J. Biol. Chem. 196, 753 (1952).

10. Patnaik, B. K., Nature (London) 218, 393 (1968).

11. Edgar, J. A., Experientia 25, 1214 (1969).

12. Ginter, E., Biologia 17, 1771 (1962).

13. Mitoma, C., and Smith, T. E., J. Biol. Chem. 235, 426 (1960).

14. Robertson, W. V. B., and Schwartz, B., J. Biol. Chem. 201, 689 (1953).

15. Stone, N., and Meister, A., Nature (London) 194, 555 (1962).

16. Law, L. W., Dunn, T. B., Trainin, N., and Levey, R. H., *in* "The Thymus" (V. Defendi and D. Metcalf, eds.), p. 105. Wistar Inst. Press, Philadelphia (1964).

17. Hays, E. F., and Alpert, P. F., J. Exp. Med 130, 847 (1969).

18. Hays, E. F., J. Exp. Med. 129, 1235 (1969).

19. Michie, D., *in* "Handbook of Experimental Immunology" (D. M. Weir, ed.), p. 969. Davis, Philadelphia (1967).

Received Sept. 2, 1970. P.S.E.B.M., 1971, Vol. 136.

2060

Proc Soc Exp Biol Med 137 (1):341-346. Illus. 1971.

Vitamin C in Lymphoid Organs of Rats and Cockerels Treated with Corticosterone or Testosterone[1] (35573)

MICHAEL P. DIETER AND ROBERT P. BREITENBACH

Laboratory of Physical Biology, National Institutes of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014; and Division of Biological Sciences, University of Missouri, Columbia, Missouri 65201

The lympholytic steroid hormones, testosterone and corticosterone, have been shown to be important factors responsible for changes in growth rate and intermediary metabolism in the developing lymphoid organs (1, 2). Recently there have been repeated demonstrations that a humoral factor elaborated by the mammalian thymus, a central lymphoid organ, influences the growth, metabolism, and immunologic function of the lymph nodes and spleen—peripheral lymphoid organs (3–7). It is probable that these hormones act in harmony to affect the physiological changes seen during ontogeny of the lymphoid organs.

Previously we have determined the concentration and oxidation state of vitamin C in the developing lymphoid organs of intact cockerels and found that the concentration of ascorbate relative to that of dehydroascorbate increased during most rapid growth, whereas during cessation of growth and ensuing involution, vitamin C concentration in the lymphoid organs decreased and the remaining portion of the vitamin was predominantly in the oxidized form (dehydroascorbate). The latter changes occurred at approximately the same period as maximum increases in comb and testis weights, and at the time of adrenal ascorbic acid depletion, suggesting that changes in testicular and/or adrenal function could have influenced the oxidation state of vitamin C in the lymphoid organs (8).

Subsequent investigations have shown an inverse correlation between the percentage of dehydroascorbate in the total vitamin C concentration of thymic preparations and the ability of thymic humoral factor to promote growth and direct glucose oxidation in the lymphoid organs (6). Additional work suggested that vitamin C was not required for the expression of thymic humoral factor *per se*, but that it was needed during ontogeny for the optimal production of the humoral factor (7).

Accordingly we examined the possibility that the lympholytic steroid hormones not only affect the growth and metabolism of the lymphoid organs directly, but may also change the oxidation state of vitamin C in the lymphoid organs, particularly in the thymus, which could indirectly affect the peripheral lymphoid organs by inhibiting the optimal production of thymic humoral factor.

Materials and Methods. Intact male Sprague-Dawley pathogen-free rats and single comb white leghorn cockerels were injected im for 14 days with 0.1 ml of testosterone propionate, corticosterone or vehicle (20% ethanol-cottonseed oil). Testosterone propionate was administered at 0.05, 0.50, and 5.0 mg/day and corticosterone at 0.05, 0.50, and 1.0 mg/day. The dose levels employed were those found in a previous study to cause near linear decreases in lymphoid organ weights; the highest dose level of corticosterone was reduced to 1.0 mg because cockerels were unable to tolerate 5.0 mg (9). Injections were begun in cockerels at 3 weeks and in rats at 5 weeks of age, because of the differences in rate of lymphoid organ development in the two species. Twenty-four hr after the last injection the cockerels were killed with an overdose of sodium Nembutal and the rats with chloroform. Body weights and weights of the lymphoid organs were recorded. Vitamin C was extracted from the lymphoid organs by homogenization in 3% metaphosphoric acid; ascorbate, de-

[1] Supported in part by U.S. Public Health Service grant AI-05643.

hydroascorbate, and total vitamin C were determined on the cleared supernate by the indophenol method (10). The data were analyzed using Student's t test with the level of probability set at 0.05.

Results. Figures 1 and 2 illustrate the decreases in relative lymphoid organ weight (closed circles) and total vitamin C concentration (ascorbate + dehydroascorbate) in the lymphoid organs (open circles) after lympholytic steroid hormone treatment. The relative weights of the central lymphoid organs in the rats (thymus) and cockerels (bursa and thymus) decreased after hormone treatment (Figs. 1 and 2, closed circles). The central lymphoid organs were more sensitive to corticosterone than to testosterone proprionate treatment and showed a linear decrease in relative weight in response to dose of hormone employed. The relative weights of the peripheral lymphoid organs in the rats (lymph nodes and spleen) and cockerels (spleen) varied in their response to hormone treatment. The maximum decline in relative lymph node weight after testosterone propionate treatment was at the 0.05-mg dose level, but at higher dose levels relative lymph node weights increased. Corticosterone treatment resulted in decreases in relative lymph node weights that were proportional to the levels of hormone administered. In rats the decreases in relative spleen weights were not proportional to dose of hormone administered as either of the lympholytic steroid hormones, at any of the dose levels employed, resulted in equivalent decreases in spleen weight. In birds there was a proportional decrease in relative spleen weight after testosterone propionate administration, whereas corticosterone treatment caused only a slight decline in relative spleen weight.

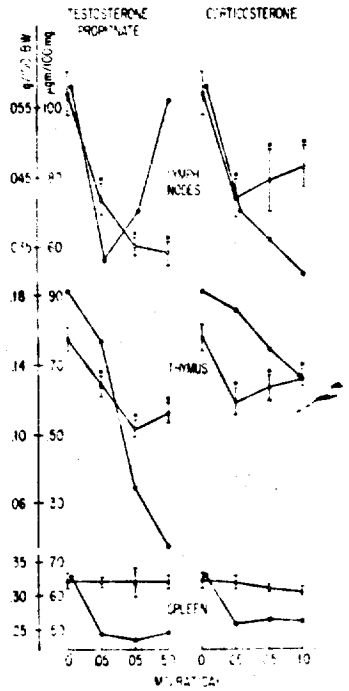


FIG. 1. The concentration of total vitamin C (ascorbate + dehydroascorbate) (μg/100 mg of tissue wt) (○); and relative weights (g/100 g of body wt) (●) of rat lymphoid organs after 14-days steroid hormone administration. Mean values for 7–10 rats, with standard errors of the mean indicated for total vitamin C concentration. *Significantly different concentration from controls, $p<0.05$, Student's t test.

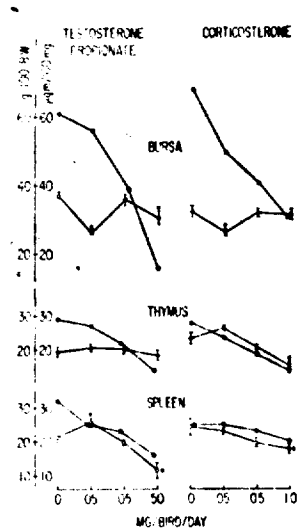


FIG. 2. The concentration of total vitamin C (ascorbate + dehydroascorbate) (μg/100 mg of tissue wt) (○); and relative weights (g/100 g of body wt) (●) of chicken lymphoid organs after 14-days steroid hormone administration. Relative weights are replotted from data submitted in a previous publication (9). Mean values for 8–16 cockerels, with standard errors of the mean indicated for total vitamin C concentration. * Significantly different concentration from controls, $p<0.05$, Student's t test.

In comparable lymphoid organs of control animals the concentration of total vitamin C was two- to threefold higher in rats than in cockerels (Figs. 1 and 2, open circles). However the content of total vitamin C (μg/organ) was 400 μg in the thymus and in the spleen of both rats and cockerels, 200 μg in the lymph nodes of rats, and 1600 μg in the bursa of cockerels.

Lympholytic steroid hormone treatment, at any dose level, caused a significant decrease in the concentration of total vitamin C in the lymph nodes and thymi of rats, but no change in the concentration in the spleens (Fig. 1). Decreases in the concentration of total vitamin C in the lymphoid organs of hormone-treated cockerels were not as marked (Fig. 2). There was a significant decrease of vitamin C in the bursa and the spleen after 5.0 mg testosterone propionate treatment, and in the thymus and spleen after 1.0 mg corticosterone treatment. In addition 0.05 mg of either hormone resulted in a significant decrease in vitamin C in the bursa, but in the spleen this level of testosterone propionate treatment significantly increased the concentration of total vitamin C.

In Tables I and II, the concentrations of oxidized (dehydroascorbate) and reduced (ascorbate) vitamin C have been expressed as percentages of the concentration of total vitamin C in the lymphoid organs of hormone-treated rats and cockerels. We presented these data in terms of concentration rather than content because of the decreases in organ weight induced by hormone treatment.

In the lymphoid organs of rats and cockerels ascorbate comprised 80–90% of the total vitamin C in the lymph nodes, bursa, and thymus, and 50–70% in the spleen. In all of the lymphoid organs except for the bursa and lymph nodes, lympholytic steroid hormone treatment decreased the percentage of ascorbate and increased the percentage of dehydroascorbate. Corticosterone treatment did not affect the oxidation state of vitamin C in the bursa, nor did testosterone propionate treatment in the lymph nodes. The greatest percentage change from controls after hormone treatment occurred in the dehydroascorbate moiety of the central lymphoid organs of the rat (thymus) and cockerel (bursa and thymus), where greater than 100% increases were seen. The highest dose level of testosterone propionate appeared to cause a slightly greater change in the percentage dehydroascorbate of the thymus than the highest dose level of corticosterone, although the thymus was much more sensitive to corticosterone treatment, since only 0.05 mg was needed to cause a significant change in the oxidation state of vitamin C. Testosterone propionate was the only hormone to affect the oxidation state of vitamin C in the bursa.

Discussion. In a previous study we observed an age-dependent decrease in the concentration of vitamin C in chicken lymphoid organs, and suggested that it could be due to steroid hormone output from the adrenals and/or testes (8). Present results support that hypothesis and show that exogenous administration of either lympholytic steroid hormone, testosterone or corticosterone, results in the reduction of vitamin C in chicken lymphoid organs. The lymphoid organs of the rat, which develop at a different rate (11), also respond to lympholytic steroid hormone treatment with vitamin C depletion. The rate of synthesis of vitamin C in these species is also affected by steroid hormones. Renal vitamin C synthesis in the chicken is inhibited by androgens (12); whereas hepatic vitamin C synthesis in the rat is augmented by androgens (13, 14). Yet corticosteroids, at least in chickens, have been shown to have no effect on vitamin C synthesis (12). The decreases in vitamin C concentration observed in the lymphoid organs of androgen-treated cockerels could thus be due to an androgenic inhibition of vitamin synthesis; however, the decreases in lymphoid organs of rats and in corticosterone-treated cockerels tend to indicate that hormone-induced vitamin depletion of the lymphoid organs is a response common to all types of lymphoid organs that is unrelated to hormonal effects on vitamin C biosynthesis.

The changes in the oxidation state of vitamin C evoked in the lymphoid organs of the hormone-treated animals were of particular interest. The potential for reversible oxidation reduction of ascorbate and dehydroascorbate, like that of reduced and oxidized glutathione (15), could assume importance in var-

TABLE I. Amounts of Dehydroascorbate (DHA) and Ascorbate (AsA) Expressed as a Percentage of Total Vitamin C in the Lymphoid Organs of Rats Injected 14 Days with Steroid Hormones.

Seven to 10 rats/group.

	Testosterone propionate			Corticosterone		
Organ	Dose (mg/day)	% DHA	% AsA	Dose (mg/day)	% DHA	% AsA
Lymph nodes	0	19.8 ± 2.1[a]	80.2	0	19.8 ± 2.1	80.2
	0.05	25.2 ± 3.3	74.8	0.05	27.8 ± 4.2	72.2
	0.5	20.9 ± 3.0	79.1	0.5	31.8 ± 5.1[b]	68.2[b]
	5.0	20.0 ± 3.1	80.0	1.0	30.7 ± 3.5[b]	69.3[b]
Change from control (%)		None	None		+55.1	−13.6
Thymus	0	11.5 ± 1.0	88.5	0	11.5 ± 1.0	88.5
	0.05	10.3 ± 1.4	90.7	0.05	19.1 ± 3.0[b]	80.9[b]
	0.5	11.9 ± 2.3	88.1	0.5	19.8 ± 3.1[b]	80.2[b]
	5.0	26.4 ± 5.4[b]	73.6[b]	1.0	23.6 ± 2.4[b]	76.4[b]
Change from control (%)		+129.6	−16.8		+105.2	−13.7
Spleen	0	29.2 ± 1.2	70.8	0	29.2 ± 1.2	70.8
	0.05	37.5 ± 2.5[b]	62.5[b]	0.05	35.8 ± 2.3[b]	64.2[b]
	0.5	39.4 ± 1.6[b]	60.6[b]	0.5	37.2 ± 2.3[b]	62.8[b]
	5.0	43.0 ± 1.7[b]	57.0[b]	1.0	40.4 ± 1.5[b]	59.6[b]
Change from control (%)		+47.3	−19.5		+38.4	−15.8

[a] Means ± SEM, standard errors for AsA same as those for corresponding DHA.
[b] Significantly different from controls at $p<0.05$, Student's t test.

TABLE II. Amounts of Dehydroascorbate (DHA) and Ascorbate (AsA) Expressed as a Percentage of Total Vitamin C in the Lymphoid Organs of Chickens Injected 14 Days with Steroid Hormones.

Eight to 16 chickens/group.

	Testosterone propionate			Corticosterone		
Organ	Dose (mg/day)	% DHA	% AsA	Dose (mg/day)	% DHA	% AsA
Bursa	0	12.9 ± 1.6[a]	87.1	0	12.9 ± 1.6	87.1
	0.05	12.7 ± 1.6	87.3	0.05	14.3 ± 2.1	85.7
	0.5	17.7 ± 2.8	82.2	0.5	14.8 ± 3.1	85.2
	5.0	32.4 ± 3.5[b]	67.6[b]	1.0	14.3 ± 2.0	85.7
Change from control (%)		+151.2	−22.4		None	None
Thymus	0	15.2 ± 2.3	84.8	0	15.2 ± 2.3	84.8
	0.05	14.8 ± 2.2	85.2	0.05	22.9 ± 1.7[b]	77.1[b]
	0.5	31.3 ± 3.6[b]	68.7[b]	0.5	35.0 ± 2.1[b]	65.0[b]
	5.0	55.6 ± 4.1[b]	44.4[b]	1.0	34.5 ± 5.0[b]	65.5[b]
Change from control (%)		+265.8	47.6		+127.0	−22.8
Spleen	0	49.5 ± 3.2	50.5	0	49.5 ± 3.2	50.5
	0.05	39.7 ± 3.3	60.3	0.05	58.6 ± 3.7	41.4
	0.5	55.4 ± 6.3	44.6	0.5	68.8 ± 7.3[b]	31.2[b]
	5.0	77.9 ± 10.7[b]	32.1[b]	1.0	68.3 ± 6.9[b]	31.7[b]
Change from control (%)		+57.4	−36.4		+38.0	−37.2

[a] Means ± SEM, standard errors for AsA same as those for corresponding DHA.
[b] Significantly different from controls at $p<0.05$, Student's t test.

ious dynamic processes that occur during lymphoid organogenesis. We found that lymphoid organ involution after hormone treatment was accompanied by a change in the oxidation state of vitamin C, such that the percentage of dehydroascorbate increased and that of ascorbate decreased. In other tissues, dehydroascorbate has also been associated with degenerative processes such as mitotic inhibition, activation of lysosomal enzymes, lipid peroxidation, and loss of control of membrane permeability (16); whereas regenerative processes such as ribonucleic acid synthesis (17) and collagen formation (18) are stimulated by ascorbate. The changes in the oxidation state of vitamin C found in the lymphoid organs of hormone-treated animals obviously must be of physiological significance. Note that the decrease in total vitamin C was paralleled by a decrease in ascorbate, and in terms of percentage change from controls, hormone treatment in some instances increased the percentage of dehydroascorbate more than 100%. Thus it could potentially reflect a greater than 2-fold change in effectiveness.

In earlier studies (6, 7) indirect evidence was obtained which indicated that dehydroascorbate inhibited the optimal production of a nonsteroidal humoral factor from the thymus. Thymic humoral factor has been shown to stimulate the growth (3, 5), metabolism (6, 7), and immunologic function (4) of the peripheral lymphoid organs. Present results demonstrate that lympholytic steroid hormone treatment affected a much greater change in the oxidation state of vitamin C in the central lymphoid organs (the thymus and bursa) than in the lymph nodes or spleen. These data collectively support the hypothesis that lympholytic steroid hormones cause an inhibition of the production of thymic humoral factor by oxidizing thymic ascorbate to dehydroascorbate, and thus affect the growth and function of the peripheral lymphoid organs.

Corticosterone was found to be far more effective in this regard than testosterone propionate, as only 0.05 mg was needed to significantly alter the oxidation state of vitamin C in the thymus. This difference in response should prove to be of value in future experiments regarding vitamin C–hormone interrelationships, since we found earlier that androgen effects on lymphoid organ growth and metabolism were more permanent than those of corticosterone (9). Also it would appear to be important in the immune development of animals that inhibitory changes evoked by adrenocortical stressor hormones, such as oxidation of ascorbate in the lymphoid organs, would be of a transient rather than a permanent nature.

Summary. Immature rats and cockerels were injected for 14 days with 0.05–5.0 mg of testosterone propionate or corticosterone, and the concentration and oxidation state of vitamin C in their lymphoid organs was measured. The total vitamin C concentration (ascorbate + dehydroascorbate) in the lymphoid organs of both animals decreased after hormone administration, but to a greater extent in rats than in cockerels. These changes appear to be independent of any direct hormonal influence on vitamin C biosynthesis. Significant and marked changes in the oxidation state of lymphoid organ vitamin C occurred after lympholytic steroid hormone treatment: the percentage of dehydroascorbate increased and that of ascorbate decreased. After hormone treatment the largest percentage change from controls (more than 100% increases in percentage dehydroascorbate) occurred in the central lymphoid organs, the bursa and thymus, rather than in the spleen or lymph nodes. Much lower dose levels of corticosterone (0.05 mg) than of testosterone propionate (5.0 mg) were required to produce this shift in the oxidation state of lymphoid organ vitamin C. We suggest the ascorbate–dehydroascorbate system in the lymphoid organs is an integral portion of the physiological mechanisms operative during hormonal modulation of the growth, metabolism, and function of the lymphoid organs.

1. Dougherty, T. F., Physiol. Rev. 32, 379 (1952).

2. Blecher, M., and White, A., Rec. Progr. Horm. Res. 15, 391 (1959).

3. Klein, J. J., Goldstein, A. L., and White, A., Proc. Nat. Acad. Sci. U.S.A. 53, 812 (1965).

4. Goldstein, A. L., Asanuma, Y., Battisto, J. R., Hardy, M. A., Quint, J., and White, A., J. Immunol. 104, 359 (1970).

5. White, A., and Goldstein, A. L., Perspect. Biol. Med. 11, 475 (1968).

6. Dieter, M. P., Proc. Soc. Exp. Biol. Med. 132, 1147 (1969).

7. Dieter, M. P., Proc. Soc. Exp. Biol. Med. 136, 316 (1971).

8. Dieter, M. P., and Breitenbach, R. P., Poultry Sci. 47, 1463 (1968).

9. Dieter, M. P., and Breitenbach, R. P., Proc. Soc. Exp. Biol. Med. 133, 357 (1970).

10. Hughes, R. E., Biochem. J. 64, 203 (1956).

11. Everett, N. B., and Tyler, R. W., Int. Rev. Cytol. 22, 205 (1967).

12. Dieter, M. P., Proc. Soc. Exp. Biol. Med. 130, 210 (1969).

13. Stubbs, D. W., and McKernan, J. B., Proc. Soc. Exp. Biol. Med. 125, 1326 (1967).

14. Stubbs, D. W., McKernan, J. B., and Haufrect, D. B., Proc. Soc. Exp. Biol. Med. 126, 464 (1967).

15. Pora, A. E., Toma, V., and Fabian, N., C. R. Acad. Sci. 255, 2207 (1962).

16. Edgar, J. A., Nature (London) 227, 24 (1970).

17. Price, C. E., Nature (London) 212, 1331 (1966).

18. Gould, B. S., Ann. N.Y. Acad. Sci. 92, 8 (1961).

Received Dec. 21, 1970. P.S.E.B.M., 1971, Vol.

Food Technol. 11(2): 109-113 1957

The Time-Temperature Tolerance of Frozen Foods. IV. Objective Tests To Measure Adverse Changes In Frozen Vegetables

W. C. Dietrich, F. E. Lindquist, J. C. Miers, G. S. Bohart, H. J. Neumann, and W. F. Talburt

Western Utilization Research Branch, Agricultural Research Service, United States Department of Agriculture, Albany 10, California

(Manuscript received September 1, 1956)

THE NUMBER OF ARTICLES PUBLISHED in the past 25 years on objective tests for measuring quality of frozen vegetables indicates the importance of this field of research. In 1937, Campbell *(1)* noted that undesirable color changes occurred in frozen peas when stored at temperatures above 0° F. and demonstrated that they were due to conversion of chlorophyll to pheophytin. Mackinney and Weast *(8)* proposed the use of chlorophyll and its degradation product, pheophytin, for measuring adverse color changes in frozen vegetables and described a method for determining the ratios of concentration of these two compounds. Kaloyereas *(5)* has suggested that the amount of liquid draining from fruits and vegetables after thawing is related to the quality of the product. Woodroof and Shelor *(16)* noted that the size of ice crystals in frozen products is correlated with freezing conditions and post-freezing storage history. Lee and Wagenknecht *(6)* stored unblanched peas at 0° F. for 5 years and showed that the peroxide number, iodine number, and acid number of the extracted lipid material changed during storage and that rancid off-flavors developed in the peas. Tressler and Pederson *(13)* suggested ascorbic acid and the number of micro-organisms present in frozen foods as indices of quality. Joslyn and David *(4)* found that raw or underblanched peas accumulated alcohol and acetaldehyde during frozen storage, which paralleled off-flavor development. Wagenknecht *et al. (15)* followed the loss of chlorophyll during storage of unblanched frozen peas and postulated that lipoxidase is involved in the destruction of chlorophyll. Total ascorbic acid and its degradation products—dehydroascorbic and diketogulonic acids—have been shown to remain constant in orange juice during storage for 36 days at 36° F. by Mills *et al. (10)*. No mention is made of using the ratios of reduced ascorbic acid to its oxidation products as an index of quality for frozen foods in the latter article, but the results obtained indicate that this may provide a useful tool for assay of quality, provided the sum of reduced ascorbic acid and its oxidation products remains constant in other foods as was demonstrated in orange juice. Other ways of determining quality of frozen vegetables have been proposed, including presence of cavity ice, ratios of chlorophylls *a* and *b*, and amount and type of organic acids and amines.

Evaluation of these tests and development of new tests for quality of frozen foods have been considered an important part of the comprehensive studies of changes in frozen foods which are presently under way in this Branch. The objectives and general plan for these studies have been described by Van Arsdel *(14)* in the introductory article of this series on the "time-temperature tolerance of frozen foods." Extensive subjective appraisal of samples under test by trained judges is an integral and important part of the work, in order to correlate changes in color, flavor, and texture obtained by objective measurements with those determined organoleptically.

In this work, a number of lots of frozen vegetables were obtained from various packers. These were selected to be representative of principal producing areas, leading commercial varieties, and processing variables encountered in the several plants. These various lots were shipped with dry ice to Albany, California, and subjected to various constant temperatures, fluctuating temperatures, and time-temperature cycles simulating those encountered in handling frozen foods. At intervals, samples were withdrawn from the various sublots undergoing test for objective and subjective appraisal.

Most of the program on frozen peas and green beans has been completed and work on cauliflower and spinach is in progress. Other frozen vegetables will be added to the program later. Data obtained by subjective appraisal and the correlation of subjective and objective test results will be reported in a later paper.

METHODS AND MATERIALS

The large number of samples involved in these tests made it mandatory to limit the number of objective tests that could be carried through the program. Since these commercial samples were all adequately blanched, acetaldehyde, alcohol, and changes in lipid material, which occur only in raw or underblanched material, were eliminated from the program as quality indices after some preliminary experiments. Test for adequacy of blanch was by the Masure-Campbell *(9)* peroxidase test on a filtered extract. Ice-crystal characteristics were difficult to measure and define and the amount of liquid draining from thawed samples did not seem to be correlated with changes during storage. Changes in the absolute amount of chlorophyll or in the ratio of chlorophylls *a* and *b* did not readily provide as much information as obtainable from the ratio of chlorophyll to pheophytin.

Four objective tests have been used to date: (1) reduced ascorbic acid, (2) total reduced ascorbic acid and its oxidation products, (3) percent chlorophyll converted to pheophytin, and (4) changes in reflectance color as measured by the Hunter Color and Color-Difference Meter.

Reduced ascorbic acid and its oxidation products. Reduced ascorbic acid, dehydroascorbic acid, and diketogulonic acid were determined by the procedure of Mills *et al. (10)*. In this procedure, the filtrate from a slurry of vegetable acidified with metaphosphoric acid is treated with H_2S gas to reduce dehydroascorbic acid (DHA) to reduced ascorbic acid (AsA), and CO_2 is bubbled through to remove excess H_2S. Diketogulonic acid (DKA) is obtained by direct coupling with 2,4-dinitrophenylhydrazine (DNPH). Thiourea is added to prevent oxidation of AsA. DHA and DKA are determined on a second portion of filtrate by coupling with DNPH. Total AsA, DHA, and DKA are determined on a third portion of filtrate by oxidizing reduced

AsA with a slight excess of bromine and coupling with DNPH after removing excess bromine by aeration. By proper subtractions, the amount of each compound is obtained. Reduced ascorbic acid determined by this procedure was found to be 2 to 5% higher than that obtained by the indophenol method as described by Loeffler and Ponting *(7)* as modified by Ponting *(11)*.

Chlorophyll-pheophytin. A modification of the method of Dutton *et al.* *(3)* to provide for a larger sample was used to determine the percent conversion of chlorophyll to pheophytin. An 80% acetone extract of frozen vegetable was made by the procedure described by Dietrich *et al.* *(2)*. The optical density of the filtered extract was measured at 534 and 556 mμ with a spectrophotometer and the ratio of chlorophyll to pheophytin determined by the method of Mackinney and Weast *(8)*.

Reflectance color. Color attributes of samples of peas and green beans were measured with a Hunter Color and Color-Difference Meter *(2)* set to a reference tile with values of *Rd*, *a*, and *b* of 31.0, −24.0, and +13.1, respectively. The frozen material was thawed in the package 3 hours at room temperature. Three readings were made on material from one or two packages. The material was mixed between readings. Samples were submersed in 0.5 to 1.0 inch of distilled water in a cell 3.3 inches in diameter and reflected light readings for values of *Rd*, *a*, and *b* were taken through a 2.25-inch circular opening in the cover plate.

The following lots of raw material, consisting of 50 to 100 dozen retail packages per lot, were used in the experiments: *Peas:* 4 lots, 3 varieties, 3 growing areas, 3 crop years. *Green snap beans:* 5 lots, 4 varieties, 3 growing areas, 3 crop years. *Cauliflower:* 2 lots, 2 varieties, one growing area, one crop year.

RESULTS

Ascorbic acid and related products. Changes in reduced ascorbic acid during storage at 20° F. are shown in Figure 1 for several lots of peas, green beans, and cauliflower. Differences in initial reduced ascorbic acid contents between the different vegetables and lesser differences between varieties of the same vegetable are readily apparent. Differences in rates of loss are clearly indicated by the slopes of the several lines. In Figure 2, losses of reduced ascorbic acid in peas stored at 0°, 10°, 20°, and 30° F. are shown.

Results of analyses for the sum of AsA, DHA, and DKA in several lots of peas, green beans, and cauliflower stored at several temperatures are shown in Figure 3. In the samples of green beans stored at 10°, 20°, and 25° F., the total amount of reduced ascorbic acid and its oxidation products remained almost constant. AsA, which is being oxidized under these conditions, is being converted into DHA and DKA with the reaction proceeding no further. Since there are marked decreases in the AsA contents of the two samples of cauliflower and a lesser decrease in the one sample of peas, it is obvious that reaction products other than DHA and DKA (perhaps involving fragmentation of the DKA molecule) are being produced.

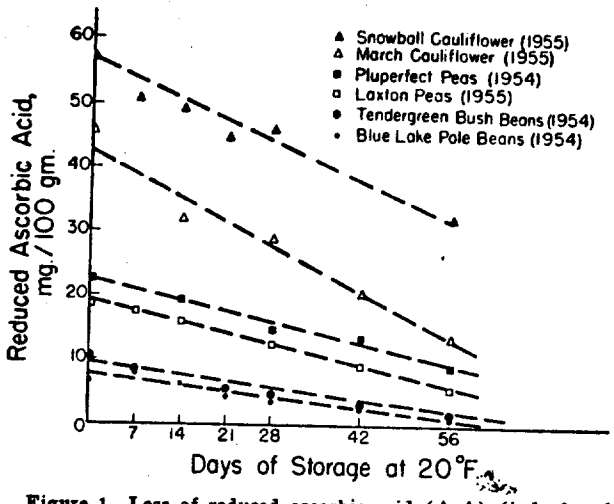


Figure 1. Loss of reduced ascorbic acid (AsA) (indophenol method) in cauliflower, peas and green beans at 20° F.

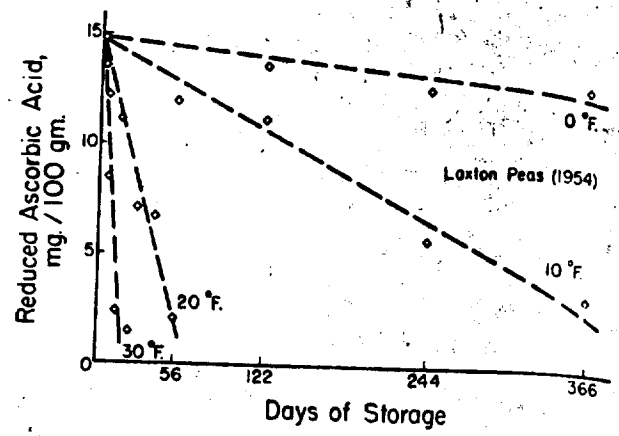


Figure 2. Loss of reduced ascorbic acid (AsA) (indophenol method) in Laxton peas (1954) at 0°, 10°, 20°, and 30° F.

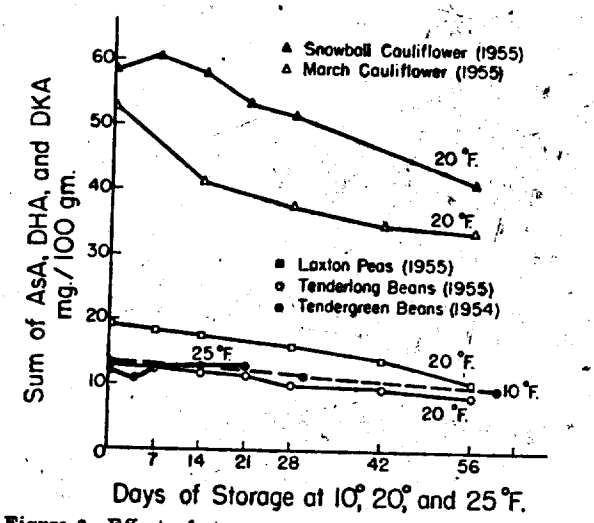


Figure 3. Effect of storage on the sums of reduced ascorbic acid, dehydroascorbic acid, and diketogulonic acid in cauliflower, peas, and bush beans at 20° F.; and at 10° and 25° F. for bush beans.

Amounts of AsA, DHA, and DKA found in one lot of Tendergreen beans stored at 25° F. are plotted in Figure 4. In this experiment, the sum of the 3 compounds remained almost constant, AsA decreased markedly, and the DHA and DKA increased by substantial amounts. Similar data for peas and cauliflower are shown in Figures 5 and 6. In both peas and cauliflower, AsA and sum of AsA, DHA, and DKA dropped sharply. DHA remained almost unchanged and fairly large increases in DKA occurred. It will be noted that initially appreciable amounts of DHA and DKA were found in the various samples. This is not surprising, since Mills *et al.* *(10)* found several mg./100 g. of DHA and lesser amounts of DKA in a number of "fresh" fruits and vegetables, particularly after holding at room

temperature. Rates of accumulation of DKA in green beans, cauliflower, and peas stored at 20° F. for periods up to 8 weeks are shown in Figure 7.

Conversion of chlorophyll to pheophytin. Data obtained for conversion of chlorophyll to pheophytin in several lots of peas and beans are plotted in Figure 8. These data show that chlorophyll in peas is considerably more stable than chlorophyll in beans during processing

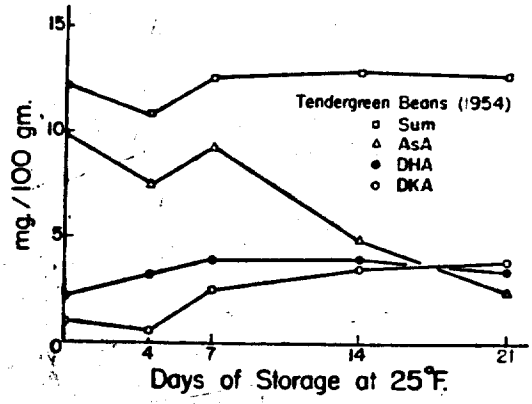


Figure 4. Effect of storage at 25° F. on the AsA, DHA, DKA content of (1954) Tendergreen bush beans graphed separately and as a sum of the three compounds.

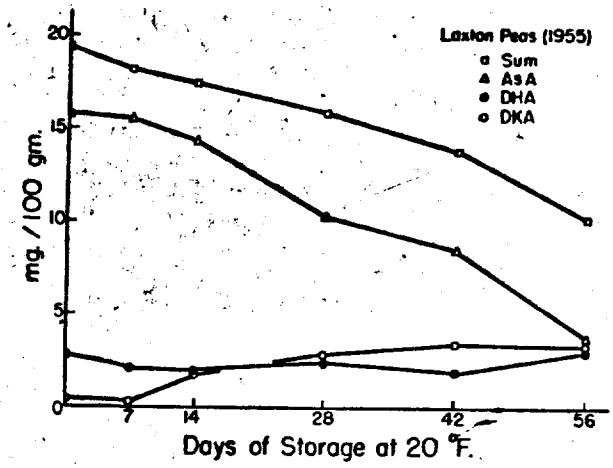


Figure 5. Effect of storage at 20° F. on AsA, DHA, DKA and the sum of these compounds in (1955) Laxton peas.

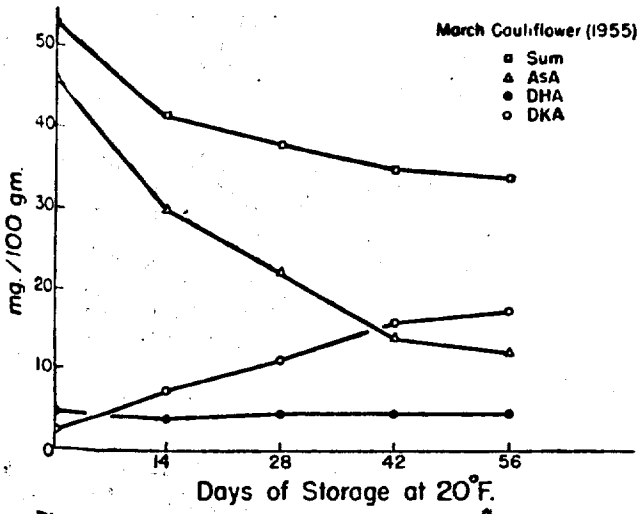


Figure 6. Effect of storage at 20° F. on AsA, DHA, DKA, and the sum of these compounds in (1955) March cauliflower.

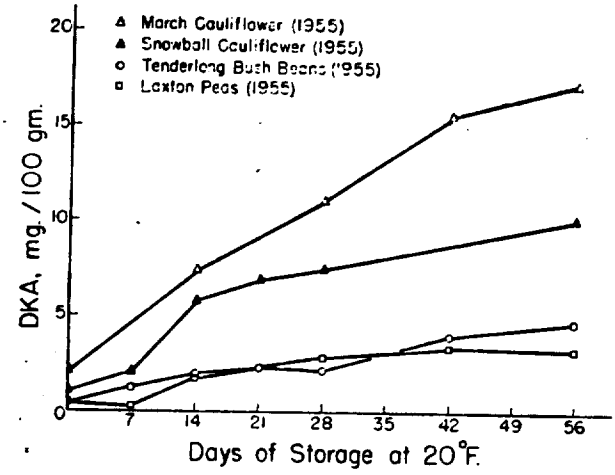


Figure 7. Effect of storage at 20° F. on the 2,3-diketogulonic acid (DKA) content of cauliflower, bush beans, and peas.

and storage. The amount of converted chlorophyll found initially is assumed to have occurred during processing, since Mackinney and Weast *(8)* and unpublished experiments in this Branch have shown that little or no pheophytin is present in freshly harvested peas or beans. It is interesting and perhaps significant from a commercial viewpoint that the rate of change of chlorophyll to pheophytin in beans proceeds more rapidly if processing has been conducted in such a way

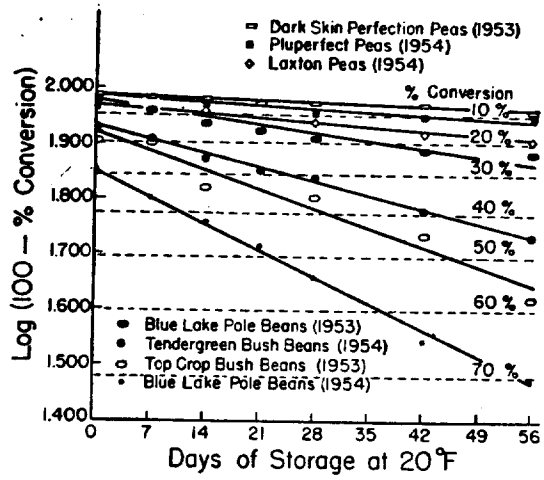


Figure 8. Effect of storage on the percentage of conversion of chlorophyll to pheophytin in peas and green beans at 20° F.

as to cause excessive chlorophyll degradation. Figure 9 illustrates the effect of storage temperature on chlorophyll degradation in green beans at 0°, 10°, 20°, and 30° F.

Reflectance color. Color changes during storage of frozen green beans and peas, as measured by the Color-Difference Meter, differ markedly. The ratio of a/b, which shows a change in hue from green towards yellow, best represents changes in color of green beans. The quantity $\sqrt{a^2 + b^2}$, which is a measure of change in saturation, best represents changes in peas. A decrease in the latter value indicates a grayer, duller, less vivid color. Robinson *et al.* *(12)* have illustrated and discussed the use of these calculations to express color in tomato juice and their conversion to other color systems.

Data obtained from bush beans and peas stored at 20° F. have been selected to illustrate respective changes in a/b and $\sqrt{a^2 + b^2}$. It is practicable to plot these values in the form shown in Figure 10, because the values fall in a favorable area in Hunter's chromaticity graph and changes in color cover only a small range of values.

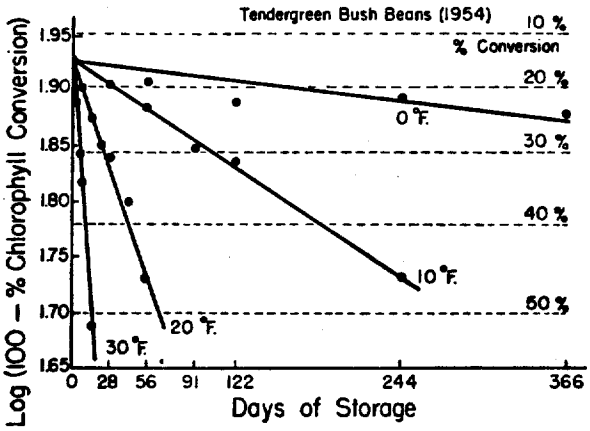


Figure 9. Increase in the percentage of conversion of chlorophyll to pheophytin in bush beans at 0°, 10°, 20°, and 30° F.

DISCUSSION

Determination of quality of frozen foods can be made by objective tests and by subjective appraisal. Theoretically, the latter method is superior, since it permits evaluation of the same quality factors—color, texture, and flavor—by the procedure that will be used by the consumer. Unfortunately, subjective appraisal is time consuming, is subject to considerable error, and is very difficult, if not impossible, to duplicate in different laboratories.

An ideal objective test for quality of frozen vegetables would produce data that would be correlated with subjective appraisal results, would be applicable to all products, would require no expensive equipment or reagents and only a short time to complete, would be reproducible with different operators, and would provide definite answers from a single sample without reference to condition of raw material or product immediately after processing, which normally is not available. It is unreasonable to expect any objective test to meet this ideal; however, the knowledge that the tests reveal is important in the current industrial effort to reduce

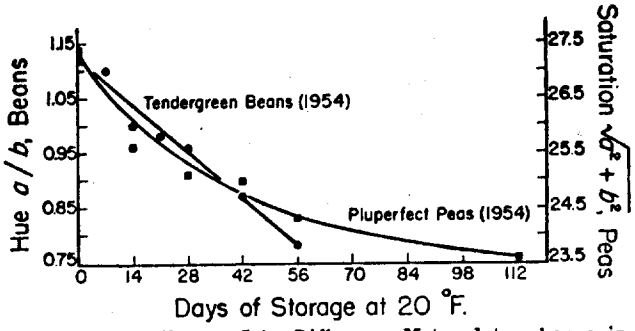


Figure 10. Hunter Color-Difference Meter data, change in hue (a/b) and saturation ($\sqrt{a^2 + b^2}$) for bush beans and peas, respectively, at 20° F. storage.

changes in quality through control of temperature and other environmental factors.

Decreases in amounts of AsA are correlated well with time-temperature history for frozen vegetables (Figures 1 and 2) and parallel changes in color as measured by change of chlorophyll to pheophytin. Data from this Branch (to be published soon) show that changes in AsA content are also closely related to flavor and color as determined by subjective appraisal. There is, however, one very serious shortcoming: determinations made on a single sample have very little value alone and are useful for determining temperature history only when retention can be calculated. This of course requires that AsA content after processing be available, since this value varies with variety, growing area, processing conditions, and other factors. Underblanching is also known to cause rapid loss of AsA.

When combined with data on amounts of DHA and DKA present, AsA becomes much more useful as a quality index and as an indicator of time-temperature history. Maximum usefulness and reliability are obtained when the change of AsA does not proceed beyond DKA or, to state this another way, while the sum of AsA, DHA, and DKA remains constant. Under these conditions, there is little need for information other than that obtainable from the sample in question. Conditions approaching this were found in the storage of green beans (Figure 4). However, in the case of peas, and cauliflower (Figures 5 and 6) there are substantial decreases in total AsA, DHA, and DKA which make this a much less valuable tool for the latter vegetables. Mills *et al. (10)* showed that in orange juice stored at 36° F. the sum of AsA, DHA, and DKA remained constant for 30 days while, under the same conditions, only 5% of the total amount remained in potato slurry stored for about 19 days. These results are consistent with the known behavior of DKA, which has been shown to undergo fragmentation of its carbon chain at pH 6.7. Differences in stability of AsA, DHA, and DKA noted for green beans, peas, and cauliflower may be due to pH differences of these commodities.

In all lots of vegetables that have been investigated to date, there has been a gradual increase of DKA proportional to storage temperature and paralleling storage time reasonably well. Much more data are needed on the increase of DKA during storage of frozen vegetables, as well as on overall changes of AsA, DHA, and DKA, before these compounds, singly or in combination, can be useful as quality indices.

Analytical procedures for AsA, DHA, and DKA are somewhat complicated and time consuming and will require considerable refinement and simplification before they can be used for purposes other than research.

For frozen vegetables containing chlorophyll, the loss of chlorophyll appears to be an excellent quality index, since it parallels adverse color changes, loss of ascorbic acid, and off-flavor development. Other investigators have shown that pheophytin is the sole degradation product of chlorophyll in vegetables that are adequately blanched. In such a test, it is not necessary to determine the absolute amount of chlorophyll or pheophytin present, since all of the required information can be

obtained from the ratios of these two compounds. This procedure is straightforward, reproducible, and takes only moderate time.

Interpretation of data is complicated somewhat, particularly for green beans, where substantial conversion of chlorophyll to pheophytin occurs during processing (Figures 8 and 9). In the samples tested to date, this conversion ranged from 7 to 30% for green beans and from 3 to 8% for peas. However, since color is one of the most important factors in determining quality, color deterioration, whether during processing or storage, is equally limiting in terms of consumer acceptance.

Differences in stability of chlorophyll between green beans and peas are quite striking (Figure 8). It is known that the conversion of chlorophyll to pheophytin —the only chlorophyll reaction that occurs in adequately blanched frozen vegetables—involves the exchange of magnesium of the chlorophyll molecule for hydrogen ion and that this reaction is favored by lower pH. The lower pH of green beans is quite likely one of the factors responsible for greater losses of chlorophyll in green beans.

Hue *(a/b)*, saturation ($\sqrt{a^2 + b^2}$), and lightness *(L)* of frozen beans and peas as measured with the Hunter Color and Color-Difference Meter were all affected by storage. Peas and beans darkened rapidly (*L* value decreased) early in storage and then remained almost unchanged. Saturation values for peas decreased with increasing storage time while hue remained nearly unchanged in the early stages of storage and then decreased rapidly. Saturation and hue of green beans changed progressively with increasing storage, but changes in hue were considerably more consistent than changes in saturation.

Reflectance measurements on frozen peas and green beans fusnish at least a qualitative measurement of changes that occur during storage. Under conditions used in these experiments, only a small amount of material was viewed by the instrument and, as a consequence, there was considerable variation between readings. When several readings were averaged, fairly consistent values were obtained.

Reflectance measurements appear to be promising as a research tool to determine influence of storage, blanching, and pre-processing conditions on color of vegetables. Certain problems are encountered here, however. The instrument does not recognize variability between color of different pieces of vegetables but merely gives an overall color measurement. It has also been noticed in this work that variation in roughness or in the amount of moisture on the surface of the vegetable has a marked effect on the values obtained.

SUMMARY

Commercial lots of frozen peas, green beans, and cauliflower obtained from the principal producing areas were held at temperatures ranging from 0° to 30° F. Various tests for changes in certain constituents were applied to determine the relationship of the results to time and temperature. Reduced ascorbic acid was found to be a reliable index of previous storage history but it has limited value unless the original content of samples is known. A summation of reduced ascorbic acid and its oxidation products (dehydroascorbic and diketogulonic acids) is more useful than reduced ascorbic acid alone, particularly for green beans. Accumulation of diketogulonic acid as storage progresses seems to parallel adverse changes reasonably well, but more data are needed before more definite conclusions can be drawn. Conversion of chlorophyll to pheophytin was found to be well correlated with temperature experience but is complicated by wide variations in amounts of chlorophyll lost during processing. Measurements of hue and saturation of color of peas and green beans by the Hunter Color Difference Meter show progressive changes related to temperature experience. In spite of rather large experimental error in the method used and initial variations in color between samples, this procedure offers considerable promise.

Acknowledgment

The suggestions and interpretation of reflectance data made by H. C. Lukens and M. M. Boggs are gratefully acknowledged.

LITERATURE CITED

1. Campbell, H. Undesirable color change in frozen peas stored at insufficiently low temperatures. *Food Research*, 2, 55-57 (1937).
2. Dietrich, W. C., Lindquist, F. E., Bohart, G. S., Morris, H. J., and Nutting, M-D. Effect of degree of enzyme inactivation and storage temperature on quality retention in frozen peas. *Food Research*, 20, 480-491 (1955).
3. Dutton, H. J., Bailey, G. F., and Kohake, E. Dehydrated spinach: Changes in color and pigments during processing and storage. *Ind. Eng. Chem.*, 35, 1173-77 (1943).
4. Joslyn, M. A., and David, J. J. Acetaldehyde and alcohol in raw and underblanched peas. *Quick Frozen Foods*, 15 (4) 51-53 (1952).
5. Kaloyereas, S. A. Drip as a constant for quality control of frozen foods. *Food Research*, 12, 419-428 (1947).
6. Lee, Frank A., and Wagenknecht, A. C. On the development of off-flavor during the storage of frozen raw peas. *Food Research*, 16, 239-244 (1951).
7. Loeffler, H. J., and Ponting, J. D. Ascorbic acid. Rapid determination in fresh, frozen or dehydrated fruits and vegetables. *Ind. Eng. Chem., Anal. Ed.*, 14, 846-849 (1942).
8. Mackinney, G.; and Weast, C. A. Color changes in green vegetables. Frozen-pack peas and green beans. *Ind. Eng. Chem.*, 32, 392 (1940).
9. Masure, M. P., and Campbell, H. Rapid estimation of peroxidase in vegetable extracts—an index of blanching adequacy for frozen vegetables. *Fruit Prod. J.*, 23, 369 (1944).
10. Mills, M. B., Damron, C. M., and Roe, J. H. Ascorbic acid, dehydroascorbic acid, and diketogulonic acid. *Anal. Chem.*, 21, 707-709 (1949).
11. Ponting, J. D. Extraction of ascorbic acid from plant materials. Relative suitability of various acids. *Ind. Eng. Chem., Anal. Ed.*, 15, 389 (1943).
12. Robinson, W. B., Wishnetsky, T., Ransford, J. R., Clark, W. L., and Hand, D. B. A study of methods for the measurement of tomato juice color. *Food Technol.*, 6, 269-275 (1952).
13. Tressler, D. K., and Pederson, C. S. A sound basis for frozen food standards. *Food Packer*, 32 (9) 20-21 (1951).
14. Van Arsdel, W. B. Time-temperature tolerance of frozen foods. I. Introduction—the problem and the attack. *Food Technol.*, 11, 28-33 (1957).
15. Wagenknecht, A. C., Lee, Frank A., and Boyle, Frank P. The loss of chlorophyll in green peas during frozen storage and analysis. *Food Research*, 17, 343-350 (1952).
16. Woodroof, J. G., and Shelor, Ethyl. Effect of storage temperature on frozen foods. *Refrig. Eng.*, 56 (6) 514-517 (1948).

Proc. Soc. Exp. Biol. Med. 139: 540-543 1972

2100

Some Anti-Inflammatory Properties of Ascorbic Acid (36181)

FRANK A. DOLBEARE AND KENNETH A. MARTLAGE
(Introduced by R. G. Herrmann)
The Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46206

Vascular and cell membrane permeability increases, the immune (allergic) response, and lysosomal hydrolase activity are interrelated in the inflammatory process. Ascorbic acid has been shown to play a stabilizing role in several of these processes indirectly in scorbutic animals. Ascorbic acid deficiency leads to separation of the cells in the vascular endothelium (1). A similar effect has been observed after histamine injection (2). The evidence for ascorbate stabilization of cellular membranes is not well documented (3). The anti-allergic effect of ascorbic acid has been demonstrated through its ability to reduce anaphylaxis in the guinea pig (4), mouse (5), and rat (6), but not in the dog (7). We have shown previously that ascorbic acid can inhibit lysosomal β-glucuronidase (8). These results led us to investigate the effect of ascorbic acid in several animal models of inflammation. This report compares the inhibitory actions of ascorbic acid, aspirin, and phenylbutazone against rat liver lysosomal β-glucuronidase (*in vitro*), UV erythema in guinea pigs, carrageenan edema in rats, adjuvant arthritis and peritonitis–polyarthritis in rats.

Materials and Methods. Animals used in these studies were (1) male, Cox/Holtzman rats for lysosomal β-glucuronidase and PPA, (2) female, Harlan/Holtzman rats for carrageenan edema and adjuvant arthritis, and (3) female albino guinea pigs for UV erythema. L-Ascorbic acid (Eastman), aspirin, and phenylbutazone (Geigy) were made up in stock solutions 20× final concentration in ethanol/isotonic saline (1/1) for *in vitro* studies and five-fold concentration in carboxymethylcellulose for animal studies. Lysosomes were prepared by the method of deDuve *et al.* (9). The lysosomal pellets from five grams of rat liver were suspended in 200 ml of Mg-free Krebs–Ringer-Phosphate buffer, pH 7.4. Fifty μl of stock solution of drug was added to 1 ml of lysosomal suspension. Control tubes contained 1 ml of lysosomal suspension and 50 μl of ethanol/saline. The tubes were incubated for 60 min at 37°. Then, 250 μl of 5 m*M* phenolphthalein-β-glucuronide (sodium salt) (Sigma) in 0.5 *M* acetate buffer, pH 5.0, was added to each tube. Incubation was continued for an additional 10 min. The enzyme reaction was stopped by the addition of 2.0 ml of 0.4 *M* glycine buffer, pH 10.7, containing 0.25% sodium dodecyl sulfate to clear the suspension. Phenolphthalein was determined with a Zeiss PMQ II spectrophotometer at 560 nm.

UV erythema was induced on guinea pigs by a modification of the method of Winder *et al.* (10). A Hanovia lamp (Kromayer model 10) was placed in contact with the skin on the back of a guinea pig for seven seconds. The animals were dosed *po* 15 min before UV exposure. The erythemas were examined at 1 hr then half hour intervals until two and one-half hours after irradiation. The severity of the burn was rated 0–3. The rating was multiplied by 4 at 1 hr, 3, 2, 1 for each of the successive intervals. Control irradiated animals rated 120 by this technique.

Edema was produced in rat paws with carrageenan by the method of Winter *et al.* (11). Drugs were given *po* two and one-half and one-half hour before the carrageenan injection. Paw volumes were determined after three hours by mercury displacement using a pressure transducer. The injected right hind paw was compared to the left, noninjected paw.

Adjuvant arthritis was produced according

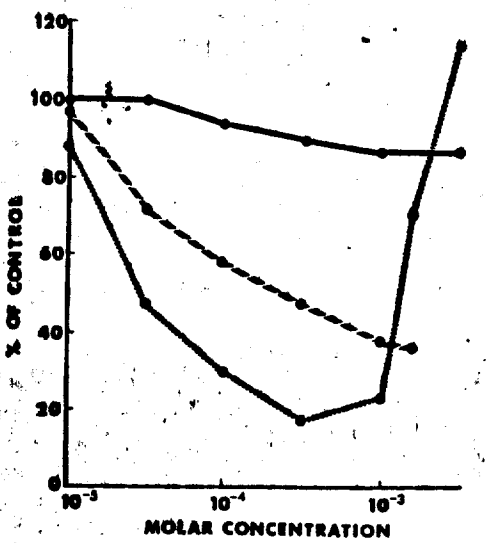


FIG. 1. The inhibition of lysosomal β-glucuronidase by ascorbic acid (●——●), aspirin (●——●), and phenylbutazone (●——●). The drugs were added in 50 μl aliquots to 1 ml of dilute liver lysosome suspension in KRP.

to the method of Pearson (12) with the injection of 0.25 mg *Mycobacterium tuberculosis* in 0.05 ml of mineral oil into the right hind foot pad. Paw volumes were determined at intervals of 0, 10, and 15 days. Animals were dosed po for 15 days.

Peritonitis–polyarthritis (PPA) was produced by the method of Dolbeare (13). A sterile peritonitis was induced by injecting 1 mg heat-killed *Mycobacterium tuberculosis* (Difco) in 0.2 ml of mineral oil. Paws begin to show first signs of swelling around day 8. Animals were dosed *po* daily for 10 days. Paw volumes were measured every 2 days for 22 days.

Results. Ascorbic acid between 0.01 and 1 m*M* incubated with lysosomal suspensions inhibited β-glucuronidase activity (Fig. 1). Higher ascorbic acid concentrations not only did not inhibit but enhanced the enzyme activity above that for controls. Phenylbutazone was less effective as an inhibitor at the lower concentrations but was more effective above 1 m*M*. Aspirin at 1 m*M* produced less than 20% inhibition of the enzyme.

Table 1 compares the anti-inflammatory actions of ascorbic acid, aspirin, and phenylbutazone in the carrageenan, erythema blocking, and adjuvant tests. Ascorbic acid did not inhibit the UV erythema response in guinea pigs. Aspirin (100 mg/kg) and phenylbutazone (50 mg/kg) produced almost complete inhibition of the erythema at 1 hr after irradiation. In the carrageenan test ascorbic acid inhibited the edema 12% compared to 29% for aspirin and 50% for phenylbutazone ($p < .05$). In adjuvant arthritis ascorbic acid (50 mg/kg) showed significantly greater activity (21.4% inhibition) than in the carrageenan edema. Aspirin (100 mg/kg) inhibited the swelling 6% and phenylbutazone (50 mg/kg) 33% ($p < .05$).

In the PPA model ascorbic acid (20 mg/kg) inhibited swelling to the same extent as phenylbutazone (50 mg/kg) during the 10–22 day period of the experiment (Fig. 2). Aspirin (100 mg/kg) was approximately 50% as effective as either ascorbic acid or phenylbutazone.

Discussion. We have demonstrated that ascorbic acid inhibits β-glucuronidase in lysosomal suspensions. Our earlier study showed that the inhibition was not due to membrane stabilization since ascorbic acid

TABLE 1. The Comparative Effects of Ascorbic Acid, Aspirin, and Phenylbutazone in Animal Models of Inflammation.

Test	Percent of Inhibition		
	Ascorbic Acid	Aspirin	Phenylbutazone
UV Erythema	0	80 ± 1.5	90 ± 1.5
Carrageenan	12 ± 1.1	29 ± 2.5	49 ± .6
Adjuvant Arthritis			
7 days	21 ± .1	12 ± .2	33 ± .1
14 days	25 ± .2	0	43 ± .1

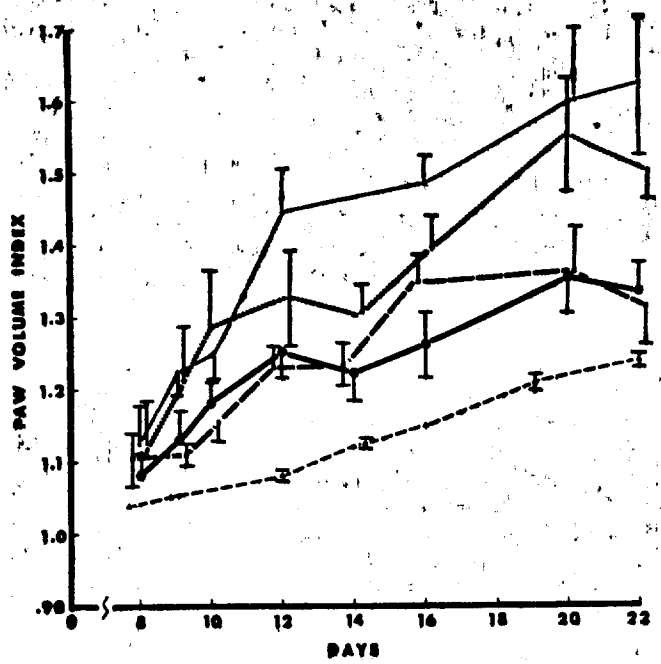


FIG. 2. Inhibition of swelling by ascorbic acid (●▬▬▬●), aspirin (●┅┅┅●), and phenylbutazone (▬ ▬ ▬ ▬) in peritonitis–polyarthritis. Control, noninjected, (△▬ ▬ ▬△). Control, TB injected (○▬▬▬○). Paw volume index—paw volume/initial paw volume. Standard error of the mean is presented.

inhibits the enzyme in sonicated and nonsonicated suspensions almost equally (8). Synovial levels of this enzyme increase during arthritis in humans (14) and the blood of rats during the development of polyarthritis (13, 15). Since many of the anti-inflammatory compounds inhibit lysosomal hydrolases (16), an anti-inflammatory role is suggested by the ascorbic acid inhibition of β-glucuronidase.

The lack of activity in the UV erythema and the good activity in the polyarthritis in the rats suggest that ascorbic acid may act as an inhibitor in the immune process. Dawson *et al.* (4) showed that ascorbic acid had synergistic effects when administered with antihistamines against antigen-induced bronchospasms in sensitized guinea pigs. In the same paper these authors proposed a possible anti-bradykinin activity for the ascorbic acid. In still another study Dawson and coworkers (17) demonstrated the inhibition of histamine induced bronchospasms in guinea pig. Other investigators have shown similar effects with the mouse (5), and rat (6).

Ascorbic acid may have important membrane action because of its reducing properties. It may behave similarly to the antioxidant, α-tocopherol, which stabilizes lysosomes at low concentrations (18) but causes hydrolase release at high concentrations (9). Chayen *et al.* (19) have shown that there is a relationship between free to bound acid hydrolase and free to total sulfhydryl groups in cultured synovial fibroblasts. Inagaki (20) has shown that ascorbate and cysteine inhibit a membrane bound K^+-Na^+-ATPase which may be involved in ion transport through plasma membrane. Tsen and Collier (21) and Jacob and Jandl (22) have shown that sulfhydryl reagents react with membrane -SH sites, lead to K^+ loss, Na^+ gain, and ultimately cell lysis. Of interest is the finding of Ignarro (23) that lysosomes in heavy mitochondrial

liver fractions were unstable in the presence of Na^+. Alteration in the oxidation state of the membrane may lead to increases in Na^+ in the cytoplasm around lysosomes. The lysosomes release hydrolases which further the damage to the plasma membrane.

Receptor sites may be affected through the adenyl cyclase system. Fassina (24) and Hynie *et al.* (25) have shown that ascorbic acid can potentiate the action of epinephrine and ACTH on fat cell lipolysis. This action appears to be via phosphodiesterase inhibition and not a direct effect on adenyl cyclase. May *et al.* (26) have shown that cyclic 3′, 5′-AMP inhibits the release of lysosomal β-glucuronidase. Other investigators (27–29) have shown that epinephrine exhibits anti-inflammatory action. Ascorbic acid may potentiate this effect.

Summary. Ascorbic acid was compared with aspirin and phenylbutazone for inhibitory action against lysosomal β-glucuronidase, UV erythema, carrageenan paw edema, adjuvant arthritis, and peritonitis–polyarthritis. The vitamin was a very effective inhibitor of β-glucuronidase activity and peritonitis–polyarthritis swelling, but a moderately effective inhibitor of adjuvant arthritis swelling. Ascorbic acid was weakly inhibitory in the carrageenan edema, and not inhibitory against UV erythema.

We appreciate the technical assistance of Mr. Charles Hillman, Mr. Norman Dininger, and Mr. Charles Brown. We are further indebted to Mr. Donald Benslay for assistance and experimental data for the UV erythema test.

1. Gore, I., Fujinami, T., and Shirahama, F., Arch. Path. 80, 371 (1965).

2. Majno, G., and Palade, G. E., J. Biophys. Biochem. Cytol. 11, 571 (1961).

3. Kryukova, L. V., Byul. Eksp. Biol. Med. 58, 1318 (1964).

4. Guirgis, H. M., J. Pharm. Pharmacol. 17, 387 (1965).

5. Hoffman, V., Arch. Exp. Path. Pharmacol. 199, 531 (1942).

6. Dawson, W., and West, G. B., Brit. J. Pharmacol. Chemother. 24, 725 (1965).

7. Csaba, B., and Toth, S., J. Pharm. Pharmacol. 18, 325 (1966).

8. Dolbeare, F. A., Fed. Proc. 30, 1092A (1971).

9. DeDuve, C., Wattiaux, R., and Wibo, M., Biochem. Pharmacol. 9, 97 (1962).

10. Winder, C V., Wax, Jr., Burr, V., Been, M., and Rosier, S. E., Arch. Int. Pharmacodyn. 116, 261 (1958).

11. Winter, C. A., Risley, E. A., and Nuss, G. W., J. Pharmacol. 141, 369 (1963).

12. Pearson, C. M., Proc. Exp. Biol. Med. 91, 95 (1956).

13. Dolbeare, F. A., Proc. Soc. Exp. Biol. Med. 138, 312 (1971).

14. Jacox, R. F., and Feldmahn, A., J. Clin. Invest. 34, 263 (1955).

15. Collins, A. J., and Lewis, D. A., Biochem. Pharmacol. 20, 251 (1971).

16. Anderson, A. J., Biochem. Pharmacol. 17, 2253 (1968).

17. Dawson, W., Hemsworth, B. A., and Stockham, M. A., Brit. J. Pharmacol. Chemother. 31, 269 (1967).

18. Guha, A., and Roels, O. A., Biochim. Biophys. Acta 111, 364 (1965).

19. Chayen, J., Bitensky, L., Butcher, R. G., and Poulter, L. W., Nature (London) 222, 282 (1969).

20. Inagaki, C., Jap. J. Pharmacol. 20, 52 (1970).

21. Tsen, C. C., and Collier, H. B., Can. J. Biochem. 38, 981 (1960).

22. Jacob, H. S., and Jandl, J. H., J. Clin. Invest. 41, 779 (1962).

23. Ignarro, L. J., Biochem. Pharmacol. 20, 2847 (1971).

24. Fassina, G., Life Sci. 6, 2191 (1967).

25. Hynie, S., Cernohorsky, M., and Cepelik, J. Europ. J. Pharmacol. 10, 111 (1970).

26. May, C. D., Levine, B. B., and Weissmann, G., Proc. Soc. Exp. Biol. Med. 133, 758 (1970).

27. Spector, W. G., and Willoughby, D. A., Ann. N.Y. Acad. Sci. 116, 839 (1964).

28. McKinney, G. R., and Lish, P. M., Proc. Soc. Exp. Biol. Med. 121, 494 (1966).

29. Brown, J. H., Mackey, H. K., Rigglio, D. A., and Schwartz, N. L., J. Pharmacol. Exp. Ther. 160, 243 (1968).

Received Sept. 1, 1971. P.S.E.B.M., 1972, Vol. 139.

Modern Hosp. 61(4): 110-115

How Cooking Affects Vitamins

A CONDENSED summary of the most recent information on vitamin losses in cooking is presented in the accompanying table. Although the data, of necessity, are incomplete, this compilation should make a major contribution to the general knowledge of the subject by indicating the gaps that remain to be filled before a really intelligent and complete picture of the problem can be anticipated.

The Army classification of foods is followed and individual data are presented under subdivisions representing different cooking utensils. No differentiation between poor and good cooking procedures is attempted but the range of values may indicate the extremes of cooking practices encountered.

It is doubtful whether too much significance can be attached to the "average" figures until a larger number of controlled experiments is available. The importance of specifying cooking time, amount of water and type of heat cannot be overemphasized and such data must be available before tables that have a wide scope of usefulness can be prepared.—C. A. ELVEHJEM, *chairman, and* PAUL L. PAVCEK, *secretary, Committee on Food Composition, Food and Nutrition Board, National Research Council, Washington, D. C.*

Vitamin Losses in Cooking Foods

	Vitamin A % Loss			*Thiamine % Loss*			*Riboflavin % Loss*			*Niacin % Loss*			*Ascorbic Acid % Loss*		
	Dis	*Tl*	*T*	*Dis*	*Tl*	*T*	*Dis*	*Tl*	*T*	*Dis*	*Tl*	*T*	*Dis*	*Tl*	*T*
MEATS, FISH, POULTRY (*Beef, lamb, mutton, pork, poultry, glandular organs*)															
Boiling				47*	15*	62*									
Broiling					25	35		10*	20*		10*	20*			
					18-43*	21-50*		0-12	10-23		4-25	18-29*			
Baking						60						55			
						50-70*			57*			49-67*			
Braising					45	45		15	20		15	35			
					27-88	15-51		10-28	19-28*		0-34	26-39*			
Roasting					35	40		10	20		10	20			
					16-55	20-56		0-15	0-61		3-19	2-37			
Frying						25		15	20		25	30			
						0-54		0-33	3-56		0-72	0-64			

(*Continued on Page* 112)

Vitamin Losses in Cooking Foods (Cont.)

	Vitamin A % Loss			Thiamine % Loss			Riboflavin % Loss			Niacin % Loss			Ascorbic Acid % Loss		
	Dis	*Tl*	*T*	*Dis*	*Tl*	*T*	*Dis*	*Tl*	*T*	*Dis*	*Tl*	*T*	*Dis*	*Tl*	*T*
Stewing					43&65*	40-82		10*	35*		10*	50*			
Steaming											10 0-15*	40* 34-46			
All methods				47*	35 6-88	40 0-82		10 0-33	20 0-61		15 0-72	30 0-67			
Eggs															
Boiled and scrambled						20*			20-25*			25*			
Cereals, Grain Products															
Bread						10-15			0			10			
Grain products						0-10			0			Negligible			
Beans, Other Legumes, Nuts (Dry)															
Baked, boiled, pressure cooked						0-32**			0			16 8-25**			
Vegetables—Leafy Green or Yellow															
Leafy Green (Beet Greens, Brussels Sprouts, Cabbage, Chard, Spinach, Turnip Greens)															
Boiled			30* 6-61	20** 8-32	25** 17-40	35** 25-53			20* 6-38	5* 0-11	20* 12-26	25* 17-37	25 12-66	15 0-56	45 15-78
Steamed									20* 14-27			15* 4-19	20 5-50	15 6-26	30 6-76
Pressure													15* 8&18	5* 2&5	30* 13-53
Sautéed															55** 49&59
All methods			30* 6-61	20 8-32	25 17-40	35 20-53			20 6-38	5* 0-11	20* 12-26	20 4-37	25 5-66	15 0-56	45 6-78
Green and Yellow (Asparagus, Green Beans, Broccoli, Kohlrabi, Okra, Peas, Peppers, Squash, Sweet Potatoes)															
Boiled			25 0-79	10 0-31	15 0-59	25 0-64			5** 0-13	5 0-13	10 2-35	20 5-42	25 12-48	10 2-45	40 6-69
Steamed												0*	10 0&18*	15 14&14*	25 14&32
Sautéed															50 0-76
Baked			10 8-11**												40 6-72**
Pressure															40** 12-58
Waterless cooker															50** 48-50
All methods			20 0-79	10 0-31	15 0-59	25 0-75			5** 0-13	5 0-13	10 2-35	15 0-42	25 0-48	10 2-45	40 0-76
Potatoes															
Boiled			7*	13*	20*	25 10-36			25* 9&43	10* 2-19	5* 4-12	20 8-31	5*	19*	25 0-46
Baked						40 16-35									35 0-73
Fried															35 27-51
All methods			7*	13*	20*	30 10-55	7*	5*	20* 9-43	10* 2-19	5* 4-12	20 8-31	5*	19*	30 0-73

(*Continued on Page* 114)

Vitamin Losses in Cooking Foods (Cont.)

	Vitamin A % Loss			Thiamine % Loss			Riboflavin % Loss			Niacin % Loss			Ascorbic Acid % Loss		
	Dis	*Tl*	*T*	*Dis*	*Tl*	*T*	*Dis*	*Tl*	*T*	*Dis*	*Tl*	*T*	*Dis*	*Tl*	*T*
Vegetables Other Than Leafy Green or Yellow (*Beets, Cauliflower, Celery, Corn, Cucumber, Eggplant, Lima Beans, Onions, Parsnips, Rhubarb, Rutabagas, Sauerkraut, Turnips*)															
Boiled			5 0&5**	10 4-12**	40 6-80**	45 9-92			20 10-53	15 0-31	15 2-26	35 8-54	25 0-41	10 0-17	35 0-77
Steamed				50*	5*	55*			10 5-17			30** 6-61	10** 0-19	10 4-14	30 14-69
Pressure													5 0-11	15 0-36	35 8-70
Waterless cooker															55 49&66*
Sautéed															50 22-60
Baked															20 12-27**
All methods			5 0&5**	20 4-51**	30 4-80**	45 9-92			20 5-53	15 0-31**	15 2-26**	30 6-61	25 0-41	10 0-36	35 0-77
Fruits, Other Than Citrus															
Apples, all methods of preparation															80 30-40
Other fruits (*i. e.* plums, bananas, apricots)															15* 0-5*
Berries															
Dried Fruits	Negligible*														

Legend:
Dis—dissolved Tl—thermal loss T—total loss *—one laboratory **—inadequate data

The Lancet Dec 5 1970 (ii): 1197

ASCORBIC ACID AND SERUM-CHOLESTEROL

SIR,—There is evidence that high doses of ascorbic acid (vitamin C) reduce the cholesterol concentration in plasma and tissues of experimental animals [1,2] and that tissue-cholesterol levels are raised in guineapigs deficient in ascorbic acid.[3] There is evidence that a high dietary-cholesterol intake reduces the life-span of guineapigs, an effect which is reduced if excess dietary ascorbic acid is given at the same time.[4] These observations led us to wonder whether, in man, there is any evidence that ascorbic acid protects against the effects of a high serum-cholesterol. A further possible aspect of such a mechanism concerns tobacco consumption. Cigarette smoking is probably associated with an increased risk of arteriosclerotic heart-disease, but the mechanism involved is unknown. Smoking lowers leucocyte and plasma ascorbic-acid,[5] and if ascorbic acid is involved in cholesterol metabolism a raised prevalence of arteriosclerosis in heavy smokers might result.

We have lately tested a hypothesis based on these considerations—namely, that there is a negative correlation between plasma-ascorbic-acid and serum-cholesterol in man. Our data relate to a representative community sample of normal women living in a Welsh mining valley. There is an association between cigarette consumption and plasma-ascorbic-acid (see below). However, we found no evidence

Cigarettes/day:	*No.*	*Plasma-ascorbic-acid (mg./100 ml.)*
None	154	0·63+0·03
<15	61	0·54+0·07
15+	39	0·45+0·08
Total	254	0·58+0·03

of a significant association (at $P<0·05$) between plasma-ascorbic-acid and serum-cholesterol. In the non-smokers the mean serum-cholesterol was 231±3·8 mg. per 100 ml., and the linear correlation coefficient (r) with plasma-ascorbic-acid is 0·02 ($P>0·05$).

M.R.C. Epidemiology Unit (South Wales)
and
University of Wales Institute of Science and Technology, Cardiff.

P. C. ELWOOD
R. E. HUGHES
R. J. HURLEY.

1. Sokoloff, B., Hori, M., Saelhof, C. C., Wrzolek, T., Imai, T. *J. Am. Geriat. Soc.* 1966, 14, 1239.
2. Ginter, E., Bobek, P., Babala, J., Barbierikova, E. *Cor Vasa*, 1969, 11, 65.
3. Ginter, E. Proceedings of Fifth Convegno Internatzonale, Rimini, 1966, p. 299.
4. Hughes, R. E., Jones, P. R. Unpublished.
5. Brook, M., Grimshaw, J. J. *Am. J. clin. Nutr.* 1968, 21, 1254.

J. Pharmacol. and Exptl Ther. 78: 215-221, 1943.

THE EFFECT OF SODIUM DIPHENYL HYDANTOINATE (DILANTIN SODIUM) ON THE UTILIZATION OF ASCORBIC ACID BY GUINEA PIGS[1]

A. D. EMMETT, EVA R. HARTZLER AND R. A. BROWN

From the Research Laboratories of Parke, Davis and Company, Detroit, Michigan

Received for publication September 10, 1942

It has been reported that sodium diphenyl hydantoinate (dilantin sodium), an effective anticonvulsant agent in epilepsy, may stimulate gingival hyperplasia in some patients when given over extended periods. Kimball (1, 2) associated this with a possible vitamin C deficiency. Frankell (3) made similar observations and reported some evidence that the plasma ascorbic acid level was lowered. Drake, Gruber and associates (4, 5) claimed that dilantin sodium definitely lowered the vitamin C of the blood in guinea pigs and also of the tissues in rats. An opposite view has been expressed by Gruhzit (6), Merritt and Foster (7), Hawke (8), Ziskin *et al.* (9), and Millhon and Osterberg (10). This report is further evidence in support of the latter opinion.

The plan of our study included feeding young guinea pigs a basal scurvy-producing diet, then giving them graded amounts of ascorbic acid and in turn supplementing this with different doses of dilantin sodium. The criteria used were: (*1*) the effect on weight increment, (*2*) gross pathological changes, (*3*) assay for the ascorbic acid plasma levels, and (*4*) the determination of the ascorbic acid content of the adrenals, brain, testes and liver.

EXPERIMENTAL. *Animals and diet:* Young guinea pigs weighing 250–300 grams were kept in groups of 4 to 11 animals each. The scurvy diet fed throughout was essentially that of Sherman (11). It consisted of rolled oats, 29.5%; wheat bran, 29.5%; heated skim milk powder, 30%; butter fat, 9%; cod liver oil, 1% and sodium chloride, 1%. In addition, water and autoclaved clover hay were given *ad libitum*. The animals were weighed at regular intervals and, as the experiment progressed, observations were recorded on the appearance and condition of the fur, eyes, teeth, gums, tenderness and enlargement of the joints. Further, on autopsy, the following were noted: firmness of teeth, presence of "rosary" on the ribs, enlargement of joints, hemorrhagic appearance of the knees, elbows and intestinal tract. The lungs, liver, kidneys and adrenals were also observed.

Experiments: Four feeding experiments were carried out. In each, the animals were divided into 5 to 7 groups, some of which served as controls while, with the other groups, the interrelationship of ascorbic acid and dilantin sodium was studied. Experiments 1 and 2 were the preventive scurvy type and experiment 3 was the curative type. In these, the weight increment, the general appearance, and autopsy findings were noted. In experiment 4, preventive type, the ascorbic acid plasma levels and the ascorbic acid content of tissues were determined.

Methods: In experiments 1, 2 and 3 the ascorbic acid was given orally by pipette and the dilantin sodium orally from capsules by emptying the powder well back in the throat and following with a few drops of water. In experiment 4 the ascorbic acid was administered subcutaneously; the concentration of the solution was such that the dose was not more than 1.0 cc. The dilantin sodium was given in the same manner as in experiments 1 and 2.

[1] Presented before the September 1942 meeting of the American Chemical Society, Buffalo, N. Y.

The samples for the blood plasma were taken by cardiac puncture from each guinea pig once a week, over a period of 7 weeks. Further, the actual time of drawing the test sample was adjusted so that it fell between 16 and 20 hours after administering the supplements. To this end, the animals from each group were always handled routinely in the same order. Immediately after taking the blood and transferring it to tubes, it was centrifuged. The plasma ascorbic acid was determined at once by the Farmer-Abt (12) micro-method.

For the tissue work, male guinea pigs were taken. They were killed by decapitation. Following the Bessey technic, (13) the tissues (the adrenals, brain, right testis and liver) were handled as rapidly as possible, weighed, ground with washed sand in the presence of 3% metaphosphoric acid, diluted with more acid, centrifuged and assayed colorimetrically, using the Evelyn photocolorimeter.

TABLE 1

Effect of dilantin sodium on weight increment

GROUP	DAILY TREATMENTS		NUMBER OF ANIMALS	BODY WEIGHT		GAIN
	Ascorbic acid (a)	Dilantin (b)		Initial	Final	
Experiment 1 (7 weeks)						
	mgm.	*mgm.*		*grams*	*grams*	*grams*
I	Lettuce	None	4	261	545	284
II	Lettuce	50	4	258	479	223
III	0.5	None	4	249	456	207
IV	0.5	50	4	264	498	234
V	0.5	100	4	258	479	223
Experiment 2 (7 weeks)						
I	Lettuce	None	5	308	544	236
II	Lettuce	50	7	284	502	218
III	0.5	None	8	308	525	217
IV	0.5	50	9	296	487	191
V	0.5	100	7	282	475	193
VI	None	None	9	316	196(c)	−120

(a) Mgm. per guinea pig per day.
(b) Mgm. per kilo. per day.
(c) Weight on 26th day.

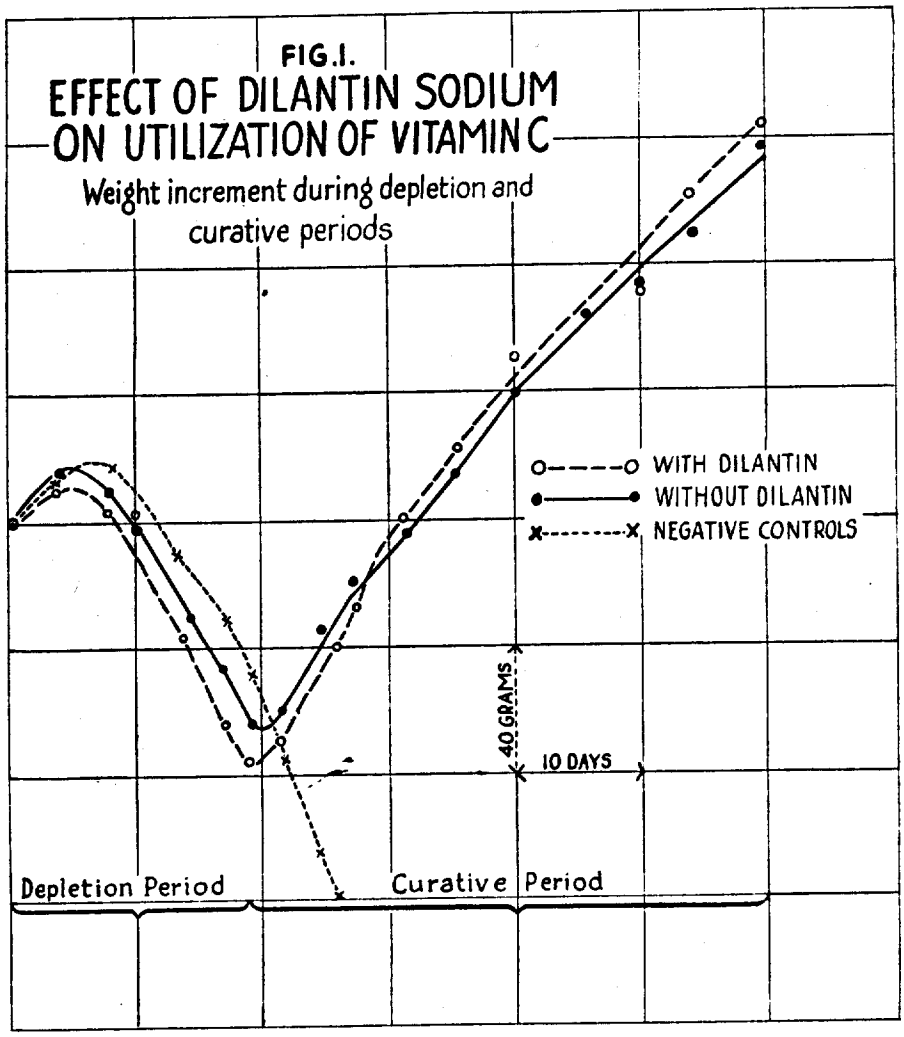
RESULTS. *Effect on weight increment:* The average weight gains for experiments 1 and 2 are given in table 1 and those for experiment 3 in figure 1. The data are arranged primarily so as to compare the effect of the two supplements, ascorbic acid and dilantin sodium. The level of vitamin C (0.5 mgm. daily) was intentionally kept low while that for the dilantin sodium was very high. It was thought that such a high ratio would tend to accentuate any effect that dilantin sodium might have.

The results indicate, except for group VI, the negative control, that all the guinea pigs made relatively good gains. All animals were active and alert and gave no outward indications of having any scurvy symptoms.

Specifically, the data in both experiments 1 and 2 indicate for groups I and II (lettuce with and without dilantin sodium) that the drug seemed to retard the gains. This difference was due in part however to variations in animals, and

in part to the handling which the guinea pigs of group II received in their daily treatment. Comparing the values in groups III, IV and V, the evidence indicates that dilantin sodium had very little effect.

Additional data in experiment 3 (fig. 1) substantiate this. Here there were three groups, having 9 to 11 animals each. All were kept on the vitamin C



depletion diet for about three weeks in order to permit scurvy to develop (as measured by weight). One group received 50 mgm. dilantin sodium per kilogram per day while the other two had no supplement. From this time on, one group was continued on the scurvy diet as negative control and two groups (one with and without dilantin sodium) were given, over a curative period of 45 days, 0.5 mgm. of ascorbic acid per day per pig. It will be seen from the curves that

the animals receiving the dilantin sodium behaved no differently than the controls throughout the depletion and curative periods. Further, examination upon autopsy of these guinea pigs showed no differences between the groups with respect to symptoms of scurvy including gingivitis.

Effect on the plasma ascorbic acid levels: Our original plan in experiments 1, 2 and 3 called for observations on the plasma ascorbic acid level. We found, however, that the normal plasma level was so very low (0.02 to 0.05 mgm. %) that it would be difficult to measure any significant differences in a decline. Later, Drake and associates (5), in studying this same problem with guinea pigs, apparently overcame the difficulty by first giving the animals an excess of ascorbic acid (5 mgm. per day). In this manner they raised the vitamin C blood level to around 0.6 mgm. %. Under these conditions they found, upon giving 13 mgm. of dilantin sodium per kilo per day, that the ascorbic acid level dropped to around 0.3 mgm. % and on withdrawing the drug, it came up to 0.6–0.7 mgm. Unfortunately the authors did not state how soon the blood samples were taken following their treatment with ascorbic acid and dilantin sodium, nor did they give any data for control groups. Since it is known that plasma vitamin C levels drop very rapidly immediately after giving ascorbic acid, the obvious question arises as to whether Drake *et al.* made their observation before the animals had time to allow the blood vitamin C level to approach a constant or maintenance phase. This rapid decline was shown by Zilva (14) who found the plasma ascorbic acid level after injecting 50 mgm. of vitamin C was 32.5 mgm. % in 30 minutes and down to 3.5 mgm. in 4 hours.

In experiment 4, we followed the plan of Drake *et al.* in raising the ascorbic acid plasma levels above that of the controls. Our procedure differed from theirs fundamentally, however, in three respects: First, the basal rations were not alike, second, Drake *et al.* studied the whole blood while we tested plasma; and third, we adjusted the time of taking the cardiac puncture samples so as to avoid the rapid falling phase. We therefore allowed appreciable time for the ascorbic acid blood levels to approach maintenance. Following our daily routine sequence of carrying out the various steps in the technic, this interval of time was 16–20 hours.

The data are presented in table 2. Groups I and II serve as the controls and give values of 0.01–0.03 mgm. ascorbic acid per 100 cc. of plasma. Group III and IV correspond with those of Drake *et al.* It will be seen that while the vitamin C levels are definitely higher than the controls they are decidedly lower than theirs. When the data are examined with respect to dilantin sodium, they indicate that the drug had no effect. In order to raise the so-called maintenance plasma level further, in group V, VI and VII, 25 mgm. of ascorbic acid were injected subcutaneously. Besides, in group VII, the daily dose of dilantin sodium was increased from 13 to 50 mgm. per kilo per day, which is 10 times the human daily dose. It will be seen that while the plasma levels were thus raised to around 0.4 mgm. %, the values for the different groups were practically identical, indicating again that dilantin sodium had no effect on the utilization of vitamin C.

Effect on the ascorbic acid content of tissues: Since we were unable to find any

effect of dilantin sodium on the plasma ascorbic acid levels, we decided to investigate its effect on the storage of this vitamin in the tissues of the animals. Determinations were accordingly made on the adrenals, brain (cerebrum), liver

TABLE 2

Effect of dilantin sodium on plasma ascorbic acid

GROUP	NUMBER OF ANIMALS	TREATMENT		AVERAGE MILLIGRAMS ASCORBIC ACID PER 100 CC. PLASMA							
		Ascorbic acid (a)	Dilantin (b)	Preliminary period	Experimental Period						
					1st week	2nd week	3rd week	4th week	5th week	6th week	7th week
I	25	Lettuce	None	0.05	0.02	0.01	0.01	0.02	0.02	0.03	0.03
II	17	None	None		0.02	0.02	0.02	0.01	0.03	0.01	
III	16	5	None	0.18	0.14	0.14	0.18	0.14	0.12	0.13	0.11
IV	24	5	13	0.19	0.15	0.13	0.12	0.12	0.11	0.11	0.10
V	20	25	None	0.51	0.36	0.36	0.31	0.27	0.31	0.32	0.33
VI	29	25	13	0.40	0.35	0.35	0.30	0.27	0.33	0.32	0.28
VII	25	25	50	0.40	0.32	0.34	0.31	0.26	0.32	0.32	0.30

(a) Mgm. per guinea pig per day.
(b) Mgm. per kilo. per day.

TABLE 3

Effect of dilantin sodium on ascorbic acid content of tissues

GROUP	TREATMENT		ADRENALS			BRAIN			TESTIS			LIVER
	Ascorbic acid (a)	Dilantin (b)	Gms. tissue two adrenals	Mgm. per gm. of tissue	Total mgm. in adrenals	Gms. tissue	Mgm. per gm. of tissue	Total mgm. in brain	Gms. tissue right testis	Mgm. per gm. of tissue	Total mgm. in testis	Mgm. per gm. of tissue
I	Lettuce	None	0.376 ±0.058(c)	0.128 ±0.022	0.046 ±0.008	3.15 ±0.20	0.044 ±.006	0.137 ±0.015	2.38 ±0.09	0.041 ±0.005	0.097 ±0.009	0.026 ±0.006
III	5	None	0.404 ±0.04	0.686 ±0.064	0.265 ±0.013	3.07 ±0.07	0.185 ±0.005	0.569 ±0.018	2.32 ±0.16	0.148 ±0.014	0.335 ±0.020	0.102 ±0.008
IV	5	13	0.527 ±0.076	0.582 ±0.047	0.300 ±0.028	3.16 ±0.08	0.173 ±0.011	0.546 ±0.034	2.40 ±0.14	0.139 ±0.009	0.333 ±0.012	0.092 ±0.010
V	25	None	0.413 ±0.020	1.351 ±0.088	0.563 ±0.060	3.14 ±0.08	0.207 ±0.004	0.649 ±0.021	2.42 ±0.02	0.289 ±0.007	0.700 ±0.012	0.217 ±0.016
VII	25	50	0.546 ±0.053	1.356 ±0.031	0.743 ±0.074	3.08 ±0.03	0.218 ±0.004	0.670 ±0.008	2.27 ±0.17	0.285 ±0.011	0.647 ±0.053	0.219 ±0.008

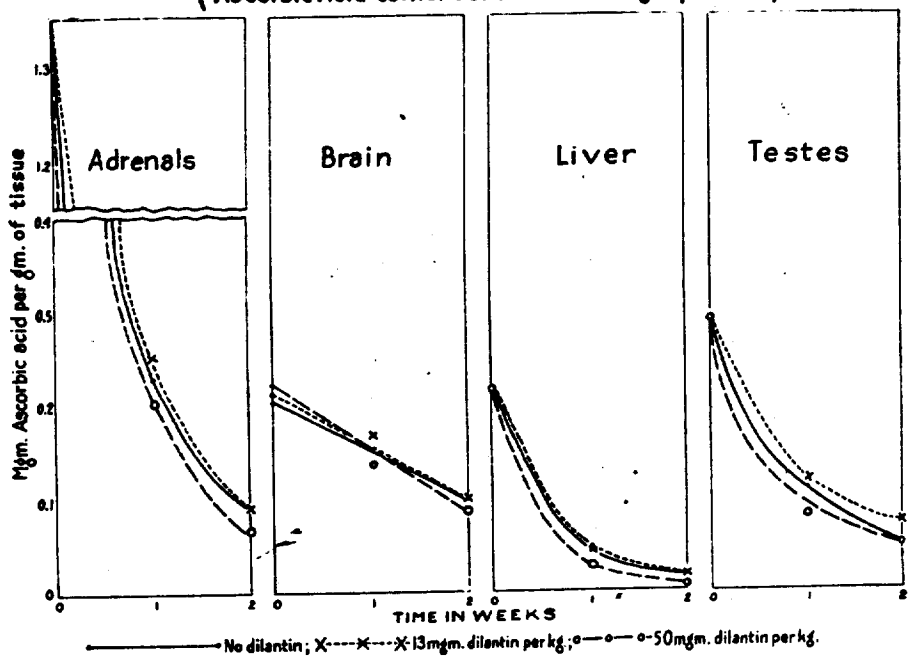
(a) Mgm. per guinea pig per day.
(b) Mgm. per kilo. per day.
(c) ± Values represent standard error.

and testes of the animals used in the plasma study. The values obtained are shown in table 3. In no case is there a significant difference between the corresponding values for the ascorbic acid content of the tissues of the dilantin sodium

treated animals and their controls. A difference which approaches statistical significance exists for the mgm. of ascorbic acid per gram of adrenal gland between groups III and IV. Further inspection of the data shows this to be due to a slight hypertrophy of the adrenals of the dilantin sodium treated animals, and that actually the total amount of ascorbic acid stored in the adrenals is greater than that of their controls. It is interesting to note that the low level of ascorbic acid in the tissues of the normal control animals, group I, is in good accordance with the low values for plasma ascorbic acid (table 2).

At the end of this stage of our study of the vitamin C content of the tissues we discontinued the injection of ascorbic acid supplement of the remaining ani-

Fig. 2. Effect of Dilantin Sodium on Vitamin C Utilization. (Ascorbic Acid Content of Tissues During Depletion.)



mals in groups V, VI and VII, and continued the daily treatments with dilantin sodium to see if some difference might arise from the effect of depletion. At the end of one week and again after 2 weeks on the vitamin C-free regime, analyses were made of the ascorbic acid content of the tissues. Three animals from each group were taken, males being selected due to the fact that most of the females were either pregnant or nursing their young. In this way we were able to observe the effect of the drug on tissue levels far below saturation. The results are shown in figure 2. Examination of the graphs reveal that there is practically no difference between the curves obtained for the dilantin-treated animals and their controls. The curves for the group receiving the higher dosage of dilantin

run a little below the curves for the other two groups in every case, but this difference is much too small to be considered significant. The striking difference between the slope of the curve obtained for the brain as compared with the curves for the three other tissues seems worthy of note. The data of table 3 show that this organ is also the first of the four to become saturated with ascorbic acid when this vitamin is available to the animal. The physiological significance of this finding remains to be pointed out.

SUMMARY AND CONCLUSIONS

In a study of the influence of sodium diphenyl hydantoinate (dilantin sodium) on the utilization of vitamin C by young guinea pigs, observations were made on the gain in weight, gross pathological changes, plasma vitamin C levels and the ascorbic acid content of tissues.

Feeding experiments were carried out with both the curative and preventive vitamin C regime. The Sherman basal scurvy ration was used, supplemented in one series with a minimum oral daily dose of 0.5 mgm. of ascorbic acid. Dilantin sodium was then given orally to some groups in massive amounts—50 and 100 mgm. per kilo (10 to 20 times the human dose). After 7 to 9 weeks the guinea pigs were autopsied. In another series (prophylactic) guinea pigs were given daily oral doses of 13 and 50 mgm. of dilantin sodium per kilo and after 1 to 2 hours injected subcutaneously with 5 and 25 mgm. of ascorbic acid per pig per day, over a period of 8 to 14 weeks.

The results indicate: (*a*) that the dilantin sodium had little or no effect on the gain or loss in weight; (*b*) that the dilantin sodium treated guinea pigs were able to breed and rear their young; (*c*) that dilantin did not raise or lower the plasma ascorbic acid levels; (*d*) that the drug had no influence on the ascorbic acid content of the adrenals, brain, testes and liver; and (*e*) that there were no gross pathological manifestations of scurvy including gingivitis.

From the above findings we feel justified in concluding that sodium diphenyl hydantoinate (dilantin sodium) has no significant effect on the utilization of ascorbic acid by the guinea pig.

REFERENCES

(1) Kimball, O. P., J. A. M. A., **112:** 1244, 1939.
(2) Kimball, O. P., and T. N. Horan, Ann. Int. Med., **13:** 787, 1939.
(3) Frankel, S. I., J. A. M. A., **114:** 1320, 1940.
(4) Drake, M. E., C. M. Gruber, and V. G. Haury, This Journal, **71:** 268, 1941.
(5) Drake, M. E., C. M. Gruber, V. G. Haury, and E. R. Hart, Ibid., **72:** 383, 1941.
(6) Gruhzit, O. M., Arch. Path., **28:** 761, 1939.
(7) Merrit, H. H., and A. Foster, Am. J. Med. Sci., **200:** 541, 1940.
(8) Hawke, W. A., Canad. M. A. J., **45:** 234, 1941.
(9) Ziskin, D. E., L. R. Stowe, and E. V. Zegarelli, Arch. Neurol. Psychiat. **46:** 897 1941.
(10) Millhon, J. A., and A. E. Osterberg, J. Am. Dent. A., **29:** 207, 1942.
(11) Sherman, H. C., Chemistry of Food and Nutrition, 4th ed. p. 423, 1932.
(12) Farmer, C. J., and A. F. Abt, Proc. Soc. Exper. Biol. & Med., **34:** 146, 1936.
(13) Bessey, O. A., J. Biol. Chem., **126:** 771, 1938.
(14) Zilva, S. S., Brit. J. Exper. Path., **18:** 449, 1937.

Indian J. Biochem 3: 196-202 1966 2369

Interdependence of Dietary Proteins & Vitamins in Metabolism: Part III—Influence of Vitamin C on Protein Metabolism in Growing Guinea-pigs

G. C. ESH & SHYAMA P. BHATTACHARYA
Bengal Immunity Research Institute, Calcutta 16

Manuscript received 30 *December* 1965

The influence of vitamin C on protein metabolism has been studied in growing guinea-pigs maintained on diets containing both casein and groundnut protein, and supplemented with high level of vitamin A. Growth performance and the rate of growth of some of the tissues, viz. liver, testes and adrenal, are greater when the diet is supplemented with high level of vitamin C. High protein and high vitamin A diet when supplemented with high level of vitamin C depresses liver storage of vitamin A with less storage of vitamin C in the liver; with groundnut protein, however, liver storage of both vitamin A and C is higher. With both the test proteins and at both low and high protein levels, total nitrogen in plasma and liver, nitrogen efficiency ratio, gain in carcass nitrogen, total plasma protein, albumin and albumin/globulin ratio are greater when the diets are supplemented with higher level of vitamin C. The level of xanthine oxidase activity in the liver, and the activity of aspartate aminotransferase and alanine aminotransferase in liver, heart, kidney and plasma increase with increase of vitamin C in the diet. The interdependence of vitamin C and protein in metabolism under high level of vitamin A feeding has been discussed.

RATS are known to synthesize vitamin C under certain dietary conditions but guinea-pigs like man depend on some dietary source for their requirement of this vitamin. Further, while rats grow quite well on diets containing 10-15 per cent casein, guinea-pigs kept on such a diet do not even survive[1] and the amino acid pattern necessary for their growth differs considerably from that of rats[2]. Thus different species of animals differ to a great extent in their requirement for these essential nutrients. Greater biosynthesis of vitamin C in rat has been indicated under high protein[3-5] feeding and when the diet is supplemented with high level of vitamin A[6,7]. Susceptibility to scurvy in the guinea-pig[8] has been found to be more when the animals are maintained on low protein diet. While variation in the growth of various tissues of guinea-pigs has been observed under the qualitative and quantitative variation in the dietary protein intake[9], changes in the free amino acids level in the blood[10] and disruption in the synthesis of blood proteins[11] have been reported during avitaminosis C. Further, vitamin C deficiency in guinea-pigs[12] and rats[13] has been shown to affect nitrogen metabolism significantly.

The precise way how vitamin A affects the metabolism of other nutrients is not known. In view of the fact that high intake of vitamin A[14] improves the utilization of dietary protein as well as stimulates the biosynthesis of vitamin C in the rat, it is of interest to study the influence of variation in the intake of vitamin C on the metabolism of protein under the condition of feeding high protein and vitamin A. Since guinea-pig does not normally synthesize vitamin C, experiments were undertaken with this animal. The influence of feeding vitamin C when protein depleted guinea-pigs are maintained on different proteins, has been reported earlier[15]. The present paper reports the results of maintaining growing guinea-pigs on diets containing high level of casein, groundnut protein and vitamin A, and supplemented with varying levels of vitamin C.

Materials and Methods

Weanling guinea-pigs (120-140 g.) bred and reared in the Institute colony on semisynthetic diets were selected for these experiments. The basal diet was essentially the same as followed earlier[15], and contained, in per cent, the following: sucrose, 10; cellulose, 10; agar, 5; salt mixture, 6; hydrogenated oil, 5; potassium acetate, 2·5; magnesium oxide, 0·5; inositol, 0·2; choline chloride, 0·2; vitamin B complex mixture, 1; arachis oil containing vitamin K and E, 1; arachis oil containing vitamin D, 1; sufficient casein to supply 25 g. protein and starch to make 100. Each animal was supplemented with 100 IU of vitamin A daily per 100 g. body weight. Vitamin C (4 mg. per 100 g. body weight) was supplied to each animal of the control group. Test diets containing 25 per cent or 10 per cent protein were prepared with casein and protein isolated from groundnut. Each of the four groups of rats maintained on the respective diets was, then, divided into three subgroups with respect to vitamin C such that each animal of first, second and third subgroups received 0·1 mg., 0·5 mg. and 4 mg. vitamin C daily per 100 g. of the body weight. Each animal was kept in individual metal cages and diets and distilled water were offered *ad libitum*. Daily food intake and biweekly body weight were recorded. At the end of 30 days, the animals were bled by cardiac puncture under light chloroform anaesthesia. Blood was collected separately in K-oxalated and anticoagulant-free tubes and immediately

TABLE 2 — TISSUE LEVELS OF VITAMINS A AND C, AND URINARY EXCRETION OF VITAMIN C WHEN YOUNG GUINEA-PIGS ARE MAINTAINED ON DIETS CONTAINING DIFFERENT LEVELS OF VITAMIN C AND DIFFERENT TYPES OF PROTEIN FOR 30 DAYS

(Values are the mean of values for 3 males and 3 females with standard error)

Daily dose of vitamin C mg./100 g. body wt	Dietary protein	Vitamin A in liver IU/g.	Vitamin C in urine, µg./animal, in 24 hr excretion	Vitamin C, mg. per 100 g. of tissue			
				Liver	Adrenal	Spleen	Duodenum
0·1	GP 25%	86·14	Traces	1·298 ±0·185	4·124 ±1·03	—	2·87 ±0·26
	CP 25%	10·4	68·7 ±3·1	2·095 ±0·217	4·331 ±0·866	3·786 ±0·841	3·02 ±0·08
0·5	GP 25%	27·04	47·6 ±2·4	7·426 ±0·535	16·047 ±2·168	17·066 ±1·148	11·39 ±1·34
	CP 25%	10·29	87·4 ±4·1	4·675 ±0·2	13·91 ±0·43	9·958 ±0·36	8·22 ±1·25
4	GP 25%	21·89	61·5 ±7·2	20·532 ±0·702	91·914 ±1·875	42·071 ±2·589	13·90 ±0·98
	CP 25%	Traces	94·53 ±7·03	9·286 ±0·28	37·05 ±1·18	26·677 ±1·321	11·56 ±1·8
0·1	GP 10%	do	Traces	0·822 ±0·164	8·251 ±0·629	4·453 ±0·742	1·84 ±0·39
	CP 10%	24·4	117·7 ±7·29	3·26 ±0·16	4·56 ±0·502	8·376 ±0·364	1·98 ±0·02
0·5	GP 10%	ND	97·27 ±8·97	4·087 ±0·118	33·128 ±1·708	25·761 ±2·016	5·03 ±0·35
	CP 10%	41·58	149·21 ±25·8	4·454 ±0·361	20·49 ±0·931	8·61 ±0·718	6·22 ±0·21
4	GP 10%	45·93	112·4 ±16·6	14·258 ±0·577	73·325 ±11·215	56·069 ±4·31	11·6 ±0·747
	CP 10%	11·02	226·5 ±23·4	8·511 ±0·236	66·891 ±3·863	30·201 ±0·816	12·35 ±2·33

GP, groundnut protein; and CP, casein protein.

in case of some organs, particularly liver, testes and adrenal, the rate of growth has been found greater with the higher intake of vitamin C.

Effect of varying vitamin C intake on tissue levels of vitamins A and C and urinary excretion of vitamin C under different conditions of protein feeding — The results presented in Table 2 show that at higher level of protein feeding, groundnut protein diet induces greater storage of vitamin A in the liver than casein diet. Increasing the vitamin C level in the diet reduces vitamin storage in the liver indicating the greater utilization of vitamin A. At lower level of protein feeding, the liver storage of vitamin A remains relatively high. As in the case of vitamin A, more vitamin C is stored in the liver when groundnut protein is fed, although greater storage is observed with higher level of protein feeding. Relatively greater retention in the liver and less urinary excretion of vitamin C observed in the case of animals fed groundnut protein diet may be attributed to less utilization of the vitamin. At lower level of protein intake, however, urinary excretion of vitamin C is higher in the case of both test protein diets. The storage of vitamin C in other tissues is not significantly different excepting that high level of protein feeding stimulates greater storage.

Effect of varying vitamin C intake on nitrogen metabolism — The results of total and non-protein nitrogen levels in plasma and liver are given in Table 3. The results indicate that under the conditions of feeding both the test proteins, the level of total nitrogen in plasma and liver increases as the level of vitamin C in the diet increases although it seems that the nitrogen content of the tissues almost levels off when 0·5 mg. vitamin C is fed to the animals; no significant increase occurs when more vitamin C is fed. Also, no significant influence of the level of feeding vitamin C is observed on the non-protein nitrogen content of plasma and liver.

Nitrogen efficiency ratio, gain in carcass nitrogen and retention of food nitrogen in carcass are recorded in Tables 4 and 5. As expected, higher nitrogen efficiency ratio is obtained with high casein protein diet. With increased vitamin C in the diet, an increase in nitrogen efficiency ratio and gain in carcass nitrogen are observed when both the test protein diets are fed at higher level. The animals maintained on diets containing lower protein level lost body nitrogen but the nitrogen loss is less with increasing vitamin C supplement in the diet. In the case of casein protein diet, retention of food nitrogen in carcass and increase in carcass nitrogen per 100 g. body weight almost level off when the diet is supplemented with 0·5 mg. of vitamin C. Further increase in food nitrogen in carcass and carcass nitrogen per 100 g. body weight is observed when groundnut protein diet is supplemented with more vitamin C (4 mg.). This indicates the higher requirement of vitamin C for optimum utilization of protein.

TABLE 3 — EFFECT OF VARYING VITAMIN C INTAKE AND THE AMOUNT AND TYPE OF DIETARY PROTEIN ON PLASMA AND LIVER NITROGEN OF YOUNG GUINEA-PIGS FED DIET FOR 30 DAYS

(Values are the mean of values for 3 males and 3 females with standard error)

Daily dose of vitamin C mg./100 g. body wt	Dietary protein %	Casein protein group				Groundnut protein group			
		Plasma nitrogen mg./100 ml.	Plasma NPN mg./100 ml.	Liver N mg./g.	Liver NPN mg./g.	Plasma nitrogen mg./100 ml.	Plasma NPN mg./100 ml.	Liver N mg./g.	Liver NPN mg./g.
0·1	25	868·96 ±7·77	60·93 ±0·62	35·36 ±0·08	4·787 ±0·107	925·39 ±5·49	44·34 ±0·68	30·197 ±0·107	3·244 ±0·077
0·5	25	920·2 ±6·2	60·51 ±0·21	36·81 ±0·16	4·799 ±0·369	987·18 ±6·86	45·55 ±0·25	30·71 ±0·07	3·114 ±0·177
4	25	973·11 ±6·22	54·72 ±0·63	36·52 ±0·19	5·75 ±0·09	985·81 ±13·73	48·32 ±0·27	32·06 ±0·14	5·028 ±0·179
0·1	10	741·46 ±4·63	33·88 ±0·93	28·76 ±0·09	4·819 ±0·108	840·27 ±2·74	31·02 ±2·09	27·04 ±0·25	3·059 ±0·096
0·5	10	781·91 ±17·1	39·79 ±0·62	—	4·256 ±0·095	859·49 ±5·49	27·87 ±0·96	28·33 ±0·09	2·796 ±0·186
4	10	811·45 ±6·22	34·29 ±1·03	30·03 ±0·06	4·679 ±0·156	871·85 ±4·12	25·67 ±0·69	27·55 ±0·13	3·264 ±0·112

TABLE 4 — EFFECT OF VARYING VITAMIN C INTAKE ON NITROGEN EFFICIENCY AND NITROGEN RETENTION IN GUINEA-PIGS MAINTAINED ON DIETS CONTAINING VARYING AMOUNTS OF GROUNDNUT PROTEIN FOR 30 DAYS

(Values are the mean of values for 3 males and 3 females with standard error)

Daily intake of vitamin C per 100 g. body wt mg.	Total nitrogen intake g.	Wt gain g.	Nitrogen efficiency*	Gain in carcass nitrogen g.	Retention of food nitrogen in carcass %	Increase in carcass N per 100 g. increase in body wt	Mortality
			GROUNDNUT PROTEIN, 25%				
0·1	16·43 ±0·894	12·0 ±3·2	0·73	0·33 ±0·04	2·01	2·75	One
0·5	18·05 ±0·761	71·3 ±12·4	3·95	1·278 ±0·029	7·08	1·79	Nil
4	19·71 ±0·889	82·0 ±6·7	4·16	2·769 ±0·128	14·05	3·37	Nil
			GROUNDNUT PROTEIN, 10%				
0·1	3·291 ±0·07	−30·0 ±3·5	Negative	−0·62 ±0·015	Negative	Negative	Two
0·5	3·73 ±0·565	−23·6 ±12·3	do	−0·416 ±0·04	do	do	One
4	3·843 ±0·296	4·3 ±3·7	1·118	0·145 ±0·059	3·77	3·37	—

*Gain in body weight per g. of nitrogen intake.

Total protein, albumin and globulin, and their ratio in plasma are recorded in Table 6. The levels of total protein, albumin and albumin/globulin ratio progressively increase with increasing supplementation of the diet with vitamin C, although the values level off at a daily intake of 0·5 mg. vitamin C. The levels of globulin and fibrinogen seem to decrease accordingly with higher vitamin intake.

Effect of varying vitamin C intake on tissue enzyme levels — The results presented in Table 7 show that in all the tissues the levels of aspartate aminotransferase and alanine aminotransferase progressively increase with both the test protein diets and at both the levels (10 and 25 per cent) of protein feeding when the diets are supplemented with increasing levels of vitamin C. In the case of alanine aminotransferase the rate of increase seems to be much higher. Vitamin C seems to have no effect on succinate dehydrogenase level either in the heart or liver. The activity of xanthine oxidase increases significantly in liver with feeding higher level of the vitamin C and a supplement of 0·5 mg. daily seems to be sufficient for maintaining optimum enzyme activity (Table 8.)

Discussion

Guinea-pigs require high protein diets for growth. Also, a daily requirement of 0·5 mg. vitamin C per 100 g. body weight has been recommended[24]. Our observations indicate that during feeding of both casein and groundnut protein diet at 25 per cent protein level, significant increase in growth occurs

TABLE 5 — EFFECT OF VARYING VITAMIN C INTAKE ON NITROGEN EFFICIENCY AND NITROGEN RETENTION IN GUINEA-PIGS MAINTAINED ON DIETS CONTAINING VARYING AMOUNT OF CASEIN PROTEIN FOR 30 DAYS

(Values are the mean of values for 3 males and 3 females with standard error)

Daily intake of vitamin C per 100 g. body wt mg.	Total nitrogen intake g.	Wt gain g.	Nitrogen efficiency*	Gain in carcass nitrogen g.	Retention of food nitrogen in carcass %	Increase in carcass N per 100 g. increase in body wt	Mortality
			CASEIN PROTEIN, 25%				
0·1	11·98 ±0·09	14·5 ±4·329	1·21	0·148 ±0·056	1·23	1·02	Nil
0·5	12·73 ±1·14	96·0 ±6·856	7·541	2·517 ±0·19	19·77	2·62	Nil
4	14·8 ±0·232	116·0 ±8·367	7·83	3·01 ±0·02	20·33	2·6	Nil
			CASEIN PROTEIN, 10%				
0·1	2·499 ±0·384	−12·3 ±3·18	Negative	−0·592 ±0·081	Negative	Negative	One
0·5	2·445 ±0·627	−17·3 ±5·8	do	−0·155 ±0·005	do	do	do
4	3·267 ±0·007	−6·75 ±5·936	do	0·306 ±0·057	9·37	o	Nil

*Gain in body weight per g. of nitrogen intake.

TABLE 6 — EFFECT OF MAINTAINING YOUNG GUINEA-PIGS ON DIETS CONTAINING DIFFERENT LEVELS OF VITAMIN C AND DIFFERENT TYPES OF PROTEINS FOR 30 DAYS ON PLASMA PROTEINS

(Values are the mean of values for 3 males and 3 females with standard error)

Dietary protein	Daily vitamin C intake mg./100 g. body wt	Total protein g./100 ml.	Albumin g./100 ml.	Globulin g./100 ml.	Fibrinogen g./100 ml.	Albumin/globulin ratio
CP 25%	0·1	5·431 ±0·048	2·006 ±0·006	2·978 ±0·023	0·446 ±0·077	0·6736 ±0·032
GP 25%		5·78 ±0·04	2·029 ±0·002	3·313 ±0·0211	0·441 ±0·015	0·6125 ±0·0028
CP 25%	0·5	5·75 ±0·04	3·016 ±0·011	2·416 ±0·035	0·318 ±0·007	1·248 ±0·014
GP 25%		6·17 ±0·042	2·987 ±0·023	2·831 ±0·065	0·334 ±0·028	1·055 ±0·016
CP 25%	4	6·081 ±0·039	3·026 ±0·016	2·744 ±0·062	0·311 ±0·039	1·103 ±0·019
GP 25%		6·16 ±0·087	3·042 ±0·036	2·779 ±0·03	0·339 ±0·02	1·093 ±0·002
CP 10%	0·1	4·634 ±0·029	1·976 ±0·01	2·172 ±0·078	0·486 ±0·039	0·891 ±0·0573
GP 10%		5·25 ±0·018	2·007 ±0·006	2·871 ±0·066	0·373 ±0·042	0·6992 ±0·0183
CP 10%	0·5	4·886 ±0·107	2·201 ±0·018	2·441 ±0·059	0·223 ±0·028	0·91 ±0·0148
GP 10%		5·37 ±0·036	2·184 ±0·009	2·8 ±0·02	0·384 ±0·008	0·7799 ±0·0023
CP 10%	4	5·07 ±0·04	2·224 ±0·015	2·496 ±0·021	0·349 ±0·000	0·891 ±0·0029
GP 10%		5·45 ±0·02	2·718 ±0·01	2·561 ±0·063	0·166 ±0·031	1·059 ±0·032
Basal diet	—	6·19 ±0·048	3·014 ±0·013	2·824 ±0·061	0·351 ±0·001	1·067 ±0·028

CP, casein protein; and GP, groundnut protein.

TABLE 7 — EFFECT OF VARYING VITAMIN C INTAKE ON TISSUE LEVELS OF ASPARTATE AMINOTRANSFERASE AND ALANINE AMINOTRANSFERASE IN YOUNG GUINEA-PIGS MAINTAINED ON DIETS CONTAINING VARYING LEVELS OF CASEIN AND GROUNDNUT PROTEINS FOR 30 DAYS

(Values expressed in µg. pyruvate/hr/mg. wet tissue or ml. plasma; values are the mean of values for 3 males and 3 females with standard error)

Dietary protein	Daily intake of vitamin C per 100 g. body wt mg.	Aspartate aminotransferase				Alanine aminotransferase			
		Liver	Heart	Kidney	Plasma	Liver	Heart	Kidney	Plasma
CP 25%	0·1	291±14	521±9	263±11	455±24	109±5	15±3	20±1	214±6
GP 25%		218±7	412±9	252±11	389±8	118±9	23±2	16±2	202±1
CP 25%	0·5	341±21	612±6	329±58	496±6	258±9	40±1	32±1	247±4
GP 25%		293±5	512±10	297±7	407±5	182±15	30±2	21±2	251±6
CP 25%	4	380±8	672±6	406±5	585±13	286±4	47±0	43±2	297±6
GP 25%		319±13	560±12	315±4	521±11	211±5	35±3	24±2	279±8
CP 10%	0·1	231±10	425±16	242±9	497±9	92±5	—	20±2	180±8
GP 10%		192±10	348±23	245±6	369±12	57±7	11±1	11±2	180±8
CP 10%	0·5	307±3	525±3	334±15	509±5	191±6	31±3	22±3	218±15
GP 10%		205±10	386±6	255±4	386±6	96±5	24±3	20±1	184±4
CP 10%	4	362±7	619±7	390±8	534±5	220±4	36±3	29±2	236±4
GP 10%		223±19	395±7	293±12	418±14	124±12	31±2	22±1	201±3

CP, casein protein; and GP, groundnut protein.

TABLE 8 — EFFECT OF VARYING VITAMIN C INTAKE ON XANTHINE OXIDASE AND SUCCINATE DEHYDROGENASE LEVELS IN YOUNG GUINEA-PIGS MAINTAINED ON DIETS CONTAINING VARYING AMOUNTS OF CASEIN AND GROUNDNUT PROTEINS FOR 30 DAYS

(Values are the mean of values for 3 males and 3 females with standard error)

Dietary protein	Daily intake of vitamin C mg./100 g. of body wt	Liver N g./100 g.	Xanthine oxidase in liver*	Succinate dehydrogenase†	
				Liver	Heart
CP 25%	0·1	3·536±0·008	6·508±0·123	1·18±0·09	4·081±0·444
GP 25%		3·019±0·011	6·639±0·511	2·842±0·072	6·232±0·138
CP 25%	0·5	3·681±0·016	11·048±0·425	0·698±0·012	4·125±0·065
GP 25%		3·071±0·007	7·04±0·000	1·202±0·064	4·6±0·07
CP 25%	4	3·652±0·019	10·106±0·006	1·945±0·059	4·342±0·028
GP 25%		3·206±0·014	6·15±0·15	1·732±0·073	5·808±0·042
CP 10%	0·1	2·876±0·009	3·366±0·919	1·424	4·967±0·033
GP 10%		2·704±0·025	3·168±0·861	1·699±0·071	4·859±0·023
CP 10%	0·5	—	9·21±1·742	2·43±0·12	5·244±0·044
GP 10%		2·833±0·009	3·845±0·375	2·877±0·123	2·69±0·33
CP 10%	4	3·003±0·006	12·597±0·663	1·963±0·076	5·337±0·064
GP 10%		2·755±0·013	6·406±0·534	2·943±0·556	5·731±0·081

CP, casein protein; and GP, groundnut protein.
*µmoles/g. liver/hr.
†Change in absorbancy at 400 mµ/g. tissue/min. at 20°C.

with higher supplements of vitamin C (4 mg.). At 10 per cent protein level, however, higher supplement of vitamin C does not improve the growth of animals on casein diet but slightly better growth occurs with groundnut protein diet. The requirement of arginine for guniea-pigs is high[1,2] and groundnut protein is very rich in this amino acid and contains almost double that present in casein. The animals were supplied diets *ad libitum*. It is probably because of the peculiar amino acid make-up of groundnut protein diets that the animals consumed this diet in greater amounts than casein diet. In exactly what way higher supplements of vitamin C can improve the utilization of groundnut protein, particularly when present in the diet at low protein level is difficult to explain at this stage. Mayfield and Roehm[25] observed in rats maintained on diets containing carotene that ingestion of high level of vitamin C decreases the vitamin A storage in the liver and Kuzminskaya[26] reported that in guinea-pigs hypervitaminosis A causes hypovitaminosis C. Our results with high protein and high vitamin A diets also indicate depression of liver storage of vitamin A when high dose of vitamin C is ingested daily followed by less liver storage of the vitamin. This indicates better utilization of the various nutrients in the diet by the animal. With low protein and high vitamin A diets, however, greater liver storage of vitamin A and greater urinary excretion of vitamin C occur. Protein is necessary for the optimum absorption, storage, transport and utilization of vitamin A. The results of the present study tend to show that vitamin C plays some role in these processes. The higher retention of vitamin A in the liver of animals fed diets containing 10 per cent groundnut protein with higher supplementation of vitamin C may be due to increasing absorption of protein and/or inhibition of protein utilization or to some other cause which is difficult to explain at this stage. The results of the present study also confirm the previous report of Reid[9] that high protein diets are necessary for the proper growth of various tissues and further indicate that the rate of growth of some tissues such as testes, adrenal and liver is greater with higher level of vitamin C in the diet. The relative weight of kidney is slightly lower in animals maintained on diets with higher supplementation of vitamin C. Both the quality and level of dietary protein influence the relative weights of tissues and although the exact mechanism regarding the possible relation of the increased size of the tissues to their maturation rate is not known, it is likely that the extent of utilization of dietary protein plays a significant role. The effects of supplementing high protein and high vitamin A diet with high level of vitamin C in increasing the nitrogen efficiency ratio, retention of food nitrogen in carcass, and plasma protein demonstrate better utilization of dietary protein, particularly when the diet contains naturally available plant proteins. Previous workers have reported that in scorbutic guinea-pigs a consistent fall[6] in serum albumin and albumin/globulin ratio and an increase in serum globulin[27] occur. The results of the present study indicate progressive increase in albumin content and albumin/globulin ratio, and a decrease in globulin fraction when high vitamin A diets are supplemented with high level of vitamin C indicating the part played by these vitamins in protein anabolic-catabolic reactions for the synthesis of blood proteins. The increase in the activity of xanthine oxidase, aspartate aminotransferase and alanine aminotransferase in liver and other tissues as a result of feeding high level of vitamin C furnishes additional evidences regarding the essentiality of this vitamin for the optimum utilization of a diet containing high level of protein and vitamin A.

Acknowledgement

The authors' thanks are due to Dr U. P. Basu, Director of this Institute, for his kind interest in this investigation and to the Ministry of Education, Government of India, for the award of a fellowship to one of them (S.P.B.).

References

1. Heinicke, H. R., Harper, A. E. & Elvehjem, C. A., *J. Nutr.*, **57** (1955), 483.
2. Reid, Marry E. & Mickelsen, Olaf, *J. Nutr.*, **80** (1963), 25.
3. Hopkins, F. G. & Slater, B. R., *Biochem. J.*, **29** (1935), 2803.
4. Yanovskaya, B. I., *Biokhimiya*, **17** (1952), 414.
5. Srinivasan, M., Rao, M. V. L. & Bhagaban, H. N., *Proc. Soc. exp. biol. Med.*, **105** (1960), 615.
6. Sure, B., Theis, R. M. & Harrelson, R. T., *J. biol. Chem.*, **129** (1939), 245.
7. Sheshadri Sastry, P., Malathi, P., Subba Rao, K. & Ganguly, J., *Nature, Lond.*, **193** (1962), 1080.
8. Cohen, B. & Mendel, L. B., *J. biol. Chem.*, **35** (1918), 425.
9. Reid, M. E., *J. Nutr.*, **80** (1963), 33.
10. Ginter, E., *Physiologia Bohemoslov.*, **7** (1958), 409.
11. Banerjee, S. & Rohatgi, Krishnasudha, *Proc. Soc. exp. biol. Med.*, **97** (1958), 234.
12. Yoshimi, Morozumi, *Med. J. Shinshu Univ.*, **5** (1960), 217.
13. Brown, F. F. & Morgan, A. F., *J. Nutr.*, **35** (1948), 425.
14. Bhattacharya, R. K. & Esh, G. C., *Ann. Biochem. exp. Med.*, **21** (1961), 215.
15. Esh, G. C., Bhattacharya, Shyama P. & Basu, U. P., *Metabolism*, **15** (1966), in press.
16. Roe, J. H. & Keuther, C. A., *J. biol. Chem.*, **147** (1943), 399.
17. Horner, A. A. & Morton, R. A., *Biochem. J.*, **74** (1960), 301.
18. Cama, H. R., Collins, F. D. & Morton, R. A., *Biochem. J.*, **50** (1951), 48.
19. Hawk, Philip B., Oser, Bernard L. & Summerson, William H., *Practical physiological chemistry* (J. & A. Churchill Ltd, London), 1954, 602.
20. Litwack, G., Bothwell, J. W., Williams, J. N. (Jr) & Elvehjem, C. A., *J. biol. Chem.*, **200** (1953), 303.
21. Slater, E. C. & Bonner, W. D., *Biochem. J.*, **52** (1952), 185.
22. Cabaud, P. R., Leeper, R. & Wroblewski, F., *Amer. J. clin. Path.*, **26** (1956), 1101.
23. Wroblewski, F. & Cabaud, P. R., *Amer. J. clin. Path.*, **27** (1957), 235.
24. Collins, Macie & Elvehjem, C. A., *J. Nutr.*, **64** (1958), 503.
25. Mayfield, Helen L. & Roehm, Richard R., *J. Nutr.*, **58** (1956), 203.
26. Kuzminskaya, U. A., *Vrach. Delo*, **259** (1959), 62.
27. Pavel, I., Campeanu, S. & Chisiu, N., *Chem. Abstr.*, **53** (1959), 4460.

for 20 min at 0°C and 21,000g. The supernate was analyzed for creatine-phosphokinase activity, fructose-1,6-diphosphate aldolase activity, and fructose-1-phosphate aldolase activity using auxiliary enzymatic reactions by following absorbance changes of respective coenzymes at 340 mm[9]. The enzymes were determined at room temperature on a recording Beckman model DU spectrophotometer at pH 9.0 (creatinephosphokinase) or pH 7.4 (aldolase). It was shown that the velocities of the reactions were directly proportional to the amount of supernate used in the assays. The protein concentration of the supernate was determined[10] and the tissue enzyme activity expressed in milliunits (mM substrate transformed/mg protein/min). It was found that experimental manipulations did not significantly alter the concentration of the supernatant protein.

The plasma was thawed and enzyme activity determined at room temperature at the same pH's noted above. Creatinephosphokinase was assayed using a Sigma kit (St. Louis, Mo.). The samples were run in duplicate with and without added substrate and the difference in initial enzyme activity expressed in Sigma units. Fructose-1, 6-diphosphate aldolase was assayed using a Boehringer kit (Boehringer Mannheim Corp., New York, N. Y.) and the units expressed as the change in absorbance x 1000/min/ml plasma. Fructose-1-phosphate aldolase activity was expressed in the same way and determined according to Bergmeyer[9].

Results

Muscle Creatinephosphokinase

Ten hours exercise significantly elevated creatinephosphokinase activity (50-55%) in both red and white muscle of untrained guinea pigs maintained on low levels of vitamin C, but did not affect creatinephosphokinase in the muscles of guinea pigs maintained on high vitamin C levels (Table I).

largely in one tissue type[7]. Creatinephosphokinase activity was determined in red and white muscle, fructose-1-phosphate aldolase in the liver, fructose-1,6-diphosphate aldolase in both tissue types, and the activity of each enzyme determined in the plasma.

Materials and Methods

Male guinea pigs of the NIH Hartley strain, 300-350g, were maintained five to a cage on vitamin C deficient diets[a] and supplemented with either 1.0% (high level) or 0.1% (low level) ascorbic acid in the drinking water. It was estimated that each of the animals consumed approximately 50 ml. of the vitamin C supplemented water per day. Animals of each dietary group were divided into 4 categories: (1) unexercised, (2) untrained and exercised, (3) only trained, and (4) trained and then exercised. The animals were weighed daily and all became accustomed to handling. Training, a daily run of four hours at 6.9 m/min as described previously[8], was carried out for six weeks. Twelve hours after the last training day, appropriate groups of animals from each dietary regime were exercised for ten hours at the same speed as before. Immediately afterward these and the remainder of the animals were killed with chloroform anaesthesia. Heart blood was taken from five animals of each group, the plasma separated, and frozen for later enzyme determinations. The liver, red muscle (M. soleus), and white muscle (anterior portion of M. biceps femoris) from each of the animals were removed and immediately used for tissue enzyme determinations.

Portions of the liver (1% w/v), red muscle (1% w/v), and white muscle (0.5% w/v) were homogenized in cold 0.05 M Tris buffer (pH 7.4) using glass tubes and motor-driven teflon pestles. The homogenates were centrifuged

---

[a] Ascorbic acid deficient diet (Guinea Pig) from Nutritional Biochemicals Corp., Cleveland, Ohio.

Life Sciences Vol 9, Part II pp 301-311, 1970.
2058

ENZYME CHANGES IN RED AND WHITE MUSCLE, LIVER, AND PLASMA OF EXERCISED GUINEA PIGS MAINTAINED ON HIGH OR LOW LEVELS OF VITAMIN C

M. P. Dieter

National Institutes of Health, NIAMD-LPB, BETHESDA, MARYLAND 20014

(Received 13 October 1969; in final form 5 December 1969)

Guinea pigs deficient in vitamin C exhibit characteristic disturbances in carbohydrate metabolism[1]. For instance, it was shown both *in vivo* and *in vitro* that the scorbutic liver converts less glucose-C^{14} into glycogen[2]. Muscle glycogen also diminished during the course of vitamin C depletion[3]. In addition, derangements in muscular contraction have been observed in the scorbutic animal, and a progressive decrease in exercise tolerance corresponding to the degree of scurvy has been repeatedly observed[1,3].

Previously it was shown that exercise increased the level of glycolytic serum enzymes[4] and that prior training prevented the rise in serum enzyme activity following strenuous exercise[5]. Isoenzyme analysis suggested that muscle was the major source of the serum enzymes[6], although the liver and other organs were not entirely excluded.

Thus, it is possible that muscular disability in exercised, scorbutic animals is partially due to increased loss of muscle enzymes to the plasma. The relationship between vitamin C and muscular function may also involve direct interaction between vitamin and enzyme, e.g., vitamin C may influence cellular enzyme activity.

These hypotheses were tested by evaluating the activity of tissue and plasma enzymes following exercise of trained and untrained guinea pigs maintained on high or low levels of vitamin C. The enzymes selected for analysis were those considered to be "tissue specific" or localized

The dose-response and time-response relationships between ascorbic acid and strychnine show that the antagonism between the two is competitive and is directly related to the plasma level of ascorbic acid.

The mechanism of action of ascorbic acid in counteracting strychnine toxicity may lie in its ability to convert strychnine to some derivative/derivatives which lack the toxic action of strychnine. Already it has been shown that a derivative of strychnine, Gumlich-Wieland aldehyde, possesses very little convulsive action and it is mainly hypotensive[6]. It has been found that an incubated mixture of strychnine and ascorbic acid (or mixture of lemon juice and ascorbic acid), when pharmacologically screened on the circulatory system, shows effects similar to the Gumlich-Wieland aldehyde (Dey, P. K., unpublished data). Chromatographic identification of the derivatives of strychnine in the above mixture was unsuccessful since the R_f values of strychnine and Gumlich-Wieland aldehyde were found to be nearly the same and no other new spot was detected. However, it is certain that some structural modifications of strychnine occur as a result of its reaction with ascorbic acid and this is borne out from the pharmacological effects of the mixture on the circulatory system (Dey, P. K., unpublished data). This structural modification of strychnine by ascorbic acid may be responsible for the *in vivo* protection of the latter against strychnine though there are other possibilities. Finally it may be assumed that the factors which lead to the increased formation of ascorbic acid are effective in counteracting strychnine toxicity and the reverse is true with factors leading to the deficiency of ascorbic acid in the body.

In view of the above finding it is pointed out that the protective action of ascorbic acid against strychnine toxicity may also be extended in cases of tetanus toxin poisoning, since it is generally suggested that the mode of action and site of action of strychnine and tetanus toxin poisoning on the nervous system are almost same. Thus the therapeutic possibility of using ascorbic acid in combating tetanus toxin deserves serious consideration. It has been shown recently that ascorbic acid can also nullify the lethal and convulsive actions of tetanus toxin in rats[7].

Acknowledgement

The author expresses his gratitude to Prof. S. R. Maitra, Head of the Department of Physiology, Calcutta University, for providing facilities for this work. Thanks are also due to Dr S. Mukherjee, Department of Applied Chemistry, for supplying ethionine and to Shri B. K. Chatterjee and Smt. Pratima Rakshit for technical assistance.

References

1. Dey, P. K., *Naturwissenschaften*, **52** (1965), 164.
2. Somogyi, J. C., *Z. Vitam. Forsch.*, **16** (1945), 134.
3. Tousler, O. & Hollman, S., *Ann. N.Y. Acad. Sci.*, **92** (1961), 318.
4. Lin, S. & Cohen, H. P., *Archs Biochem.*, **88** (1960), 256.
5. Vercellana, G., cited by Browning, E., in *Vitamins* (Williams & Wilkins Co., Baltimore), 1931, 341.
6. Dey, P. K., Chatterjee, M. & Chatterjee, A., *Indian J. Physiol. all. Sci.*, **16** (1962), 99.
7. Dey, P. K., *Naturwissenschaften*, **53** (1966), 310.

15 min. strychnine (2 mg./kg.) was given subcutaneously and the convulsive and lethal actions noted.

Efficacy of ascorbic acid precursors in counteracting strychnine toxicity in ethionine treated mice — Ethionine (100 mg./kg.) was given orally to 2 groups of mice along with milk diet for 9 days. One group was given 2 g./kg. of L-gulonolactone and the other 2-ketogulonolactone intraperitoneally. After 15 min. strychnine (2 mg./kg.) was administered subcutaneously and the toxicity noted.

Results

In vivo action of ascorbic acid precursors — L-Gulonolactone and 2-ketogulonolactone provided 100 per cent protection against the toxic effect of strychnine.

Dose-response relationship—The percentage protection against strychnine toxicity increased with the increasing concentration of ascorbic acid (Table 1).

Time-response relationship and plasma ascorbic acid level — The protective action of ascorbic acid against strychnine toxicity was directly proportional to plasma ascorbic acid concentration (Table 2).

In vitro study — Only ascorbic acid showed marked protection against the toxic action of strychnine (Table 3) whereas the precursors of ascorbic acid showed one-fifth of the protective action of ascorbic acid. The control group showed 100 per cent mortality and mean survival time was 5·6 min.

Efficacy of ascorbic acid against Metrazol and Picrotoxin — No protective action was shown by ascorbic acid against the convulsant drugs Metrazol and Picrotoxin and the mortality was 100 per cent.

TABLE 1 — PROTECTIVE ACTION OF DIFFERENT DOSES OF ASCORBIC ACID AGAINST STRYCHNINE TOXICITY IN MICE

(Number of animals used for each dose are 10)

Ascorbic acid mg./kg. body wt	Mean survival period min.	Protection %
Control (2 mg./kg. strychnine)	4·2	nil
50	7·8	40
100	8·0	60
500	9·0	80
1000	—	100
2000	—	100

TABLE 2 — ASCORBIC ACID CONCENTRATION IN PLASMA AND ITS PROTECTION AGAINST STRYCHNINE TOXICITY IN MICE AT DIFFERENT INTERVALS OF TIME FOLLOWING ADMINISTRATION OF ASCORBIC ACID

[Ascorbic acid (1 g./kg. body weight) administered intraperitoneally]

Time min.	Ascorbic acid conc. in plasma mg./100 ml.	Protection %
15	6·0	100
30	2·4	100
60	1·04	50
120	0·86	nil
Control	0·66	do

TABLE 3 — *In vitro* ACTION OF ASCORBIC ACID AND ITS PRECURSORS ON STRYCHNINE TOXICITY IN MICE

(Number of animals used for each dose are 10)

Dose 1 g./kg. body wt	Mean survival time min.	Protection %
L-Gulonolactone	8·0	20
2-Ketogulonolactone	6·5	20
Ascorbic acid	—	100
Ascorbic acid (alkaline)	4·8	nil

The degree and duration of convulsion using the lower doses of Metrazol and Picrotoxin have not been abolished or minimized by ascorbic acid, indicating that the latter is incapable of acting against these convulsant drugs.

Efficacy of pentobarbital against strychnine in ethionine treated mice — Sodium pentobarbital was ineffective against strychnine in ethionine treated animals. Only the mean survival time was increased to 150 per cent.

Efficacy of L-gulonolactone and 2-ketogulonolactone in counteracting strychnine toxicity in ethionine treated mice — The precursors of ascorbic acid are incapable of counteracting strychnine toxicity in ethionine treated animals. Mean survival period with L-gulonolactone was 3·5 min. and with 2-ketogulonolactone 3·2 min.

Discussion

From the present and previous studies[1] it appears that ascorbic acid and its metabolic precursors markedly nullify the convulsive and lethal actions of strychnine *in vivo*. It is evident from the results that ascorbic acid only possesses protective action against strychnine and not its oxidized product, since ascorbic acid made alkaline is quickly oxidized to diketogulonic acid which is devoid of protective action against strychnine toxicity. Ascorbic acid level is reported to be increased greatly in pentobarbital anaesthesia[4]. Pentobarbital is known to counteract strychnine toxicity and the present study shows that the protective action of pentobarbital is due to the elevated level of ascorbic acid. Tousler and Hollman[3] have shown that ethionine can block rise in ascorbic acid as a result of pentobarbital treatment. In the present investigation, it is observed that in ethionine treated animals, barbital is incapable of counteracting strychnine toxicity.

Ethionine is known to be an inhibitor of protein synthesis and blocks the synthesis of ascorbic acid in the body[3]. The protective action of ascorbic acid precursors (L-gulonolactone and 2-ketogulonolactone) in mice has been observed to be exerted through the formation of ascorbic acid since these precursors are not effective in ethionine treated mice. This has also gained support from the fact that in guinea-pig, which is unable to synthesize ascorbic acid, these precursors are not effective against strychnine (Dey, P. K., unpublished data). Also, in scorbutic guinea-pigs, strychnine toxicity is greatly increased[5].

2012

INDIAN J. EXPTL. BIOL., 5: 110-112, 1967.

Protective Action of Ascorbic Acid & Its Precursors on the Convulsive & Lethal Actions of Strychnine

P. K. DEY

Department of Physiology, University College of Science, Calcutta 9

Manuscript received 22 *March* 1966

Ascorbic acid completely counteracted the convulsive and lethal actions of strychnine, and this protective action is directly dependent on the plasma ascorbic acid level. Ascorbic acid failed to show any protective action against other convulsive agents like Picrotoxin and Metrazol. The precursors of ascorbic acid, L-gulonolactone and 2-ketogulonolactone, were also effective against strychnine toxicity and they exert protection through the formation of ascorbic acid. It is suggested that ascorbic acid interacts directly with strychnine converting it to some non-toxic derivative.

STRYCHNINE incubated with lemon juice at 37°C. loses its lethal and convulsive properties and at 50°C. it retains its lethal property, suggesting that the lemon juice rich in ascorbic acid acts as a protective or detoxicating agent[1]; the loss of protective action at 50°C. can be ascribed to the destruction of ascorbic acid. It was shown that ascorbic acid is normally prevented from oxidation due to the presence of a protective agent in lemon juice which is thermolabile[2].

In view of the above findings the present investigation was undertaken to study, *in vivo* and *in vitro*, the mechanism of protective action of ascorbic acid and its precursors, L-gulonolactone and 2-ketogulonolactone, against the lethal and convulsive actions of strychnine.

Materials and Methods

Albino mice weighing between 18 and 25 g. were used in the study. The protective action of ascorbic acid and its precursors was studied as follows in different groups of mice each containing 10 animals. The lethal and convulsive properties of strychnine were evaluated on the basis of number of animals died and the absence of or very feeble convulsions respectively.

Control group — Strychnine (2 mg./kg. body weight) was administered subcutaneously and its effect noted.

In vivo protective action of L-*gulonolactone and* 2-*ketogulonolactone* — Both the precursors were administered separately intraperitoneally in doses of 1 and 2 g./kg. body weight into 2 groups of mice. Strychnine was injected 15 min. following the administration of ascorbic acid precursors and the lethal and convulsive properties noted.

Dose-response relationship between ascorbic acid and strychnine — Ascorbic acid was administered intraperitoneally in doses of 50, 100, 500, 1000 and 2000 mg./kg. body weight respectively into 5 groups of mice. After 15 min. following ascorbic acid administration strychnine was administered and the lethal and convulsive effects noted.

Time-response relationship between the effective dose of ascorbic acid and strychnine — Ascorbic acid, 1 g./kg. body weight (effective dose), was administered intraperitoneally into 4 groups of mice. The lethal and convulsive properties of strychnine were noted at 15, 30, 60 and 120 min. intervals after ascorbic acid administration.

Ascorbic acid concentration in plasma at different time intervals — Ascorbic acid (1 g./kg. body weight) was administered intraperitoneally into 4 groups of mice and blood was collected at 15, 30, 60 and 120 min. intervals respectively. Ascorbic acid concentration in plasma was determined by the method of King using 2,6-dichlorophenol indophenol as indicator.

In vitro study — Strychnine sulphate (2 mg.) was dissolved separately in 10 ml. solutions (10 g./100 ml.) of ascorbic acid, ascorbic acid made alkaline (*p*H 8·0), L-gulonolactone and 2-ketogulonolactone. All the solutions were incubated at 37°C. for 40 min. Lethal and convulsive properties of strychnine were tested by subcutaneous administration of respective mixtures in doses containing 2 mg. strychnine/kg. body weight.

Counteraction of strychnine toxicity by ascorbic acid and its precursors in urethanized cats — Lethal doses of strychnine were administered to urethanized (1·8 g./kg. body weight, intramuscularly) cats and within 3 min. violent tetanic convulsions and respiratory arrest occurred; the protective action of ascorbic acid, L-gulonolactone and 2-ketogulonolactone was separately tested by quick intravenous administration of the compound at a concentration of 30 mg./ml., the total dose given being 300 mg.

Efficacy of ascorbic acid against Metrazol and Picrotoxin — Ascorbic acid was administered intraperitoneally into 2 groups of mice. After 15 min., to one group Metrazol (100 mg./kg.) and to the other group Picrotoxin (20 mg./kg.) were given subcutaneously, and the convulsive and lethal actions studied.

In another experiment the above procedure was followed using Metrazol 50 mg./kg. and Picrotoxin 10 mg./kg.

Efficacy of pentobarbital in counteracting strychnine toxicity in ethionine treated mice — Ethionine (100 mg./kg.) was given orally with milk diet to a group of mice for 9 days. Then pentobarbital was administered intraperitoneally (40 mg./kg.). After

Naturwissenschaften 53 (12): 310. 1966

Efficacy of Vitamin C in Counteracting Tetanus Toxin Toxicity

P. K. Dey

Department of Physiology, University College of Science, Calcutta

The author has shown [1] that ascorbic acid is most effective as prophylactic and therapeutic agent in nullifying the lethal and convulsive properties of strychnine. He now examined the efficacy of ascorbic acid in counteracting the toxic action of tetanus toxin since Sherrington [2] observed that the effects of strychnine poisoning are similar to those appearing in tetanus toxin toxicity and Brooks et al. [3] confirmed the findings of Sherrington that the action of tetanus toxin in the spinal cord closely resembles that of strychnine. Also, Jungblut [4] has shown that the toxin is destroyed *in vitro* by vitamin C.

Adult rats were used in all the experiments. Diet, temp. and space allowed for movement were kept uniform. The gastrocnemius muscle was the site used for the intramuscular administration of toxin.

Group 1: 5 rats were given 2MLD (minimum lethal dose) of tetanus toxin. The symptoms of toxicity were then noted. — *Group 2*: 5 rats were given simultaneously 2MLD of toxin and 1 gm/kg of vitamin C intraperitoneally. Then for subsequent three days, vitamin C (1 gm/kg) was only administered twice daily i. p. — *Group 3*: 5 rats were administered ascorbic acid 1 gm/kg twice daily for three days. Then 2MLD of toxin was given, followed again by administration of vitamin C for subsequent three days at the previous dose. — *Group 4*: 5 rats were given 2MLD of toxin. Usally after 16 to 26 hours, local tetanus appeared in the affected leg. When such beginning of symptoms were noted, vitamin C (1 gm/kg) was given i. p. twice daily for 3 days. — *Group 5*: 10 rats were given 2MLD of toxin. After 40 to 47 hours, general tetanic symptoms markedly developed, vitamin C (300 mg) was administered intravenously after anaesthetizeing the animal with Na-thiopental.

Results. *Group 1*: Following tetanus toxin, local tetanus appeared in 16 to 26 hours. The affected leg was in fixed position and toes were extended. Within 27 to 30 hours, the tail, extremity and hip deviated to the injection side. Both extremities assumed parallel extended position. In 40 to 47 hours, spasticity of the abdominal and thoracic musculature and flexor muscles of the spine and neck was seen. Tachycardia, dyspnoea, and convulsions were oberved. Death followed in 47 to 65 hours. — *Group 2*: All the animals survived. Only very mild local tetanus were seen at the affected leg after 18 hours. — *Group 3*: All the animals survived. No symptoms of toxicity appeared. — *Group 4*: When the initial symptoms of local tetanus appeared, administration of vitamin C prevented the further spread of the symptoms and they finally survived. — *Group 5*: Administration of vitamin C through

intravenous route at an advanced stage of tetanus led to the survival of the animals.

From the above results, it definitely appears that vitamin C can be effectively used as a simple prophylactic and therapeutic tool to combat the neurotoxic effects of tetanus toxin.

Thanks are due to Prof. S. R. Moitra for his interest in this work.

Eingegangen am 31. März 1966

[1] Dey, P. K.: (a) Naturwissenschaften 52, 164 (1964); — (b) Ind. J. Exptl. Biol. (communicated, 1966). — [2] Sherrington, C. S.: The Integrative Action of Nervous System, p. 303, 112. New York: Yale University Press 1906. — [3] Brooks, B. V., D. R. Curtis, and J. C. Eccles: Nature 175, 120 (1955). — [4] Jungblut, C. W.: J. Immunol. 33, 203 (1937).

NATURWISSENSCHAFTEN 52(7), 164, 1965

Protective Action of Lemon Juice and Ascorbic Acid Against Lethality and Convulsive Property of Strychnine

We have observed that acetyl radical[1a, b]) is the functional group of strychnine (indoline) and Kopsine (indole) alkaloids. This observation has prompted to investigate whether the process of deacetylation can occur in the biological kingdom enzymatically or non-enzymatically as a normal process of the metabolism of strychnine. Incidentally, it has been observed[2]) that ascorbic acid plays some role in the metabolism of indole compound. Hence the role of ascorbic acid on the metabolism of strychnine has also been evaluated.

Enzymatic deacetylation of strychnine was effected by incubating solution of strychnine with lemon juice. Initially, strychnine soln. was added to lemon juice and the whole mixture was divided into four equal parts, A, B, C and D which were then incubated accordingly as follows: A-incubated at room temp. (25° C) for 15 min, B-incubated at 37° C for 15 min, C-incubated at 37° C for 45 min, D-incubated at 50° C for 15 min. As a control study, strychnine soln. *per se* was heated at 50° C for 45 min. Then the lethality and convulsive property of strychnine of each sample were tested in mice by subcutaneous administration of incubated mixture containing 2 mg/kg of body-weight of strychnine.

Similarly, strychnine soln. was incubated with plasma of cat, rat, pigeon and toad's blood for 20 min at 37° C. The lethality and convulsive property of strychnine of each plasma sample was then tested as stated above. Ascorbic acid was given intraperitoneally in the dosage of 1 gm and 2 gm per kg body-weight respectively in groups of mice. After 15 min following ascorbic acid administration, strychnine (2 mg/kg bodyweight) was given subcutaneously and the lethality and convulsive activity were noted.

Table. *Results of the experiments*

No. of mice used: in the experiment with strychnine without lemon juice 20, in all other cases 10.

Samples	mean survival time	mortality (%)
strychnine*) without lemon juice	5	100
A (10 ml/kg)	8.3	60
B (10 ml/kg)	11.0	20
C (10 ml/kg)	33.0	20
D (10 ml/kg)	11.6	100
strychnine*) per se heated at 50° C	6.0	100
cat plasma (10 ml/kg)	12.0	100
rat plasma (10 ml/kg)	10.0	100
pigeon plasma (10 ml/kg)	6.0	100
toad's plasma (10 ml/kg)	9.0	100
ascorbic acid (1 gm/kg)	—	nil
ascorbic acid (2 gm/kg)	—	nil

*) strychnine soln. 2 mg/kg.

The convulsion was absent or very feeble with ascorbic acid and lemon juice in survived animals. It appears from above that incubation of strychnine with lemon juice (containing acylase enzyme and ascorbic acid) at 37° C leads to profound loss of lethal and convulsive property of strychnine but heating of the mixture at 50° C retains the lethal and convulsive property of strychnine completely. Incubation of strychnine with plasma of various species could not exhibit any protection against strychnine. It thus appears that deacetylation of strychnine is most probably the chief pathway of metabolism of strychnine. Ascorbic acid in very high doses shows protection against strychnine which perhaps manifests its biologic action by combining with some enzyme system in central nervous system and ascorbic acid may considerably protect that enzyme system from strychnine binding.

Department of Physiology, University College of Science, Calcutta 9. India

P. K. DEY

Eingegangen am 26. November 1964

[1]) MUKHERJEE, S. R., A. MAITI and P. K. DEY: a) Nature 180, 916 (1957); b) J. Expt. Med. Sc. 1, 179 (1958). — [2]) HOFFER, A. in: Psychotropic Drugs, Ed. S. Garattini and V. Ghetti, p. 10. New York: Elsevier Publisher C. (1957).

to cold. The surviving animals show a characteristic alarm reaction with loss of weight, thymus involution ($p < 0.001$), and adrenal enlargement ($p < 0.05$). Ascorbate administration shows no beneficial effects in the absence of adequate thyroid function replacement therapy.

In contrast, in the operated rats receiving 10 μgm. of thyroxine, exposure to cold does not seem to have any deleterious effects. This confirms our previous observations (7) as well as those of Leblond and Eartly (12).

In the thyroidectomized rats given only 3 μgm. of thyroxine, we observe the usual signs of an alarm reaction with a survival of only 55%. This again is a confirmation of the findings of Leblond and Eartly (12).

The most striking results were obtained in the thyroidectomized rats simultaneously treated with ascorbate and 3 μgm. of thyroxine. We have seen that, used separately, these treatments provide an entirely inadequate protection against exposure to cold. It is quite clear that ascorbate enhances the effect of a low dose of thyroxine in animals exposed to cold, which behave almost as intact animals or thyroidectomized animals receiving adequate amounts of thyroxine.

3. *Discussion*

In a previous paper (7), we discussed at some length the relations between the thyroid hormones and adrenaline. Since that time, other workers have accumulated evidence to show the role of epinephrine and nor-epinephrine on the thyroidal release of thyroid hormones (1, 13), as well as the importance of the combined effects of the thyroid hormones and those of the adrenal medulla in thermogenesis during exposure to cold (4, 6, 17, 19). It must also be noted that some relationship has been found (11) between the thyroid hormones and the metabolism of ascorbic acid. It is not surprising to find that ascorbate administration in animals exposed to cold seems to bear a definite relation to the thyroid hormones.

Acknowledgments

We are greatly indebted to Dr. L. P. Dugal for his continuous support in this work, and to Dr. C. P. Leblond of McGill University for his fruitful advice. We also wish to extend our appreciation to Hoffman-LaRoche, Ciba, and Merck & Co., who generously supplied the ascorbate, DCA, and cortisone needed for these experiments.

Résumé

Nous avons étudié les effets de l'administration d'ascorbate sur la résistance au froid et la fonction thyroïdienne de rats surrénaloprives traités à la cortisone et/ou à la DCA en doses variables, alors que chez le rat sans thyroïde traité ou non à la thyroxine, nous avons évalué la résistance au froid et la fonction de la cortico-surrénale.

Chez le rat sans surrénales exposé au froid et recevant de fortes doses de cortisone (2.5 mg.), l'administration d'ascorbate (150 mg.), ou de DCA

(2.5 mg.) augmente la survie, réduit le degré de thymolyse, et modère l'activation de la thyroïde. Chez le rat surrénalectomisé ne recevant ni cortisone ni DCA ou ne recevant que de faibles doses (0.4 et 0.1 mg.) de ces hormones, l'administration d'ascorbate conserve des effets bienfaisants: la croissance et la survie sont meilleures. Si la DCA est administrée seule à faible dose (0.1 mg.), c'est-à-dire non associée à la cortisone, l'ascorbate semble devenir nocif: la croissance et la survie des animaux exposés au froid sont diminuées, et ce phénomène s'accompagne d'une activation considérable de la thyroïde.

Chez le rat éthyroïdé exposé au froid, l'administration d'ascorbate (150 mg.) est sans effet en l'absence d'hormones thyroïdiennes, mais augmente l'efficacité de faibles doses (3 μg.) de thyroxine sur la croissance et la survie, supprimant en même temps certains signes typiques de la réaction d'alarme: la thymolyse et l'hypertrophie des surrénales.

Ces résultats semblent montrer que le rôle des hormones corticales dans la résistance au froid se limiterait à une action de support, et que l'effet bienfaisant de l'ascorbate serait plutôt relié à l'action des hormones de la thyroïde.

References

1. Ackerman, N. B. and Arons, W. L. Federation Proc. 15 : 1. 1956.
2. Axelrad, A. A. and Leblond, C. P. Can. Med. Assoc. J. 67 : 675. 1952.
3. Bacchus, H., Toompas, C. A., and Heiffer, M. H. Federation Proc. 10 : 7. 1951.
4. Bernstein, H., Goldstein, M. S., and Ramey, E. R. Federation Proc. 15 : 18. 1956.
5. Booker, W. M., DaCosta, F. M., Tureman, J. R., Froix, C., and Jones, W. Endocrinology, 56 : 413. 1955.
6. Cottle, W. and Carlson, L. D. Federation Proc. 15 : 41. 1956.
7. DesMarais, A. Can. J. Biochem. Physiol. 33 : 1018. 1955.
8. DesMarais, A. Compt. rend. soc. biol. 149 : 214. 1955.
9. DesMarais, A. and Gagnon, P. M. Compt. rend. soc. biol. 149 : 1714. 1955.
10. Dugal, L. P. and Thérien, M. Endocrinology, 44 : 420. 1949.
11. Gemmill, C. L. Am. J. Physiol. 167 : 349. 1951.
12. Leblond, C. P. and Eartly, H. Endocrinology, 51 : 26. 1952.
13. Menof, P. Lancet, 267 : 996. 1954.
14. Salgado, E. Rev. can. biol. 12 : 449. 1953.
15. Salgado, E. Endocrinology, 55 : 377. 1954.
16. Selye, H. and Bois, P. *In* 4th Annual report on stress. *Edited by* Selye and Heuser. 1954. p. 533.
17. Swanson, H. E. Federation Proc. 15 : 183. 1956.
18. Thérien, M., Leblanc, J., Héroux, O., and Dugal, L. P. Can. J. Research, E, 27 : 349. 1949.
19. Weiss, A. K. Federation Proc. 15 : 197. 1956.

1981

considered as having nothing but a "permissive" or "conditioning" action on the beneficial effects of ascorbate, provided the reactive ability of the organism towards cold stimulation is not exceeded.

As far as the thyroid effect of DCA is concerned, when it is administered alone at low dosage level, we would like to recall the work of Axelrad and Leblond (2). They found that an adequate supply of sodium chloride, as such, is indispensable for the development of goitrous thyroids under conditions of iodine deficiency in mice. The analogy between the present results, obtained with DCA treatment, and those found by Axelrad and Leblond using sodium chloride seems to be of a particular interest in the light of the recent observations of Salgado (14, 15) on the role of the thyroid gland in the production of cardiovascular diseases induced by DCA.

II. Thyroidectomized Animals

These last results appeared sufficient to support the hypothesis of an extra-adrenal effect of ascorbate in animals exposed to cold, since our previous findings (7, 8, 9) had already suggested some relation between the thyroid and ascorbate.

1. *Experimental*

Sixty-four male albino rats of the Wistar strain, weighing between 145 and 164 gm., were used in this experiment. Ten were kept as non-operated controls and the others were thyroidectomized at day 0 of the experiment. They were all fed the usual Purina diet, supplemented by calcium lactate 1% in the drinking water of the operated animals. The treatments were initiated 24 hr. after operation and maintained throughout according to the distribution indicated in Table III. The thyroxine was administered in one daily subcutaneous injection as thyroxine-Na (BDH) in a slightly alkaline solution. The ascorbate was administered orally as in the previous experiments. All the animals were kept at 24 ± 1.0° C. for the first seven days of the experiment; they were then transferred into a cold room at 14 ± 1.0° C. for another seven days, after which the remaining animals were sacrificed. The thymus and adrenals were weighed in the fresh state; the thyroid of the non-operated controls was fixed and prepared for epithelium measurements as described previously, and comparisons were made with the thyroids removed at the onset of the experiment.

2. *Results* (*Table III*)

At this temperature of exposure, intact rats become readily acclimatized to cold. Their thyroids exhibit a definite and significant ($p < 0.001$) activation, as compared to the thyroid glands removed 14 days previously in the operated animals. They otherwise show no sign of alarm reaction, the adrenal and thymus weights being quite normal for rats of this age.

Thyroidectomized rats given ascorbate without thyroxine have a very low growth rate at room temperature and are almost all eliminated during exposure

TABLE III

EFFECTS OF ASCORBATE ADMINISTRATION AND THYROXINE IN THYROIDECTOMIZED RATS EXPOSED TO COLD

Treatment	No. animals at autopsy	Growth in gm. 0–7th d.	Growth in gm. 7–14th d.	% survival	Thymus, fresh wt., mgm.	Adrenals, fresh wt., mgm.	Thyroid, height of epithelium, μ
Untreated, non-operated rats	10	32.3	30.4	100	366.7 ± 14.22*	27.8 ± 1.93*	12.82 ± 0.36*
Thyroidectomized							
Ascorbate (150 mgm.)	3	5.0	−15.2	20	83.4 ± 9.17†,‡	32.6 ± 1.19†	
Thyroxine (10 μgm.)	10	15.6	31.3	100	348.2 ± 17.34	29.7 ± 2.12	
Thyroxine (3 μgm.)	10	18.2	3.5	55	207.3 ± 23.68†	35.9 ± 1.32†	7.97 ± 0.18* at operation
Thyroxine (3 μgm.) + ascorbate (150 mgm.)	10	19.1	21.7	91	316.2 ± 29.73	28.6 ± 2.06	

*Standard error of the mean.
†Significantly different from untreated, non-operated rats.
‡Significantly different from thyroxine (3 μgm.) treated animals.

1981

TABLE II

EFFECTS OF ASCORBATE ADMINISTRATION IN ADRENALECTOMIZED RATS RECEIVING LOW DOSES OF CORTICOSTEROIDS AND EXPOSED TO COLD

NaCl 1%	Cortisone, 0.4 mgm.	DCA, 0.1 mgm.	Ascorbate, 150 mgm.	No. animals at autopsy	Temperature and duration of exposure	Growth: Absolute in gm.	Growth: % of controls at room temp.	% survival	Thymus, fresh wt., mgm.	Thyroid, height of epithelium, μ
+				8	24° C. (0–20 days)	61.2 ± 5.2*		100	628.7 ± 52.9*	12.5 ± 0.34*
+			+	8	24° C. (0–20 days)	62.0 ± 5.8		100	626.8 ± 57.6	11.3 ± 0.29
+				6	14° C. (0–16 d.); 9° C. (16–20 d.)	35.0 ± 6.5	57.2†	46	550.5 ± 51.8	14.4 ± 0.32†
+			+	10	14° C. (0–16 d.); 9° C. (16–20 d.)	46.6 ± 8.7	75.0	83	520.8 ± 53.7	12.1 ± 0.32
	+	+		8	24° C. (0–20 d.)	91.1 ± 6.6		100	602.1 ± 48.3	12.6 ± 0.27
	+	+	+	8	24° C. (0–20 d.)	84.1 ± 6.8		100	654.9 ± 50.9	12.3 ± 0.30
	+	+		10	10° C. (0–8 d.); 1.5° C. (8–20 d.)	46.8 ± 6.7	51.4†	71	494.4 ± 46.6	13.8 ± 0.32†
	+	+	+	13	10° C. (0–8 d.); 1.5° C. (8–20 d.)	60.8 ± 7.3	72.4	100	670.3 ± 54.5	14.3 ± 0.34†
		+		8	22° C. (0–20 d.)	60.0 ± 5.5		100	618.2 ± 51.4	12.9 ± 0.28
		+	+	8	22° C. (0–20 d.)	72.1 ± 6.2		100	582.8 ± 49.3	15.8 ± 0.33
		+		10	10° C. (0–16 d.); 1.5° C. (16–20 d.)	32.2 ± 6.1	53.7†	83	561.2 ± 41.7	17.0 ± 0.33†
		+	+	8	10° C. (0–16 d.); 1.5° C. (16–20 d.)	31.5 ± 7.8	43.7†	67	530.5 ± 47.5	17.0 ± 0.35†

*Standard error of the mean.
†Significantly different from controls at room temperature.

exhibit a maximal thyroid response, but those kept at room temperature and given ascorbate have a significantly ($p < 0.001$) higher epithelium than their controls at room temperature without ascorbate. This effect of DCA at low dosage level is particularly striking if we recall that the normal height of the thyroidal epithelium in rats of this age is around 8.0 μ.

C. Discussion

As we already noted in previous publications on this subject (7, 8), the administration of ascorbate to adrenalectomized rats receiving large doses of cortisone does not particularly change the growth rate, though it has some effect in reducing the extent of thymolysis and thyroid activation. The present results also confirm our previous observation (8) that large doses of DCA have effects similar to those of ascorbate administration as far as the same targets are concerned: thymus weight and thyroid activity.

However, from our last experiments (7) and this one, we gained evidence that the doses of cortical hormones used were too high. In order to see whether ascorbate administration still had some effect on resistance and adaptation to cold in animals with normal or subnormal corticoadrenal function, it was necessary to use smaller doses of cortical hormones or none at all in adrenalectomized rats. The doses used were suggested by the extensive studies of Selye and Bois (16).

From our results, it can be seen that ascorbate administration still retains some of its beneficial effects (better growth and survival) in adrenalectomized animals entirely deprived of cortical hormones. This strongly suggests that ascorbate might act, at least partly, through other systems than those directly influenced by changes in corticoadrenal function. This is further substantiated by the finding that, although the growth and survival are enhanced by the administration of minimal amounts of cortisone and DCA, ascorbate administration has the same relative effect on the growth and survival of animals exposed to cold as compared to their controls at room temperature. This again strongly indicates that ascorbate has some effects in animals exposed to cold which do not depend upon an increase in secretion of corticoadrenal hormones. It might be of interest to note that Booker *et al.* (5) have made a similar observation in their work on the effect of ascorbic acid on the survival of adrenalectomized mice exposed to cold without corticosteroid treatment. However, our results are in sharp contrast with those of Bacchus *et al.* (3), who reported a complete loss of action of ascorbic acid in adrenalectomized rats exposed to cold without cortical hormones, particularly on the survival. It seems to us that these results do not invalidate our findings, since the discrepancy may be explained through differences in experimental conditions.

It is true that adrenalectomized animals exposed to cold and given ascorbate are in a much better condition when they receive minimal amounts of cortical hormones. However, at the present time, it is difficult to see whether ascorbate has any direct effect, *in vivo*, on the peripheral action of cortical hormones as far as adaptation to cold is concerned. The cortical hormones might be

1981

TABLE I

EFFECTS OF ASCORBATE ADMINISTRATION IN ADRENALECTOMIZED RATS RECEIVING HIGH DOSES OF CORTICOSTEROIDS AND EXPOSED TO COLD

Treatment	No. animals at autopsy	Growth in gm. 0–7th day	Growth in gm. 7–14th day	% survival	Thymus, fresh wt., mgm.	Thyroid, height of epithelium, μ
Untreated, sham-operated rats	8	35.0	31.4	100	367.8 ± 24.72*	8.24 ± 0.18*
Adrenalectomized						
Cortisone (2.5 mgm.)	10	12.1	17.3	100	182.7 ± 28.53†	8.31 ± 0.14
Cortisone (2.5 mgm.) + cold: 14° C. (7–14 d.)	9	11.6	18.1	69	62.3 ± 9.41†,‡	12.72 ± 0.32†
Cortisone (2.5 mgm.) + ascorbate (150.0 mgm.) + cold: 14° C. (7–14 d.)	14	11.0	14.2	78	123.6 ± 31.32†	9.78 ± 0.28§
Cortisone (2.5 mgm.) + DCA (2.5 mgm.) + cold: 14° C. (7–14 d.)	10	14.2	14.6	91	176.8 ± 25.63†	10.65 ± 0.33†,§

*Standard error of the mean.
†Significantly different from untreated, sham-operated rats.
‡Significantly different from cortisone-treated rats at room temperature.
§Significantly different from cortisone-treated rats exposed to cold.

Table II. The sodium chloride was administered at 1% concentration in the drinking water; cortisone and DCA were given in one daily subcutaneous injection at the respective dosage levels of 0.4 and 0.1 mgm.; the sodium ascorbate was administered by mouth in two daily doses of 75 mgm. In all these experiments, the changes in temperature and time of exposure to cold were made so that the animals exposed to cold without ascorbate treatment would have approximately the same relative difference in growth rate as compared to their controls at room temperature.

2. *Results (Table II)*

NaCl With or Without Ascorbate

Analysis of results show that ascorbate treatment has no particular effect at room temperature. In animals exposed to cold, the impairment of growth is lessened by ascorbate treatment, the decrease in growth being significant only for those without ascorbate ($p < 0.01$). It can also be seen that ascorbate administration increased the survival and prevented the activation of the thyroid. No definite effect was observed on the thymus weight.

Cortisone and DCA With or Without Ascorbate

Here again, there is no effect of ascorbate administration in animals kept at room temperature. It has to be noted that in order to obtain the same relative impairment of growth in these animals, where low doses of both cortisone and DCA are substituted for NaCl, we had to use lower temperatures. Despite this, the survival is much better (100%), although ascorbate treatment did not affect the relative growth more than it did in the previous experiment with only NaCl administration. No effect of ascorbate treatment can be observed on the thyroid; exposure to cold increases the activity of this gland in both groups, whether or not they are given ascorbate. As far as the thymus is concerned, there is a small but non-significant decrease in its weight when no ascorbate is given to rats exposed to cold.

DCA With or Without Ascorbate

Substituting DCA for NaCl seems to afford a greater degree of resistance to cold, since the survival is better (83% as compared to 46%) at lower temperatures. Moreover, DCA alone appears to be as effective as DCA and cortisone as far as survival is concerned, though the absolute growth is decreased by omission of cortisone.

The most striking observation is the apparent deleterious effect of ascorbate administration in animals exposed to cold and given DCA without cortisone. Again, it is only in the cold that this effect of ascorbate administration is disclosed; at room temperature, ascorbate treatment slightly increases the growth rate of DCA-treated animals.

Another impressive observation is the great increase in thyroid activity in those animals exposed to cold and given this low dose of DCA with or without ascorbate administration. Not only the DCA-treated rats exposed to cold

1981

FURTHER STUDIES ON THYROID – ADRENAL – ASCORBIC ACID RELATIONS IN ANIMALS EXPOSED TO COLD[1]

By André DesMarais[2]

Abstract

The effect of ascorbate administration on resistance to cold and thyroid function was studied in adrenalectomized rats receiving various amounts of cortisone and/or DCA; in thyroidectomized rats given different doses of of thyroxine, the effect of ascorbate administration was evaluated on both resistance to cold and adrenal function.

In adrenalectomized rats given large doses of cortisone (2.5 mgm.) and exposed to cold, the administration of either ascorbate (150 mgm.) or DCA (2.5 mgm.) enhances the survival, reduces the extent of thymolysis, and decreases the activation of the thyroid. In adrenalectomized rats receiving no cortisone or DCA or low doses (0.4 and 0.1 mgm.) of these hormones, ascorbate administration (150 mgm.) still retains some of its beneficial effects on resistance to cold (better growth and survival); when low doses of DCA (0.1 mgm.) are given without cortisone, ascorbate administration seems to have a deleterious effect on the growth and survival during exposure to cold, with a greater increase in thyroid activity.

In thyroidectomized rats exposed to cold, ascorbate administration (150 mgm.) has no effect in the absence of thyroxine, but increases the efficiency of low doses (3 μgm.) of thyroxine, preventing at the same time some of the typical signs of an alarm reaction: thymolysis and adrenal enlargement.

These results are interpreted as showing that the role of the cortical hormones in resistance to cold might be limited to a "conditioning" action and that the beneficial effects of ascorbate administration would be mediated through the thyroid hormones.

In a recent paper (7), we reviewed the work of Dugal *et al.* on the beneficial effects of ascorbic acid administration in animals exposed to cold, and we confirmed their findings concerning the reduction in thymic involution and the prevention of adrenal hypertrophy in rats exposed to cold and given ascorbic acid (10, 18). In the same paper, we confirmed our earlier results (8) showing that these particular effects of ascorbic acid administration were accompanied by a decrease of thyroid activation. We also presented evidence to show that the administration of thyroxine in adequate amounts to thyroidectomized rats exposed to cold duplicates the beneficial effects of ascorbate in normal rats exposed to cold, namely a better growth and the prevention of both thymolysis and adrenal hypertrophy. When given to thyroidectomized–adrenalectomized rats exposed to cold and receiving large doses of cortisone, thyroxine completely reversed the catabolic effect of cortisone. In the same paper, it was shown that ascorbic acid administration had no effect on the growth rate of thyroidectomized–adrenalectomized rats exposed to cold and given large doses of cortisone but no thyroxine.

The question then arose whether the presence of the thyroid or its hormones was necessary to obtain the desired beneficial effect of ascorbate administration in cold-exposed animals. The present paper will deal with some aspects of this problem.

[1]*Manuscript received July 9, 1956.*
Contribution from the Department of Experimental Physiology, Laval University, Quebec, Que.
This work was supported by a Grant from the Defence Research Board, Canada.
[2]*Present address: Department of Biology, University of Ottawa, Ottawa, Canada.*

I. Adrenalectomized Animals

A. High Doses of Cortisone and DCA

1. Experimental

Sixty male albino rats of the Wistar strain were used. Their weight at the onset of the experiment ranged from 142 to 158 gm. On day 0 of the experiment, 52 animals were operated on and the treatments were initiated 24 hr. later, according to the distribution shown in Table I. All adrenalectomized rats were given 1% sodium chloride in tap water to drink ad libitum. Cortisone and DCA were administered daily at the dosage level indicated, in one subcutaneous injection, while the sodium ascorbate was given by mouth in two daily doses of 75 mgm. in 0.5 ml. of redistilled water. The animals were kept in a constant temperature room at 24 ± 1.0° C., except when they were exposed to cold in a room maintained at 14 ± 1.0° C. The body weights were recorded on the 7th and the 14th days of the experiment. At autopsy, the thymus was weighed to 0.1 mgm. on a torsion balance, the thyroid was fixed in Bouin's, and 5 μ sections were stained with Mallory's triple stain. The height of the epithelium was measured with an ocular micrometer in 10 follicles chosen at random.

2. Results (Table I)

In the cortisone-treated rats kept at room temperature, the growth is about one third of normal, the weight of the thymus is significantly reduced ($p < 0.001$), indicating an excessive dose of cortisone, but the thyroidal epithelium remains unchanged.

When cortisone-treated rats are exposed to cold, the growth rate is not further reduced, but the survival is decreased to 69%, the thymus weight is reduced to a significantly greater extent ($p < 0.001$), and the height of the epithelial cells of the thyroid is increased ($p < 0.001$).

In the cortisone-treated, cold-exposed animals, the administration of either ascorbate or DCA enhances the survival, reduces the extent of thymolysis, and significantly ($p < 0.001$) decreases the height of the thyroidal epithelium, as compared to the cortisone-treated animals exposed to cold, without ascorbate or DCA, though the thymus weight and the epithelium height remain significantly different from normal.

B. Low Doses of Cortisone and DCA

1. Experimental

On day 0 of the experiment, 124 male albino rats of the Wistar strain were adrenalectomized. Their initial weight was between 138 and 164 gm. All animals were killed after 20 days; the thymus was weighed and the thyroid removed, prepared, and examined as described in the first experiment. The different treatments, temperature, and time of exposure are indicated in

Can. J. Biochem. Physiol. 34(6): 1251-1260 1981

and the Ontario Department of Agriculture and Food. Data presented in this paper are taken from a thesis presented to the Faculty of Graduate Studies, University of Guelph, by the senior author as partial fulfillment of the degree of Master of Science.

BLAXTER, K. L., BROWN, F. and WOOD, W. A. 1953. Some effects of natural and synthetic antioxidants on the incidence of muscular dystrophy induced by cod-liver oil. Brit. J. Nutr. 7: 337–349.

BROWN, R. G., SHARP, B. A., YOUNG, L. G. and VAN DREUMEL, A. 1971. Serum ascorbate levels in swine with cardiac muscular dystrophy (mulberry heart disease) and hepatosis dietetica. Can. J. Anim. Sci. 51: 537–539.

DEMILLE, F., MILLER, R. B. and BUCHANAN-SMITH, J. G. 1972. Effects of vitamin E or ethoxyquin and rumen development upon muscular dystrophy in lambs. Can. J. Anim. Sci. 52: 351–361.

HJAIRE, A. and LILLEENGEN, K. 1936. Muscle degeneration in calves affected with avitaminosis C. Nord. Med. Tidskr. p. 472.

KNIGHT, C. A., DUTCHER, R. A., GUERRANT, N. B. and BECHDEL, S. I. 1941. Utilization and excretion of ascorbic acid by the dairy cow. J. Dairy Sci. 24: 567–577.

NATIONAL ACADEMY OF SCIENCES. 1968. Nutrient requirements of sheep. Publ. 1693. p. 13. Washington, D.C.

RICHMOND, M. S., SATTERFIELD, G. H., GRINNELLS, C. D. and DANN, W. J. 1940. Ascorbic acid content of goat's milk and blood: influence of ascorbic acid injection and diet. J. Nutr. 20: 99–108.

ROE, J. H. and OESTERLING, H. J. 1944. The determination of dihydroascorbic acid and ascorbic acid in plant tissues by the 2,4,dinitrophenylhydrazine method. J. Biol. Chem. 152: 511–517.

STEEL, R. G. D. and TORRIE, J. H. 1960. Principles and procedures of statistics. McGraw-Hill Book Co., Inc., New York, N.Y.

WILLMAN, J. P., McMAY, C. M., MORRISON, F. B. and MAYNARD, L. A. 1940. The relation of feeding and management to the cause of the stiff-lamb disease. Proc. Amer. Soc. Anim. Prod. p. 185–192.

F. DEMILLE, R. G. BROWN, and J. G. BUCHANAN-SMITH

Department of Animal and Poultry Science, University of Guelph, Guelph, Ontario. *Received Oct. 4, 1971, accepted Mar. 3, 1972.*

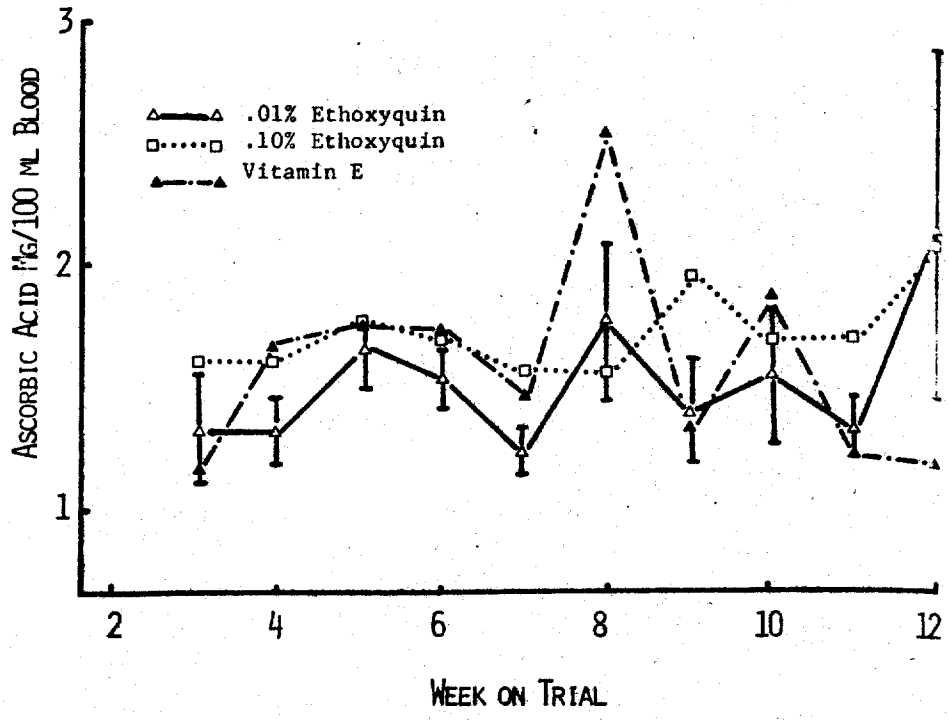


Fig. 1. Effects of ethoxyquin or vitamin E on weekly blood ascorbate levels in lambs (each point is the mean for surviving lambs; range of one standard error equivalent to all values within weeks shown by vertical lines).

have a persistent effect upon blood ascorbate levels and is in accord with recent findings in swine (Brown et al. 1971).

Furthermore, levels of ascorbate in other soft tissues of these lambs were not affected ($P > 0.05$) by the nutritional supplement fed. Mean levels of ascorbate, mg/g dry matter (standard deviation in parentheses), were as follows: adrenal 6.46 (4.46); liver 1.30 (0.53); kidney 0.78 (0.32); skeletal muscle 0.73 (0.46); and cardiac muscle 0.70 (0.39). This lack of any consistent relation between the occurrence of dystrophy and tissue levels of ascorbic acid suggests that ascorbic acid metabolism was not profoundly affected by muscular dystrophy.

Rumen development did not affect ($P > 0.05$) tissue ascorbate concentrations either. Evidence indicating that rumen development was successfully manipulated as intended is presented elsewhere (deMille et al. 1972). The level of ascorbic acid in the basal diet is probably extremely low. This finding does not, therefore, refute the idea that ascorbic acid is metabolized in the rumen (Knight et al. 1941). It is, however, unlikely that significant amounts of ascorbic acid are recycled from the host tissues to the rumen, or that metabolism in the host tissues that is generally associated with rumen function profoundly affects ascorbate metabolism.

The authors thank Mr. L. McKeown for technical assistance. Financial support was provided by the National Research Council of Canada (Grant no. A5934)

Can. J. Anim. Sci. 52(2), 441-3 (Eng.) 1972

1890

NOTES

ASCORBIC ACID METABOLISM IN LAMBS AND THE EFFECTS OF VITAMIN E OR ETHOXYQUIN AND RUMEN DEVELOPMENT

Ascorbic acid (vitamin C) is not recognized as an essential nutrient for sheep (National Academy of Sciences 1968). Although Hjaire and Lilleengen (1936) reported that the compound could prevent enzootic nutritional muscular dystrophy in these animals, others found it was ineffective (Willman et al. 1940). If effective, the role of ascorbic acid may be as an antioxidant and as such Blaxter et al. (1953) described it as partially effective compared with vitamin E. These workers tested ascorbic acid in calf diets that contained unsaturated fat. Although there are some data on ascorbic acid supplementation of sheep diets, few are apparent for ascorbic acid metabolism in sheep. The purpose of this report is to present data on the effects of muscular dystrophy and rumen development on ascorbic acid levels in lamb tissues.

The basal diet consisted of torula yeast (50%), glucose (35.7%), lard (10%), minerals, and vitamins to provide a complete ration except for vitamin E (deMille et al. 1972). Twenty-seven crossbred lambs (ewes and wethers) that were weaned at 1 week of age were fed the basal diet supplemented with vitamin E (68 ppm as α-tocopheryl acetate) or ethoxyquin (0.01 or 0.1 ppm). The lambs were fed the diets as liquid or pellets, thus constituting a 2 × 3 factorial arrangement of treatments (four–five lambs per treatment). The pelleted diets contained solka-floc (10%) and cornstarch (15%) replacing glucose. Blood samples in EDTA were collected weekly from the 3rd week on trial and taken from either the orbital plexus or jugular vein. Samples of other tissues were taken at slaughter from surviving lambs after 12 weeks on trial. Other details regarding the experimental procedures are given elsewhere (deMille et al. 1972). Ascorbic acid was analyzed in whole blood and other tissues using the 2,4-dinitrophenylhydrazine procedure of Roe and Oesterling (1944). Tests of significance were performed by analyses of variance (Steel and Torrie 1960).

Plasma ascorbic acid values as affected by vitamin E or ethoxyquin are presented in Fig. 1. Wide weekly fluctuations of blood ascorbate found in these sheep were also apparent in a previous report on blood ascorbate in goats (Richmond et al. 1940). Although these fluctuations have been observed, no explanation for them has been forthcoming. It is our opinion that they probably reflect rather loose control of the ascorbic acid biosynthetic mechanism. This would also suggest that rigid control of ascorbic acid metabolism may not be essential to the animal's well-being. The blood concentration of ascorbate appears to be similar in these two species.

At the 8th week, ascorbic acid was higher ($P < 0.05$) in vitamin E-supplemented than ethoxyquin-supplemented animals. However, this pattern was not consistently observed and values at the 9th and 11th week were higher in lambs fed 0.1% ethoxyquin than in lambs from the other two groups. Lambs fed ethoxyquin, particularly at the 0.01% level, were dystrophic by the conclusion of this experiment (deMille et al. 1972), which indicates muscular dystrophy did not

QUADRO VII

Evolução do peso de ratos alimentados com dieta com 10 % de ácido ascórbi e 10 % de bicarbonato de sódio

Rato / Peso g	1	2	3	4	5	6
Inicial	109	65	73	80	55	67
Aos 3 dias	87	54	61	66	48	59
Aos 6 dias	85	Morreu	60	52	Morreu	55

QUADRO VIII

Evolução do peso de ratos alimentados com dieta com 2,5 % de ácido cítri

Rato / Peso g	1	2	3	4	5	6
Inicial	75	80	70	80	60	70
Aos 3 dias	65	72	60	75	55	70
Aos 6 dias	70	80	60	75	55	72
Aos 9 dias	80	90	70	85	65	86

BIBLIOGRAFIA

(1) CARVALHO GUERRA, F. J. A.: Tumefacção Mitocondrial, Tese Dout. Farm., Por 1964.

(2) CHAPPEL, J. B.: Integrated Oxidations in Isolated Mitochondria — Biologic Structure and Function. Vol. II, Ed. Academic Press 1961.

(3) STEPP; KÜHNAU e SCHROEDER: As Vitaminas e Seu Emprego Terapêutico. — Ed. Me lhoramentos, São Paulo.

(4) VELÁZQUEZ, B. LORENZO: Terapeutica con sus fundamentos de Farmacologia Exper mental 7.ª Ed., Ed. Cientifico-Medica, Barcelona.

(Trabalho realizado no Centro de Estudos de Bioquím da Faculdade de Farmácia do Porto)

QUADRO V

Evolução do peso de ratos alimentados com dieta com 10 % de ácido ascórbico

Rato / Peso g	1	2	3	4	5	6
Inicial	60	65	68	65	65	80
Aos 3 dias	52	64	68	52	64	80
Aos 6 dias	Morreu	69	67	Morreu	57	69

QUADRO VI

Evolução do peso de ratos alimentados com dieta com 5 % de ácido ascórbico e 5 % de bicarbonato de sódio

Rato / Peso g	1	2	3	4	5	6
Inicial	98	75	80	75	80	65
Aos 2 dias	80	60	62	60	65	55
Aos 4 dias	65	53	55	—	57	45
Aos 6 dias	57	45	45	52	Morreu	Morreu
Aos 8 dias	Morreu	Morreu	Morreu	Morreu	—	—

1863

QUADRO III

Evolução do peso de ratos alimentados com dieta com 1 % de ácido ascórbi

Rato / Peso g	1	2	3	4	5	5
Inicial	80	80	80	80	80	80
Aos 3 dias	80	85	78	80	80	78
Aos 6 dias	85	85	87	85	83	87
Aos 9 dias	90	90	90	90	95	95

QUADRO IV

Evolução do peso de ratos alimentados com dieta com 5 % de ácido ascórbi

Rato / Peso g	1	2	3	4	5	6
Inicial	85	75	85	75	85	55
Aos 3 dias	80	75	82	70	80	55
Aos 6 dias	75	75	90	70	80	55
Aos 9 dias	85	80	100	68	84	61

QUADRO I

Evolução do peso de ratos alimentados com dieta normal

Rato / Peso g	1	2	3	4	5	6
Inicial	72	70	80	65	70	65
Aos 3 dias	90	85	87	88	100	83
Aos 6 dias	100	100	104	100	117	100

QUADRO II

Evolução do peso de ratos alimentados com dieta normal, pulverizada

Rato / Peso g	1	2	3	4	5	6
Inicial	65	75	65	85	70	90
Aos 3 dias	70	70	70	80	70	90
Aos 6 dias	70	67	70	80	75	95
Aos 9 dias	70	68	80	82	80	105

mal, occorrendo a morte por paralisia respiratória e cardíaca. Pela nossa parte injectamos, no rato, por via intravenosa, doses superiores a 4 mg por grama sem consequências fatais.

Os resultados da administração de Vitamina C por via oral encontram-se distribuídos nos quadros III, IV, V, VI e VII. Nos quadros I e II inscrevemos a evolução de animais alimentados normalmente, com a particularidade de uma série ter recebido o alimento pulverizado. Esta atitude justifica-se na medida em que sempre que se adicionaram fármacos aos alimentos, estes foram prèviamente reduzidos a pó para assegurar um boa distribuição. Admitindo, por outro lado, a possibilidade de os efeitos que verificamos terem por base fenómenos de acidez, submetemos um lote de animais a uma dieta enriquecida em ácido cítrico (quadro VIII) e em duas séries com ácido ascórbico adicionamos bicarbonato de sódio à água. Como alimentação básica utilzamos uma dieta composta 41-B Inecil e água, sem restrições.

Não consideramos os resultados dos presentes ensaios suficientes para se poderem tirar conclusões causais. Entretanto, permitem algumas observações curiosas:

— os animais alimentados com as «rolhas» reduzidas a pó acusam um desenvolvimento inferior aos que receberam o produto em fragmentos volumosos. Uma vez que a constituição da dieta não variou em consequência da pulverização, só encontramos como explicação para o fenómeno a maior dificuldade que os ratos encontrem em tomar o alimento;

— pequenas doses de ácido ascórbico (da ordem do 1 %) não tem efeitos desfavoráveis sobre o desenvolvimento dos ratos; com 5 % o peso cai inicialmente, mas mostra, depois, tendência para a recuperação e com 10 % os animais sofrem uma deterioração física notória que os pode levar à morte ao fim de poucos dias. Deve assinalar-se que não houve quebra de apetite, pelo menos enquanto o estado físico não ficou sèriamente perturbado; pelo contrário, estes animais ingeriram, em média, quantidades de alimento superiores aos animais normais;

— a administração simultânea de bicarbonato de sódio aos animais tratados com ácido ascórbico teve, nas dosagens utilizadas, um efeito marcadamente prejudicial;

— a adição de 2,5 % de ácido cítrico aos alimentos levou a uma estagnação ou mesmo quebra, de peso dos ratos nos primeiros dias. Ao fim de uma semana de tratamento e mantendo-se as mesmas condições alimentares, os animais mostraram tendência para normalizar o desenvolvimento. Conclusão semelhante se pode tirar dos animais alimentados com 5 % de ácido ascórbico.

A autópsia não mostrou, em qualquer grupo de animais, sinais de lesão visceral macroscòpicamente visível. Há, entretanto, nos animais tratados com doses elevadas de ácido ascórbico, e sobretudo nos que receberam simultâneamente bicarbonato de sódio, liquefacção do conteúdo cólico acompanhada de distensão gasosa. Este achado concorda com a tendência para a dejecção de fezes líquidas que se verifica nestes animais. Um efeito laxativo e perturbações da absorção dos alimentos poderiam, de resto, estar na base dos fenómenos de alteração de desenvolvimento descritos. É uma hipótese que tencionamos esclarecer.

Rev. Port. Farm. 20(2): 41-46

1863

VOL. XX ★ 1970 ABRIL-JUNHO ★ N.º 2

TRABALHOS ORIGINAIS

ENSAIOS SOBRE A TOXICIDADE DO ÁCIDO ASCÓRBICO (*)

A. DE ALBUQUERQUE e M. ARMANDA HENRIQUES

No decurso de ensaios de tumefacção de mitocondrias de fígado de rato segundo técnica descrita por Carvalho Guerra [1], associando o estudo polarográfico do processo respiratório com o eléctrodo de Clark [2], a nossa atenção foi chamada para a possibilidade de se verificarem alterações no animal vivo que pudessem ser imputadas a um efeito tóxico do ácido ascórbico.

A observação, no microscópio electrónico, de fragmentos de fígado e supra-renal de ratos alimentados com doses elevadas de Vitamina C, ou injectados com fortes proporções do fármaco, mostrou alterações que parecem justificar uma interessante linha de trabalho. Foi precisamente o primeiro tipo de ensaios que, ao revelar perturbações do comportamento e do desenvolvimento dos animais, deu lugar à apresentação desta nota.

É raro encontrar na literatura referências a ensaios de toxicidade do ácido ascórbico; se aquela é pródiga na descrição de fenómenos de carência, outrotanto não acontece quando se trata de consequências de hiperdosagem. Na realidade, esta vitamina pode considerar-se como não tóxica. Dentro das poucas observações que a este respeito encontramos podemos referir as de STEPP [3], e de VELASQUEZ [4]. Este último autor descreve síndromes de hiperdosagem e cita ARMIJO que estabeleceu, no ratinho, uma dose letal de cerca de 3,7 mg por grama de ani-

(*) Trabalho apresentado às VII Jornadas Farmacêuticas.

Bibliography

1. Carvalho Guerra F.J.A. Mitochondrial tumefaction, Doctoral Pharmaceutical Thesis (dissertation), Porto, 1964 (in Portuguese).

2. Chappel, J.B. Integrated Oxidations in Isolated Mitochondria - Biologic Structure and Function. Vol. II, Ed. Academic Press 1961.

3. Stepp, Kuhnau and Schroeder. Vitamins and their therapeutic use, Ed. Melhoramentos, Sao Paulo (in Portuguese).

4. Velasquez B. Lorenzo. Therapeutics with its foundations of experimental pharmacology, 7th ed. Ed. Cientifico-Medica, Barcelona (in Spanish).

Table 5 - Weight changes of rats fed a diet containing 10% ascorbic acid

Weight, g \ Rat	1	2	3	4	5	6
Initial	60	65	68	65	65	80
After 3 days	52	64	68	52	64	80
After 6 days	Died	69	67	Died	57	69

Table 6 - Weight changes of rats fed a diet containing 5% ascorbic acid and 5% Na Bicarbonate

Weight, g \ Rat	1	2	3	4	5	6
Initial	98	75	80	75	80	65
After 2 days	80	60	62	60	65	55
After 4 days	65	53	55	--	57	45
After 6 days	57	45	45	52	Died	Died
After 8 days	Died	Died	Died	Died	--	--

Table 7 - Weight changes of rats fed a diet containing 10% ascorbic acid and 10% Na bicarbonate

Weight, g \ Rat	1	2	3	4	5	6
Initial	109	65	73	80	55	67
After 3 days	87	54	61	66	48	59
After 6 days	85	Died	60	52	Died	55

Table 8 - Weight changes of rats fed a diet containing 2.5% citric acid

Weight, g \ Rat	1	2	3	4	5	6
Initial	75	80	70	80	60	70
After 3 days	65	72	60	75	55	70
After 6 days	70	80	60	75	55	72
After 9 days	80	90	70	85	65	86

Table 1 - Weight changes of rats fed a normal diet

Weight, g \ Rat	1	2	3	4	5	6
Initial	72	70	80	65	70	65
After 3 days	90	85	87	88	100	83
After 6 days	100	100	104	100	117	100

Table 2 - Weight changes of rats fed a pulverized normal diet

Weight, g \ Rat	1	2	3	4	5	6
Initial	65	75	65	85	70	90
After 3 days	70	70	70	80	70	90
After 6 days	70	67	70	80	75	95
After 9 days	70	68	80	82	80	105

Table 3 - Weight changes of rats fed a diet containing 1% ascorbic acid

Weight, g \ Rat	1	2	3	4	5	6
Initial	80	80	80	80	80	80
After 3 days	80	85	78	80	80	78
After 6 days	85	85	87	85	83	87
After 9 days	90	90	90	90	95	95

Table 4 - Weight changes of rats fed a diet containing 5% ascorbic acid

Weight, g \ Rat	1	2	3	4	5	6
Initial	85	75	85	75	85	55
After 3 days	80	75	82	70	80	55
After 6 days	75	75	90	70	80	55
After 9 days	85	80	100	68	84	61

we have added sodium bicarbonate to the water. As a basic food, we used a diet consisting of 41-B Inecil and water, in an unlimited amount.

We do not consider the results of the present tests sufficient to allow us to draw any causal conclusions. In the meantime, we are permitted to make some curious observations.

Animals which were fed "pellets" reduced to a powder showed a development inferior to that of animals receiving a product consisting of voluminous fragments. Since the composition of the diet does not vary as a result of pulverization, the only explanation we can provide for this phenomenon is the greater difficulty which the rats have in ingesting their food.

Small doses of ascorbic acid (of the order of 1%) do not exert any unfavorable effects on the development (growth) of rats; with 5% the weight drops initially, but later shows a tendency to restore itself, and with 10% the animals suffer a substantial physical deterioration which can result in death within a few days. It must be pointed out that there is no loss of appetite as long as the physical condition of the animals does not become seriously disturbed; on the contrary, these animals ingest, on the average, amounts of food superior to those taken by normal animals.

The simultaneous administration of sodium bicarbonate to animals treated with ascorbic acid has, in the dosage used, a clearly adverse (detrimental) effect.

Addition of 2.5% citric acid to the food leads to a stoppage or even a decrease of the weight of rats during the first few days. At the end of one week, when the animals are kept under the same feeding conditions, their growth shows a tendency to become normal. Similar conclusions can be drawn in regard to animals fed a diet with 5% ascorbic acid.

Autopsy did not disclose, in any group of animals, any macroscopically visible signs of visceral injury. However, a liquefaction of the bowel (colon) contents accompanied by gaseous distention was noted in animals treated with high doses of ascorbic acid, and especially in those animals which received simultaneously sodium bicarbonate. This finding agrees with the tendency to excrete liquid feces which was found in these animals. A laxative effect and disturbances in the absorption of food may, moreover, constitute the basis of the altered growth phenomena described here. This is a hypothesis which we intend to clarify.

Source: Revista Portuguesa de Farmacia 1970, 20(2): 41-46

1863

Tests on the Toxicity of Ascorbic Acid

A. de Albuquerque, M. Armanda Henriques (Center of Biochemical Studies of the Porto Pharmaceutical Faculty).

In the course of tests on the tumefaction of rat liver mitochondrias according to the technique described by Carvalho Guerra (1), associated with a polarographic study of the respiratory process with the Clark (2) electrode, our attention was directed to the possibility of checking alterations in a living animal which could be attributed to the toxic effect of ascorbic acid.

Electron microscope observations of sections of the liver and adrenal glands of rats fed high dosages of vitamin C or injected with large amounts of this drug showed the presence of alterations which would appear to justify an interesting line of study. It was precisely the first type of tests (oral feeding of vitamin C), which disclosed disturbances in the behavior and development of animals, that prompted the presentation of this report.

References to tests on the toxicity of ascorbic acid are seldom found in the literature; the latter contains numerous descriptions of deficiency phenomena, but the situation is different in regard to the after effects (consequences) of vitamin overdosage. In fact, this vitamin can be considered as nontoxic. Among the few observations encountered in this respect, we can refer to those of Stepp (3) and Velasquez (4). The latter author describes overdosage syndromes and cites Armijo who established a lethal dose of about 3.7 mg/g for small rats or mice, with death occurring as a result of respiratory and cardiac paralysis. On our part, we have injected rats intravenously with doses superior to 4 mg/g without any fatal results.

The results obtained after oral administration of vitamin C are listed in Tables III-VII. The development (growth) of animals fed a normal diet, and particularly of an animal series receiving a pulverized diet, is shown in Table I and II. This approach is justified to the extent that when drugs are added to food, the latter is always reduced in size in order to ensure a good distribution of the drugs in the food. On the other hand, while acknowledging the possibility of effects which we will have to check on the basis of acidity phenomena, we have submitted a batch of animals to a diet enriched with citric acid (Table VIII), and in two series of tests with ascorbic acid

1839

5. HOFFBRAND, A. V., B. F. A. NEWCOMBE AND D. L. MOLLIN. Method of assay of red cell folate activity and the value of the assay as a test for folate deficiency. J. Clin. Pathol. 19: 17, 1966.
6. SPRAY, G. H. An improved method for the rapid estimation of B_{12} in serum. Clin. Sci. 14: 661, 1955.
7. DENSON, K. W., AND E. F. BOWERS. The determination of ascorbic acid in white blood cells. Clin. Sci. 21: 157, 1961.
8. International Committee for Standardisation in Haematology. Proposed recommendations for measurement of serum iron in human blood. Brit. J. Haematol. 20: 451, 1971.
9. WEICHSELBAUM, T. E. An accurate and rapid method for the determination of proteins in small amounts of blood serum and plasma. Am. J. Clin. Pathol. 10: 40, 1946.
10. Report of a Joint FAO/WHO Expert Group. Requirements of ascorbic acid, vitamin D, vitamin B_{12}, folate and iron. World Health Organ. Tech. Rept. Ser. No. 452. Geneva, 1970.
11. GRIFFITHS, L. L. Biochemical findings of the Farnborough survey. In: Vitamins in the Elderly, edited by A. N. Exton-Smith and D. L. Scott. Bristol: John Wright and Sons Ltd., 1968, p. 34.
12. PEARSON, W. N. The evaluation of nutrition status in population groups. In: Modern Nutrition in Health and Disease (4th ed), edited by M. G. Wohl and R. S. Goodhart. Philadelphia: Lea & Febiger, 1968, p. 578.
13. WAPNICK, A. A., T. H. BOTHWELL AND H. SEFTEL. The relationship between serum iron levels and ascorbic acid stores in siderotic Bantu. Brit. J. Haematol. 19: 271, 1970.
14. CHANARIN, I. The Megaloblastic Anaemias. Oxford: Blackwell, 1969, p. 320.
15. HO, K.-J., K. BISS, B. MIKKELSON, L. A. LEWIS AND C. B. TAYLOR. The Masai of East Africa: some unique biological characteristics. Arch. Pathol. 91: 387, 1971.
16. HOLMES, E. G., M. W. STANIER AND M. D. THOMPSON. The serum protein pattern of Africans in Uganda: relation to diet and malaria. Trans. Roy. Soc. Trop. Med. Hyg. 49: 376, 1955.
17. LEONARD, P. J., AND A. G. SHAPER. Serum proteins in African and Asian subjects in Kampala, Uganda. E. African Med. J. 42: 689, 1965.
18. Great Britain, Medical Research Council, Accessory Food Factors Committee. Vitamin C Requirements of Human Adults. Special Rept. Ser. No. 280. London: H. M. Stationery Office, 1953.

for plasma ascorbate be accepted as 0.2 mg/100 ml, then that for leukocyte ascorbate should be 15 $\mu g/10^8$ WBC. Wapnick et al. (13) have shown that nonsiderotic Bantu people in southern Africa may have leukocyte values as low as 12 $\mu g/10^8$ WBC without evidence of either scurvy or anemia. Our experimental data on the characterization of anemia in baboons with ascorbate deficiency show that scurvy is not the sole pathological process produced by reduced stores of the vitamin, and that at reduced dietary intakes of ascorbic acid, ferrokinetic abnormalities may be present in the absence of scurvy or anemia (Gatenby Davies and Newson, unpublished observations). Should these Masai show evidence of normal ferrokinetics, then it would be reasonable to suppose that the ascorbate levels found in this study were adequate under the circumstances.

The slight reduction in folate levels of the Masai group compared with the nonpastoral group might be expected from the low consumption of vegetable foodstuffs, though adequate quantities of folate should be available from their milk staple, irrespective of how this is consumed. The mean folate levels of both groups at Kajiado are low by comparison with those found in people living in developed countries (14). The elevated serum B_{12} levels in the Masai group may be associated with the high intake of animal protein.

High serum protein levels have been noted before in Masai and have been attributed to an increase in the IgA fraction of the immunoglobulins (15). The nonpastoral Bantu group also showed higher mean levels than previously reported from East Africa (16, 17).

The dietary habits of pastoral tribes differ considerably from those of agriculturists in Kenya, many of whom subsist upon a monoculture diet consisting of maize. Milk is the staple of the Masai, as it is of pastoralists generally in Kenya, and it is drunk in large quantities. A moran may consume two to three liters at a sitting, twice daily, although the mean consumption among women and elders is probably close to three liters daily. However, milk is not plentiful throughout the year, and during the drier months, meat supplemented by blood is consumed besides.

Cattle are milked directly into dried vegetable gourds which are seldom cleaned, except to be rinsed in cattle urine or water if available, inverted to dry, and then scoured by the insertion of a flaming branch. Milk may be drunk either fresh or fermented, boiled or unboiled, though by preference after fermentation. Filled gourds are left in the darkened corner of a enkaji (hut) for at least 3 days to ferment. The data in Table 3 show that little ascorbate may be consumed daily, despite the adequate initial levels in fresh milk.

A daily intake of approximately 10 mg ascorbic acid will prevent scurvy in most individuals (18), but a higher intake is considered to be desirable because of the biological variation between individuals. WHO (10) recommends a daily intake of 30 mg for the normal adult of either sex. The Masai in this study, however, appear to have a daily intake well below this without the appearance of either clinical scurvy or anemia. Further work is being undertaken to elucidate the biological significance of these ascorbate levels in the Masai of Kenya.

Summary

Masai on a traditional diet showed significantly lower plasma and leukocyte ascorbate levels than a comparable nonpastoral group of Bantu living on a mixed diet in the same area. Masai had lower red cell folate levels, and higher serum B_{12} and total protein levels. Neither scurvy nor anemia was present.

We are grateful to the Ministry of Health, Kenya, and in particular to Dr. G. Montfrans and his staff at Kajiado District Hospital, for permission to undertake this study at Kajiado. We thank Professor P. Hill of the Faculty of Medicine, University of Nairobi, for permission to investigate patients under his care at the Kenyatta National Hospital, Nairobi, and Miss P. Lall of the Wellcome Trust Research Laboratories for the folate and B_{12} assays.

References

1. WHO/FAO/UNICEF. Nutrition survey and campaign against malnutrition in Kenya, 1964-1968. Kenya Government Printer, 1969, p. 170.
2. BOTHWELL, T. H., B. A. BRADLOW, P. JACOBS, K. KEELEY, S. KRAMER, H. SEITEL AND S. ZAIL. Iron metabolism in scurvy with special reference to erythropoiesis. Brit. J. Haematol. 10: 50, 1964.
3. Food and Agriculture Organization. Food Consumption Table for Use in Africa. Food Agr. Organ., Rome, 1968.
4. HERBERT, V. The assay and nature of folic acid activity in human serum. J. Clin. Invest. 40: 81, 1961.

mean for the Kenyatta National Hospital (KNH) inpatients is not significantly different from that of the Kajiado nonpastoral Bantu group. The regression of mean leukocyte ascorbate (x) on mean plasma ascorbate (y) is given by the equation $x = 38.7y + 7.4$.

Table 2 gives the data for four individuals whose classification by diet differed from that of their ethnic origin, comparing those parameters which differed between the two groups. Their parameters reflect their diet more closely than their ethnic origin.

No clinical evidence of scurvy was seen in any individual at Kajiado. Perifollicular hemorrhages are the best indicators of scurvy in individuals, but are difficult to assess readily in pigmented peoples. Thus, our reliance on gingival changes.

The data obtained from the ascorbate analysis of cow's milk (Zebu, *Bos indicus*) under specific conditions are given in Table 3. Approximately 80% of the ascorbate content is lost from milk that is left to ferment for 4 days.

Discussion

The biochemical parameters obtained from the two groups of Kajiado (Table 1) substantiate the classification by diet; furthermore, evidence from Table 2 is suggestive that the ascorbate levels are unlikely to be due to genetic factors. Our results for the nonpastoral group at Kajiado, and also for the sample of inpatients at KNH, show that at least certain sectors of the indigenous population in Kenya may have ascorbate levels similar to those of people living in temperate climates.

The generally accepted lower figure of normality for plasma ascorbate is 0.2 mg/100 ml (10), and for leukocyte ascorbate 24 $\mu g/10^8$ WBC (11). Plasma levels below 0.2 mg/100 ml are associated with low hemoglobin values (12). However, the subjects in the Masai group did not show evidence of scurvy nor of anemia at mean plasma levels of 0.16 mg/100 ml and leukocyte levels of 13.7 $\mu g/10^8$ WBC.

Figure 1 shows that low plasma ascorbate levels reflect low leukocyte levels, and therefore stores. Furthermore, the narrower 95% confidence limits for the Masai reflect a more constant daily intake of ascorbic acid at that time of year than that of the nonpastoral groups. Examination of the regression line indicates that if the lower figure of normality

TABLE 3
Ascorbate content of milk treated in different ways, and the potential daily ascorbate intake

Milk sample	Ascorbic acid content, mg, from: 100 ml	2 liters	3 liters
Commercial whole milk:[a] Pasteurized, homogenized	1.8	36	44
Fresh Masai cow's milk			
Milked into sterile beaker	2.2	44	66
Milked into gourd, mixed, sampled into sterile beaker	2.2	44	66
Milked into gourd, left to stand in hut 12 hrs (unclotted)	1.6	32	48
Milked into gourd, left to stand in hut 4 days (clotted)	0.4	8	12

[a] Kenya Co-operative Creameries, P.O. Box 30131, Nairobi.

TABLE 2
Data on four individuals according to diet and ethnic group

	Group mean		Individual values			
Ethnic group	Masai	Bantu	Bantu	Masai	Masai	Masai
Diet	Masai	Mixed	Masai	Mixed	Mixed	Mixed
Sex	Both	Both	♀	♂	♂	♀
Plasma ascorbate, mg/100 ml	0.16	0.57	0.14	0.29	1.38	0.38
WBC ascorbate, $\mu g/10^8$ WBC	13.7	29.0	12.2	18.3	28.7	56.9
Serum folate, ng/ml	5.5	6.1	7.6	7.8	4.4	2.5
RBC folate, ng/ml	297	404	331	293	360	234
Serum B_{12}, pg/ml	1,188	607	786	682	360	634
Serum protein (total), g/100 ml	8.4	7.9	10.2	8.4	8.0	7.4

Plasma and leukocyte ascorbate levels in blood were determined by the method of Denson and Bowers (7). Plasma samples were separated and stabilized with 5% trichloroacetic acid within 0.5 hr of taking the blood, and leukocyte samples within 1 to 2 hr as soon as sedimentation was complete. Samples were kept at 4 C until assayed the following day. Determination of ascorbate content of cow's milk differed from the method for plasma. Samples were kept at 4 C for 12 hr before being stabilized with 5% metaphosphoric acid. After extraction, the supernatants were filtered, then the color reagent was added and the tubes incubated in the usual way. The ascorbic acid osazones were dissolved in 85% sulfuric acid (the stronger acid being necessary to decompose other interfering substances) and allowed to stand for 0.5 hr. Then each sample was read at 520 nm against its own milk blank, which was prepared in exactly the same way as the samples except that the color reagent was not added until after the 85% sulfuric acid. Assays were made in quadruplicate from single samples of milk.

Serum iron was determined using bathophenanthroline sulfonate as the color reagent (8). Total serum protein was determined by the biuret method (9).

Results

As there were no statistically significant differences between the sexes, data were pooled in order to compare the Masai and Bantu groups (Table 1). Comparisons between means were carried out by Student's t test. Highly significant differences ($P < 0.001$) were found for both plasma and leukocyte ascorbate, and significant differences for serum B_{12} and total protein ($P < 0.01$), and red cell hemolysates ($P < 0.02$). Despite their low ascorbate levels, the Masai group showed comparable hematological values to the nonpastoral Bantu group, and their serum iron levels also were not significantly different. Age can be discounted as a significant variable in these comparisons.

The ascorbate levels of the two groups differ in two respects (Fig. 1), firstly in their absolute values, and secondly in their ranges, as the variance ratios were significantly different for plasma ascorbate ($P < 0.001$) and for leukocyte ascorbate ($P < 0.002$). The mean for the Masai group has narrower limits than the others. The

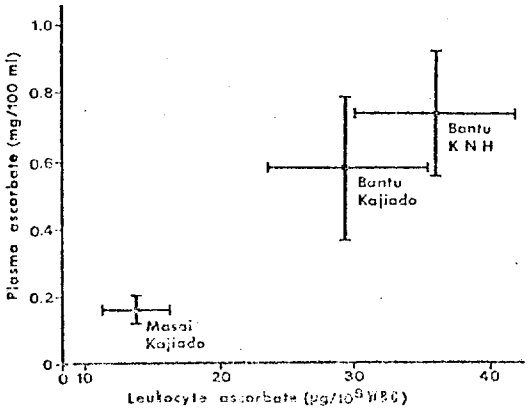


FIG. 1. Relationship between plasma and leukocyte ascorbate levels: population means with their 95% confidence limits.

TABLE 1
Means and SD for hematological and biochemical parameters for Masai (n = 21) and Bantu (n = 24) dietary groups

Parameter	Masai Mean	Masai SD	Bantu Mean	Bantu SD	Significance[a]
Age, years	33.9	13.5	32.6	13.5	NS
Hemoglobin, g/100 ml	14.8	1.6	15.1	1.8	NS
RBC, $\times 10^6/mm^3$	4.92	0.54	5.08	0.51	NS
MCV, μ^3	87	4	88	7	NS
PCV, %	42.8	4.5	44.5	4.6	NS
MCHC, %	34.4	0.8	33.9	1.4	NS
MCH, pg	30.0	1.8	29.9	3.1	NS
Platelets, $\times 10^3/mm^3$	222	56	236	60	NS
Leukocytes, $/mm^3$	7,072	2,479	7,101	2,219	NS
Plasma ascorbate, mg/100 ml	0.16	0.08	0.57	0.49	++
WBC ascorbate, $\mu g/10^8$ WBC	13.7	5.8	29.0	12.9	++
Serum iron, μg/100 ml	111	43	123	62	NS
Serum folate, ng/ml	5.5	2.2	6.1	2.7	NS
RBC folate, ng/ml	297	80	404	138	+
Serum B_{12}, pg/ml	1,188	943	607	322	+
Serum protein (total), g/100 ml	8.4	0.8	7.9	0.6	+

[a] The final column gives statistical significance levels of differences between the means of the two groups (NS = not significant; + = $P < 0.02$; ++ = $P < 0.001$)

Clin. Nutr. 27(3): 310-314

1839

editor: Robert B. Bradfield, Ph.D.

international nutrition

Low ascorbate status in the Masai of Kenya[1]

J. D. Gatenby Davies,[2] M.B., B.S., and Janet Newson,[3] B.A., D.Phil.

Comparatively little information is available from developing countries of the tropics on the prevalence of scurvy among indigenous peoples, and even less on the laboratory evidence of ascorbate deficiency. Nutritional surveys conducted in different parts of Kenya by the WHO/FAO/UNICEF group (1) provided evidence of a low intake of ascorbic acid in many of the families concerned. Biochemical studies were not undertaken. Pastoral tribes were not evaluated in these surveys as they were considered to represent a small percentage of the whole population and to suffer from few nutritional problems.

That there is an association between ascorbic acid and iron metabolism is known (2). Moreover, preliminary work on ascorbate deficiency in baboons has shown that at low ascorbate levels, ferrokinetic abnormalities may be present before either clinical scurvy or anemia are apparent (Gatenby Davies and Newson, unpublished observations). As pastoral tribes living in arid areas on traditional diets of milk, sometimes supplemented by blood or meat, would be expected a priori to have low intakes of ascorbic acid, we considered that the findings on a lack of ascorbate in baboons might have relevance to such people.

To assess the ascorbate status of a typical pastoral tribe, a sample of Masai, a Nilo-Hamitic seminomadic tribe of Kenya and Tanzania, were examined at Kajiado, 75 km south of Nairobi. A comparable sample, drawn from nonpastoral Bantu people eating a mixed diet and living in the same area, was also investigated.

The study included routine hematological parameters, and estimations of ascorbate, folate, B_{12}, iron, and total protein. No detailed clinical studies were undertaken, and only gingival changes as evidence of scurvy were sought.

Subjects and methods

Selection of subjects

Kajiado District Hospital. Twenty-three pastoral Masai and twenty-two people from nonpastoral Bantu tribes attending either as outpatients or visitors were sampled between mid-November 1972 and mid-January 1973 during the dry season. No selection was involved in sampling other than that approximately equal numbers were obtained in each group, and that individuals were willing to cooperate.

All individuals were questioned and examined by one of us and their approximate age was assessed. It was decided that the criterion for placing people in either group should be dietary rather than ethnic. Thus, three Masai who had lived on a nontraditional mixed diet for many years were placed in the nonpastoral group, and one elderly Kikuyu woman who had lived as a Masai from the age of 10 years was classified as a Masai. Thus, although 23 Masai and 22 nonpastoral Bantu people were sampled, the dietary classification gives 21 in the Masai group and 24 in the Bantu group. All were adults. Four Masai and one non-Masai woman were known to be pregnant. Masai were either women or elders; no moran (warriors) were included.

Kenyatta National Hospital, Nairobi. Twenty-three inpatients from nonpastoral Bantu tribes, all of whom were eating a balanced diet containing an estimated mean ascorbic acid intake of 75 mg daily (3), were sampled from medical beds during October 1972. The criteria for selection were that individuals were eating well, had been in the hospital for a minimum of 5 days, and were not considered to have either generalized neoplasia or infection. All were adults. None was known to be pregnant.

Hematology and biochemistry

Erythrocyte and leukocyte counts were performed by Coulter counter, model Z_{BI}: Hemoglobin was determined by a Coulter hemoglobinometer using cyanmethemoglobin. Platelets were counted manually.

Folate levels in serum and red cell hemolysates were assayed using *Lactobacillus casei* (4, 5). Serum B_{12} levels were assayed using *L. leichmannii* (6).

---

[1] From the Wellcome Trust Research Laboratories, P.O. Box 43640, Nairobi, Kenya.

[2] Clinician, Director. Present address: Nairobi Laboratories, P.O. Box 45789, Nairobi, Kenya.

[3] Research Assistant.

2. The rate of healing, epithelialization and contraction of the wound were greatly retarded in animals receiving respectively 0·5, 0·7 or 1·0 mg. ascorbic acid daily. Severe adhesions to the base of the wound in these groups were noted. At the 2 mg. level healing was more normal, and phosphatase activity, though subnormal, was increased.

3. The optimum level of vitamin C as assessed by its action on rate of healing, differentiation and phosphatase activity, is not less than 5 mg. of ascorbic acid a day, and possibly even higher.

The authors are indebted to the Medical Research Council, by whom the expenses of the investigation were in part defrayed, and to Dr. A. Glücksmann for valuable suggestions and advice. They also wish to thank Mrs. Danielli and Mr. G. Winter, assistant at the Strangeways Research Laboratory, for their help with the histological technique, and Mr. S. Impey, assistant at the Dunn Nutritional Laboratory, for his care of the animals. The photomicrographs were taken by Mr. V. C. Norfield, Senior Assistant at the Strangeways Research Laboratory.

REFERENCES.

Biesele, J. J., and Biesele, M. M.—(1944) *Cancer Res.*, **4**, 751.
Bourne, G. H.—(1942) *Lancet*, ii, 661.—(1943) *Quart. J. exp. Physiol.*, **32**, 1.—(1944) *Lancet*, i, 688.—(1945) *Proc. Nutrit. Soc.* (in press).
Dann, L., Glücksmann, A., and Tansley, K.—(1941) *Brit. J. exp. Path.*, **22**, 1.
Fell, H. B., and Danielli, J. F.—(1943) *Ibid.*, **24**, 196.
Gomori, G.—(1939) *Proc. Soc. exp. Biol. N.Y.*, **42**, 23.—(1943) *Amer. J. Path.*, **19**, 197.
Harrer, C. J., and King, C. G.—(1941) *J. biol. Chem.*, **138**, 111.
Harris, L. J., and Oliver, M.—(1942) *Biochem. J.*, **36**, 155.
Hines, H. M., Larere, B., Thomson, J. D., and Cretzmeyer, C. H.—(1944) *J. Nutrit.*, **27**, 303.
Hunt, A. H.—(1941) *Brit. J. Surg.*, **28**, 436.
Kuether, C. A., Telford, I. R., and Roe, J. H.—(1944) *J. Nutrit.*, **28**, 347.
Russell, W. O., Rouse, E. T., and Read, J. A.—(1944) *Arch. Path.*, **38**, 40.
Shwachman, H., and Gould, B. S.—(1942*a*) *J. Nutrit.*, **23**, 271.—(1942*b*) *Amer. J. Physiol.*, **135**, 485.—(1943) *J. biol. Chem.*, **151**, 439.
Takamatsu, H.—(1939) *Trans. Soc. Path. Japan*, **29**, 492.
Wolbach, S. B.—(1933) *Amer. J. Path.*, Supp., **9**, 689.
Idem and Bessey, O. A.—(1942) *Physiol. Rev.*, **22**, 233.
Idem and Howe, P. R.—(1926) *Arch. Path.*, **1**, 1.

DISCUSSION.

In the normal guinea-pig, as in the rat, two peaks of phosphatase activity are seen during the healing of a wound, one due to the invading leucocytes, and a second which is associated with the differentiation of collagenous fibrous tissue. In vitamin C-deficiency the first peak is almost or quite as great as in the controls, but the size of the second peak depends on the degree of deficiency. Thus, in acute scurvy there is no second peak, and at different levels of partial deficiency the amount of phosphatase activity during the later stages of healing is correlated with the degree of differentiation of the scar. Our results show a marked contrast between the effect of 1 mg. of vitamin C per day, at which level differentiation is poor and phosphatase activity slight, and that of 2 mg., at which differentiation begins to approach the normal, and phosphatase activity, though much below that at higher levels of dosage, is considerable.

In our experiments the optimum level of vitamin C, as assessed by its beneficial action on rate of healing, on differentiation and on phosphatase activity, is not less than 5 mg., and possibly even higher. These findings agree well with the critical levels of vitamin C necessary for the repair of other types of tissue and for producing a scar of normal tensile strength (*cf.* Bourne, 1943, 1944; Hines, Larere, Thomson and Cretzmeyer, 1944; Kuether, Telford and Roe, 1944). The slightly lower level which some authors report as optimal is probably due to the fact that in our study the guinea-pigs were subjected to a preliminary period of acute deficiency to deplete their stores of vitamin C.

As regards the function of alkaline phosphatase in wound-healing, we suggested in our earlier paper "that the phosphatase is connected, directly or indirectly with the metabolic processes more intimately concerned in the laying down of collagen." The fact that at different levels of vitamin C-deficiency there is such a close correlation between the degree of differentiation of the scar tissue and its phosphatase activity is consistent with such a view. In this connection it is interesting that Wolbach and Bessey tentatively suggest that the mechanism by which ascorbic acid promotes the formation of collagenous intercellular substances "may be involved in the chemical mechanisms (enzymes) of the cells responsible for the synthesis of this protein product (collagen)."

Our results on the scorbutic animals, however, cannot be regarded as direct proof of a connection between alkaline phosphatase and the formation of collagen. It is conceivable that there may be no causal relationship between the absence of the enzyme and the poor differentiation of the collagen fibres, but that both phenomena may be due to some unknown third factor. For example, it is possible that the phosphatase in the wound may be supplied at an undiminished rate, but that for some reason it is not retained by the abnormal scorbutic scar tissue. A similar suggestion has been made by Bourne (1945) in connection with the healing of bone wounds in scurvy.

Alternatively there may be less phosphatase available in the scar, either because the cells of the regenerating tissue are producing less or, if the enzyme is derived from the blood stream, because the blood-phosphatase is lowered in scurvy (Shwachman and Gould, 1942*a*, *b*, and 1943), and the scars are poorly vascularized. Finally, it is not yet known whether this association between phosphatase activity and the regeneration of fibrous tissue exists in species other than rodents.

SUMMARY.

1. The effect of different degrees of vitamin C-deficiency has been studied on the rate of healing, phosphatase activity and differentiation of the scar tissue in small open skin wounds in guinea-pigs.

almost colourless, though there is sometimes a slight reaction round the proliferating hair follicles at the margin of the wound.

The histological character of the scar remains almost unchanged even 21–24 days after operation. The argyrophil network becomes more abundant than before, and the thick, irregular strands (Fig. 12), which are as plentiful as ever, assume a curious bloated appearance which makes them even more conspicuous than at an earlier stage. The scar still shows almost no phosphatase activity (Fig. 6). The much smaller, compact fibrous scars of the corresponding controls with their intense phosphatase reaction present a startling contrast to the vitamin C-deficient scars (*cf.* Figs. 5 and 6).

c. *The effect on healing of different levels of partial deficiency* (Experiments 3 and 4).

Observations were made on 16 guinea-pigs of the 0·5 mg. group, 2 of which were killed after 3 weeks, 10 after 1 month, and 4 after 5 weeks. The scar tissue closely resembles that of the acutely deficient animals except that the intercellular material is rather more abundant and less abnormal. After 4 and 5 weeks a few fine, wavy collagen fibres are present in addition to the irregular argyrophil network, and the thick strands, so conspicuous in acute deficiency, are much fewer and less swollen.

While some of the scars show very little phosphatase activity, others fixed after 4–5 weeks contain areas in which not only do the nuclei give a strong reaction, but the intercellular material also shows slight but definite activity. The tissue adjacent to the proliferating hair follicles and at the margin of the wound nearly always gives a reaction, which in some of the older wounds is intense and quite extensive. But although many of the wounds of the 0·5 mg. group give a stronger phosphatase reaction than we ever observed in acute deficiency, the reaction is nevertheless conspicuously weaker than at the highest levels of vitamin C, in which the fibrous scar is nearly black.

Wounds from 6 guinea-pigs of the 0·7 mg. group and 4 of the 1·0 mg. group were examined. All the animals of these and subsequent series were killed 28 days after operation. In histological appearance and phosphatase activity the scars of these groups (Figs. 7 and 13) show little improvement on the 0·5 mg. series.

The 4 scars of the 2·0 mg. group, however, are much nearer to the normal. They are smaller than those from the preceding groups, though usually larger than those from animals kept at the higher levels of vitamin C, the orientation of the fibroblasts is much more orderly than at the lower levels of vitamin C, and the intercellular fibres are more abundant and better differentiated (*cf.* Figs. 13 and 14); there is still some haemorrhage. The stout, irregular strands of precollagen seen in the previous series are absent. All four scars show considerable phosphatase activity (Fig. 8), which is intense immediately beneath the regenerated epidermis and rapidly diminishes towards the depths of the wound, where the nuclei only are dark and the intercellular material nearly colourless.

The three scars of the 5 mg. group are almost normal. As compared with those of the 2 mg. group they are more contracted, the intercellular fibres are thicker and more numerous, and phosphatase activity (*cf.* Figs. 8 and 9) is much more intense.

There was no significant histological difference between the four scars of the 10 mg. group and two which were examined from the group given 10 mg., but without a preliminary vitamin C-deficiency. As in the other series, there was some individual variation, but healing was advanced in all. The scars were much contracted, the cut surfaces of the old dermis being almost in contact at the base of some of the wounds, and were composed of well-developed fibrous tissue. The phosphatase reaction, though still intense in most of the scars, especially below the new epidermis, had probably passed its peak and was slightly less than in the 5 mg. group.

have also begun to invade the wound from the cut edge of the dermis. Fine, wavy collagen fibres taking the characteristic blue colour with Mallory's stain have appeared between the cells, and are largest and most deeply stained in the tissue proliferating from the cut surface of the dermis. This dermal outgrowth gives a reaction for phosphatase, which is intense immediately beneath the growing edge of the epidermis, but elsewhere only moderate. In the central mass of the scar only a few capillaries and the invading leucocytes, now greatly reduced in number, react strongly, but some of the fibroblastic nuclei show slight activity.

As healing proceeds the dermal and subcutaneous outgrowths become indistinguishably welded and the wound slowly shrinks. The wavy collagenous fibres increase in number and thickness, and stain more deeply with aniline blue and less with silver than at earlier stages of repair. Fibre-formation continues to be most advanced near the cut edge of the old dermis and beneath the regenerating epithelium. The epithelium spreads completely over the wound, and new hair follicles and sebaceous glands begin to develop round the margin of the scar. A progressive increase in phosphatase activity accompanies these histological changes. By the 16th day (Fig. 4) the whole scar gives a positive reaction, which is most intense at the margin of the wound, and particularly in the space which intervenes between the regenerated epithelium and the young dermis, where both the cells and the growing fibres are nearly black.

The scar continues to contract, and the cut surfaces of the original dermis converge funnel-wise from the epidermis towards the bottom of the wound, where after 3–4 weeks the sides are nearly in contact. The parallel layers of abundant, fairly thick collagen fibres which now fill the cavity of the wound give an intense phosphatase reaction (Fig. 5), except in the much contracted region at the base of the scar, where the activity is only moderate.

In the two wounds fixed 5 weeks after operation the phosphatase reaction, though still pronounced, is less than at 4 weeks, and had probably begun to decline as in the fully healed rat wound (Fell and Danielli, 1943).

B. *Healing in acute vitamin C-deficiency* (Experiments 1 and 2).

The intact skin of the deficient animals differs little from that of the normal: the sebaceous glands and hair follicles both give a positive reaction for phosphatase.

Wounds from 17 guinea-pigs suffering from acute deficiency were examined histologically at intervals of from 1–24 days after operation.

As in the controls, the subcutaneous tissue bulges into the cavity of the deficient wound, and during the first day becomes invaded with leucocytes and covered by a scab. Both leucocytes and scab give an intense phosphatase reaction.

The small subcutaneous bulge seen in the 1–3-day wounds rapidly enlarges into a dome-shaped mass of haemorrhagic, oedematous tissue which is very poorly vascularized. The intercellular material consists entirely of a scanty, irregular argyrophil network of fine fibres and coarse fibrin-like strands composed of fine fibres embedded in amorphous material (Fig. 11). Some of the fibres, especially in the depths of the wound, stain blue like collagen with Mallory's method, but much of the fibrous network remains colourless or is coloured greenish orange. This intercellular material appears to be identical with the "precollagen" described by Hunt (1941) in C-deficient wounds. The orientation of both cells and fibres is chaotic (*cf.* Figs. 10 and 11), and there is very little outgrowth from the old dermis at any stage of healing. Only the scab and leucocytes give a strong phosphatase reaction and the regenerating connective tissue is

of leucocytic debris is nearly black. The fibroblastic nuclei of the surrounding tissue also give a positive but weaker reaction.

The subcutaneous tissue plays a more important part in healing than is usual in similar wounds in the rat, and by the 6th day the subcutaneous bulge seen in the day-old wound is larger, richly vascularized and, except in the depths of the scar, is closely packed with fibroblasts orientated parallel with the surface of the w ound. Fibroblasts

Fig. 3–15. Abbreviations: b., blood; b.p. blood pigment; mi., mitosis; o.d., original dermis; p.r., phosphatase reaction.

Fig. 3–9.—The preparations were made by Gomori's method from the half-wounds fixed in alcohol and were photographed at a magnification of × 18.

Fig. 3.—Normal guinea-pig: wound fixed 3 days after operation. At this stage almost all the phosphatase activity is due to the infiltrating leucocytes. In the subcutaneous tissue bulging into the cavity the reaction is slight except near the surface, but the scab, which contains much polymorph debris, is nearly black.

Fig. 4.—Normal guinea-pig: wound fixed 16 days after operation. Epithelialization is complete, and the second peak of phosphatase activity associated with the differentiation of fibrous tissue has begun to appear. The reaction is intense at the margin of the wound, but still only moderate in the interior.

Fig. 5.—Normal guinea-pig: wound fixed 21 days after operation. The phosphatase reaction in the scar is now maximal.

Fig. 6.—Acute vitamin C-deficiency: animal received one 2 mg. dose of ascorbic acid 8 days before death: wound fixed 24 days after operation. The scar gives no appreciable phosphatase reaction (the dark deposit in the centre is blood pigment).

Fig. 7.—Partial vitamin C-deficiency; 1·0 mg. level; wound fixed 28 days after operation. There is a slight phosphatase reaction in the deeper parts of the scar tissue. Both the scab and a small pocket of pus beneath it react intensely owing to the leucocytes present, but the superficial scar tissue below the pus gives no reaction (the apparent darkening is due to extravasated blood under the epidermis).

Fig. 8.—Partial vitamin C-deficiency: 2 mg. level. Wound fixed 28 days after operation. The scar is much more normal than that shown in Fig. 5. In the regenerating fibrous tissue the phosphatase reaction is intense immediately beneath the epidermis, but only moderate elsewhere.

Fig. 9.—Partial vitamin C-deficiency: 5 mg. level. Wound fixed 28 days after operation. The scar appears normal, is greatly contracted, and gives an intense phosphatase reaction throughout (*cf.* Fig. 3).

Fig. 10–15. The preparations were made from the half-wounds fixed in Zenker's solution and were photographed at a magnification of × 300. All the photographs are orientated in the same way, i.e. the top of each field is roughly parallel to the epidermis.

Fig. 10.—Normal guinea-pig: wound fixed 12 days after operation. Note the thin, regularly arranged argyrophil fibres. (Wilder's method: carmalum and light green mixture.)

Fig. 11.—Acute vitamin C-deficiency: wound fixed 12 days after operation. Note the chaotic arrangement of the argyrophil fibres, some of which form stout irregular strands. (Wilder's method: carmalum and light green mixture.)

Fig. 12.—Acute vitamin C-deficiency: section of the same wound as that shown in Fig. 4, fixed 24 days after operation. Note the swollen irregular masses of abnormal collagen and the chaotic arrangement of the cells. (Haematoxylin and van Gieson's stain.)

Fig. 13.—Partial deficiency: 1 mg. level. Section of the same wound as that shown in Fig. 5, fixed 28 days after operation. The intercellular fibres, though relatively scanty, are much more normal than those shown in Fig. 10, but the arrangement of both cells and fibres is very irregular, and in this part of the scar they are orientated at right angles to the epidermis. (Haematoxylin and van Gieson's stain.)

Fig. 14.—Partial deficiency: 2 mg. level. Section of the same wound as that shown in Fig. 6, fixed 28 days after operation. The fibres, though rather fine, are nearly orientated parallel to the epidermis. (Haematoxylin and van Gieson's stain.)

Fig. 15.—Partial deficiency: 5 mg. level. Section of the same wound as that shown in Fig. 7, fixed 28 days after operation. Note the abundant, well-developed fibres and orderly arrangement of the tissue. (Haematoxylin and van Gieson's stain.)

It can be seen that the rate of healing went parallel with the degree of phosphatase activity and of differentiation, but inversely to the formation of adhesions and haemorrhages in the scar tissue.

The wounds in the 10 mg. group without a preliminary acute deficiency were nearly healed two weeks after operation, and those in the other 10 mg. and in the 5 mg. groups were fully and normally healed after three weeks. At the lower levels of vitamin C, however, healing was still incomplete when the guinea-pigs were killed 28 days after operation; at this stage the scars in the 0·5 and 0·7 mg. series showed large adhesions, occasional scabs and little or no hair at the site of injury. In the 1·0 mg. group the wounds were partially epithelialized, but displayed adhesions and haemorrhages, while at the 2 mg. level, though epithelialization was complete, haemorrhages were seen in about 50 per cent of the scars.

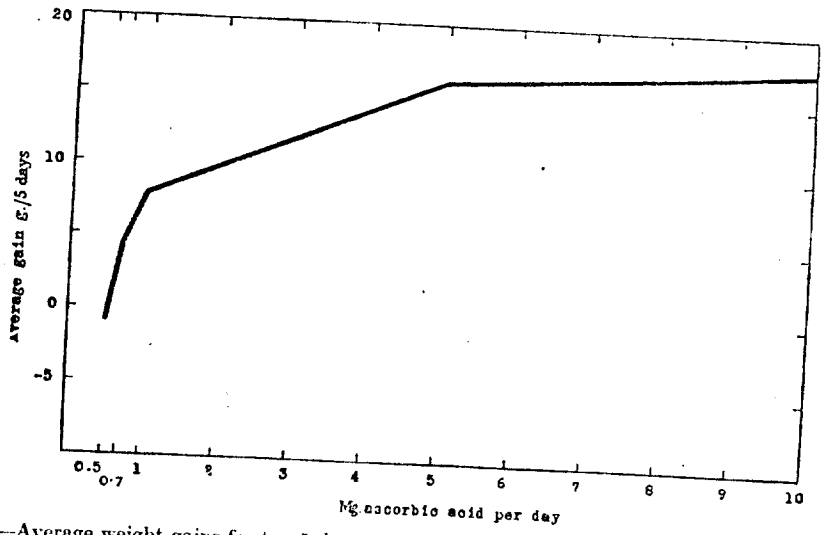


FIG. 2.—Average weight gains for ten 5-day periods throughout the experiment at different levels of vitamin C.

2. *Histological Observations.*

A. *Healing in the normal guinea-pig.*

In preparations of uninjured skin made by Gomori's method, the sebaceous glands, the proximal part of the hair follicles and a few capillaries give a strong positive reaction for phosphatase, but the rest of the skin and the subcutaneous tissue show almost no reaction.

Wounds from 18 control guinea-pigs belonging to all four experiments have been examined histologically, and the results are included in the following account of healing in the normal animal. The wounds were fixed at intervals of from 1 day to 5 weeks after operation.

In the 3-day wound (Fig. 3) the cavity is partly filled by an oedematous bulge of subcutaneous tissue which is densely infiltrated with leucocytes, mainly polymorphs, and covered by a scab. Gomori's method demonstrates an intense phosphatase activity both in the wound itself and in the interstices of the dermis and muscle, which is localized in the leucocytes and particularly in the polymorphs (*cf.* Gomori, 1943; Fell and Danielli, 1943). As in rat wounds, the scab with its very high content

TABLE I.— *Summary of Clinical and Histological Findings in Experiment 4.*

Dose of ascorbic acid mg./day (after 10 days' depletion).	Clinical.			Histological.	
	Rate of healing.	Adhesions.	Haemorrhages.	Phosphatase.	Differentiation.
0·5	±	++++	+	+	+
0·7	±	++++	+	+	+
1·0	+	++++	+	+	+
2·0	++	0	+	+	+
5·0	+++	0	0	++	++
10·0	+++	0	0	++++	+++
10·0*	++++	0	0	+++	++++
				+++	++++

* This group was dosed with 10 mg. ascorbic acid daily without a previous acute deficiency of vitamin C.

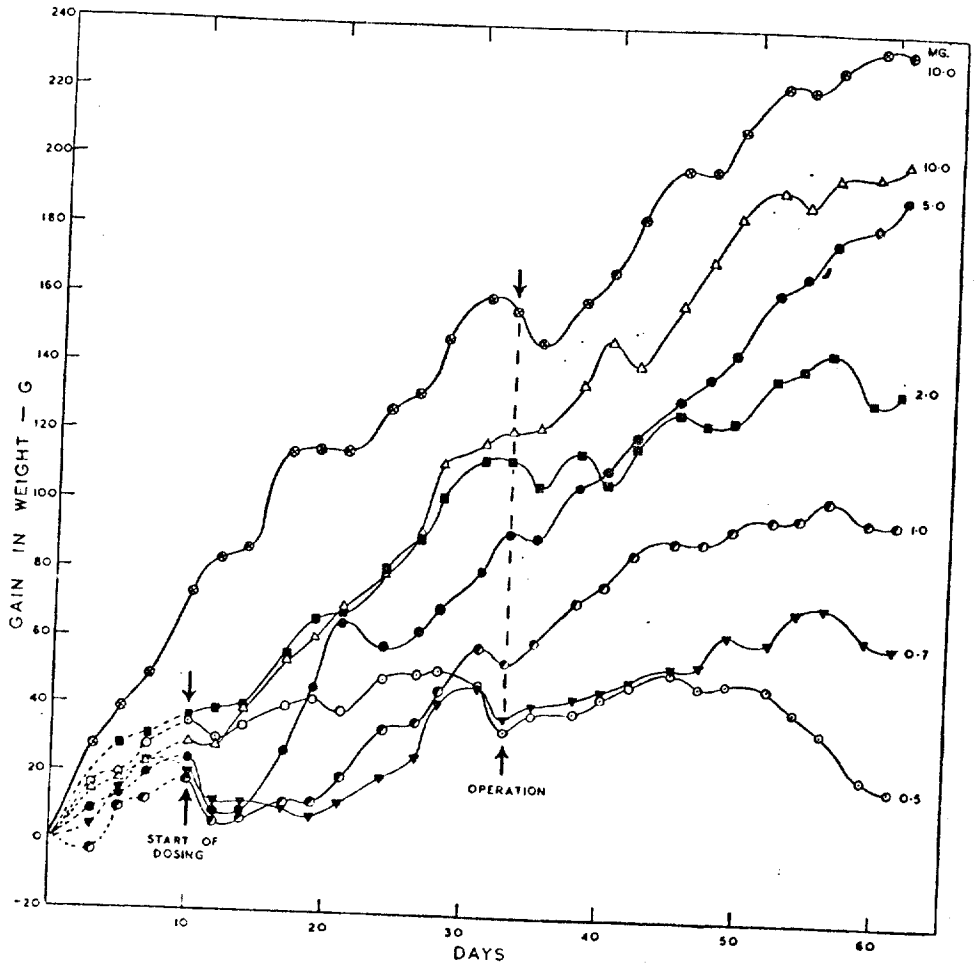


FIG. 1.— Weight gains with various intakes of vitamin C.

To control the possible effect on healing of the 9-day period of acute deficiency, a group of 3 guinea-pigs received 10 mg. ascorbic acid from the beginning of the experiment; the operations were made at the same time as in the other groups, and the animals were killed 28 days after the operation.

Surgical Methods.

Standard wounds were made under ether anaesthesia by the method described by Dann, Glücksmann and Tansley (1941). An area of dorsal skin was closely shaved and wiped with 80 per cent alcohol. A circle was cut in this area with a rotating cylindrical blade 6 mm. in diameter, and the skin was excised with fine scissors. The wounds were not covered.

Histological Methods.

Immediately the guinea-pig had been killed, a flap of skin containing the wound was removed, spread on clean filter-paper, and with a safety razor blade cut in half across the transverse axis of the scar. One half, which was used to study the distribution of phosphatase, was fixed in 80 per cent alcohol, dehydrated in absolute alcohol, cleared in cedar wood oil, and infiltrated first in soft wax at 40° C. and then in hard wax at 58° C. The other half, which was fixed in 3 per cent acetic Zenker for 5 hours and then treated in the usual way, was used to investigate the histological structure of the scar by other methods. Sections were cut at 10μ.

As in our previous experiments (Fell and Danielli, 1943), the preparations made by Gomori's method (Gomori, 1939) were incubated in the glycerophosphate solution for 24 hours instead of 2–3 hours as originally prescribed by Gomori. Slides made from the Zenker-fixed material were stained by haematoxylin followed by chromatrop or van Gieson's stain, by a modification of Mallory's triple stain in which carmalum is substituted for the acid fuchsin, and by Wilder's silver method counterstained with carmalum and a mixture of light and naphthol green.

RESULTS.

1. *Clinical Observations.*

In acute deficiency the animals in both Experiments 1 and 2 lost weight from the 13th day onwards, and when the last were killed on the 24th day after operation they showed characteristic signs of scurvy, and a large scab still covered the incompletely epithelialized wounds.

In Experiment 3 (0·5 mg. ascorbic acid) the initial weight was maintained, but epithelialization was greatly delayed. The control wounds healed during the second week, but in the deficient animals even 4 and 5 weeks after operation the wounds were swollen and had a large scab and extensive adhesions. There were usually haemorrhages round the knee-joints and scapulae.

In Experiment 4 (see Table I) the gain in weight was related to the size of the dose almost up to the 10 mg. level (see Fig. 1 and 2), but there were not sufficient animals in each group for a statistical test of the significances of these differences to be made. The results, however, agree well with the clinical and histological findings described below. One animal in the 10 mg. group deprived of vitamin C for a preliminary period of 9 days behaved erratically, its weight curve failed to rise and it was discarded.

Healing proceeded normally only at the highest level, i.e. the 10 mg. level with and without a previous acute deficiency. In the whole experiment one wound only showed obvious signs of infection and was suppurating slightly.

EXPERIMENTAL.

Diet.

Guinea-pigs of both sexes and about 300 g. in weight were used. In Experiments 1 and 2 the effect of an acute vitamin C-deficiency was studied; in Experiments 3 and 4 the guinea-pigs were divided into groups supplied with various levels of vitamin C. Four animals died shortly after operation and were discarded.

The basal diet (Harris and Oliver, 1942) without vitamin C was composed of 720 g. oatmeal, 90 g. dried egg yolk, 160 g. bran, 20 g. salt mixture, 100 g. dried yeast, and 6 drops of radiostoleum weekly to supply vitamins A and D. When vitamin C was given it was prepared immediately before use and administered daily by mouth with a dropping pipette before feedings.

Experiment 1 (18.ii.43–11.iii.43).

Acute vitamin C-deficiency. Six guinea-pigs were fed on the basal diet without ascorbic acid and five controls were given 10 mg. ascorbic acid daily. Wounds (see below) were made on the 7th day of the experiment, and animals were killed 1, 3, 6, 9, 13 and 16 days later.

Experiment 2 (18.viii.43–16.ix.43).

Acute vitamin C-deficiency. Twelve animals were fed on the basal diet without vitamin C and 6 controls received 10 mg. ascorbic acid daily. The operations were done on the 6th day of the experiment. One, two or three deficient guinea-pigs were killed respectively on the 8th, 10th, 12th, 16th, 18th, 22nd and 24th day after operation, and one control was killed on the 8th, 12th, 22nd and 24th day.*

Experiment 3 (22.iii.44–30.v.44).

Partial vitamin C-deficiency; 0·5 mg. level. Nine animals were fed on a completely deficient diet for 10 days to deplete their reserves of vitamin C and were then given 0·5 mg. ascorbic acid daily. Six controls were dosed with 10 mg. ascorbic acid daily from the beginning of the experiment. The operation was performed on the 24th day of partial deficiency. Two, three or four animals were killed respectively on the 22nd, 28th and 36th day after operation, i.e. 46, 52 and 60 days after being put on 0·5 mg. ascorbic acid daily.

Experiment 4 (6.x.44–6.xii.44).

Partial vitamin C-deficiency; 0·5–10 mg. levels. Thirty-one guinea-pigs were divided into groups of 3–7 animals, and after a preliminary period of 9 days without vitamin C were dosed daily with 0·5 (7 animals), 0·7 (6 animals), 1·0 (4 animals), 2·0 (4 animals), 5·0 (3 animals), and 10 mg. ascorbic acid (4 animals). All the wounds were made on the 24th day of partial deficiency and the guinea-pigs were killed 28 days after operation (i.e. after 9 days of acute deficiency + 52 days of partial deficiency).

* Since the maximal phosphatase activity of wounds in normal guinea-pigs is not reached until about the 3rd week after operation, an attempt was made to prolong the life of a few of the acutely deficient animals by giving doses of vitamin C during the latter part of the experiment. One of the two animals killed after 18 days received two doses of 10 mg., the last dose 2 days before death, one of the animals killed after 22 days received 5 doses of 10 mg., the last dose 5 days before death, and both the animals killed after 24 days received one dose of 2 mg. 8 days before death. The condition of the animals and the histological picture of the scar seemed almost unaffected by the few doses given at this late stage of deficiency, although, as will be seen from Experiment 4, dosage with ascorbic acid after a shorter period of acute deficiency has a very pronounced effect.

Brit. Jour. Exptl. Path. 26(6): 367-376. 1945

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.

J. F. DANIELLI, H. B. FELL* AND E. KODICEK.

From the Sir William Dunn Institute of Biochemistry, Strangeways Research Laboratory and Dunn Nutritional Laboratory, Cambridge.

Received for publication October 12, 1945.

USING the histochemical method described by Takamatsu (1939) and Gomori (1939), Fell and Danielli (1943) showed that the regenerating connective tissue in healing skin wounds in the rat contained alkaline phosphatase, the activity of which increased as healing advanced, reached a maximum when collagen-formation was greatest, and declined during the later stages of repair to the low level normal for the adult skin. It was suggested that the enzyme might be concerned in collagen-formation.

Recently Biesele and Biesele (1944) observed phosphatase activity in the skin of mice during carcinogenesis produced by methylcholanthrene, and they also noted a phosphatase reaction, similar to that described by Fell and Danielli, in an accidental wound in one of their treated animals.

It has long been known that in vitamin C-deficiency wounds heal slowly and the scar when formed is liable to break down. Histological studies (reviewed by Bourne, 1942; Wolbach and Bessey, 1942) have shown that this is due to the "inability of the supporting tissues to produce and maintain intercellular substances" (Wolbach and Howe, 1926). The histological details of wound healing in C-deficiency have recently been clearly described by Hunt (1941). In view of the association between the appearance of phosphatase and the differentiation of fibrous tissue previously observed in the rat (Fell and Danielli, 1943), it seemed desirable to find whether vitamin C-deficiency affected the phosphatase activity in healing wounds as well as the regeneration of fibrous tissue.

Histochemical observations have already been made by Bourne (1943) on the phosphatase activity of regenerating skeletal tissue in normal and C-deficient guinea-pigs. Holes were bored in the femora and allowed partly to heal. In the non-scorbutic controls the cells and fibres of the regenerating tissue gave a strongly positive reaction with Gomori's method, but "microscopically there was scarcely any phosphatase reaction in the holes in the femora of the scorbutic animals." The alkaline phosphatase reaction of kidney and other tissues and organs in normal and vitamin C-deficient guinea-pigs has been investigated by Harrer and King (1941), and Russell, Rouse and Read (1944). Shwachman and Gould (1942*a*, *b*; 1943) found a lowered blood phosphatase in vitamin C-deficiency.

In our present study we have observed the effect of different degrees of vitamin C-deficiency on the phosphatase activity and differentiation of the scar tissue in small open skin wounds in guinea-pigs.

* Foulerton Research Fellow, Royal Society.

adrenal cortical stimulation. This does not rule out the possibility that chronic (i.e., prolonged or repeated) intoxication may act as an adrenocortical stimulus, but the point must be established by direct investigation and not by unjustifiable extrapolation from presently available experimental results.

References

1. J. J. Smith. J. Clin. Endocrinol. **11**, 792 (1951).
2. J. C. Forbes and G. M. Duncan. Quart. J. Studies Alc. **12**, 355 (1951).
3. J. C. Forbes and G. M. Duncan. Quart. J. Studies Alc. **14**, 22 (1953).
4. H. B. Haag, G. I. Klingman, and R. Bane. Federation Proc. **16**, 303 (1957).
5. G. I. Klingman, H. B. Haag, and R. Bane. Quart. J. Studies Alc. **19**, 543 (1958).
6. H. W. Newman. Stanford Med. Bull. **13**, 98 (1955).
7. M. Feldstein and N. C. Klendshoj. Can. J. Med. Technol. **16**, 48 (1954).
8. I. I. Geschwind, B. S. Williams, and C. H. Li. Acta Endocrinol. **8**, 247 (1951).
9. E. Knobil, M. G. Hagney, E. J. Wilder, and F. N. Briggs. Proc. Soc. Exptl. Biol. Med. **87**, 48 (1954).
10. G. Sayers. Physiol. Revs. **30**, 241 (1950).
11. I. A. Miksky, P. Piker, M. Rosenbaum, and H. Lederer. Quart. J. Studies Alc. **2**, 35 (1941).
12. M. G. Eggleton. Brit. J. Psychol. **32**, 52 (1941).
13. H. W. Newman. Acute alcoholic intoxication. Stanford University Press. 1941. p. 169.
14. W. S. Spector (*Editor*). Handbook of toxicology. Vol. I. Natl. Acad. Sci. Natl. Research Council. 1955. pp. 128–130.
15. A. Arvola, L. Sammalisto, and H. Wallgren. Quart. J. Studies Alc. **19**, 563 (1958).

Two possible explanations were considered for the fact that only intraperitoneal administration of alcohol caused adrenal stimulation. First, it was thought that some of the effect on the adrenals might be due to pain caused by the presence of the alcohol in the peritoneal cavity rather than to the alcohol itself. However, when a local anaesthetic was given prior to the injection of alcohol, there was no change in the net adrenal response to alcohol, although the ascorbic acid and cholesterol were not decreased as much as in the unanaesthetized animals. This would seem to indicate that the presence of alcohol in the peritoneal cavity, at the concentration used in this experiment (20% v/v solution), did not act as a pain stimulus.

The effect of the intraperitoneal injection of alcohol could also be explained by the more rapid absorption of alcohol from the peritoneal cavity. The blood alcohol curves demonstrated this very rapid and high rise in blood alcohol after intraperitoneal administration as compared to the slower and lower rise found after oral ingestion of alcohol. It has been shown that both the rate and extent of rise of blood alcohol are important in regard to the degree of intoxication (11, 12, 13). This was confirmed by the observed behavior in the rats. The animals which received alcohol by the intraperitoneal route appeared much more intoxicated than those given it orally. Furthermore, it was only in those animals exhibiting this profound rapid intoxication that there was evidence of any adrenal stimulation. When the same total dose was divided and given in three spaced injections, in an attempt to make the absorption rate of the injected alcohol comparable with that found after oral ingestion, the adrenal response was less marked. Even the divided doses may not have given a slow enough rate of absorption, since the effect was still different from that observed after gastric administration. It appears, then, that the observed adrenal changes are more likely due to secondary effects of profound intoxication (e.g. possible circulatory disturbances) rather than to alcohol per se. In other words, the adrenal effect is not proportionate to the dose of alcohol at all levels, but is seen only when a state of profound intoxication is produced.

The dose of alcohol used, 2 g/kg body weight, is equivalent to 140 g of alcohol in an average man. If the specific gravity of alcohol is taken as 0.8, this means 175 ml of absolute alcohol. Assuming whisky to be 40% alcohol, this is equivalent to 430 ml of whisky in one dose. Even this, the most modest dose in any of the reported experiments, is far in excess of the rate of consumption by any drinker, except perhaps a suicidal one. It is extremely improbable, then, that such blood alcohol curves as those seen in rats after intraperitoneal injection of alcohol could ever be achieved after oral ingestion. Although there is some degree of species variation in sensitivity to alcohol in terms of lethal doses (14), studies at lower doses show that signs of intoxication appear in rats at blood alcohol levels of about 100 mg/100 ml, not far removed from those levels which produce similar degrees of intoxication in man (15). It is therefore evident that acute non-lethal intoxication by alcohol given via the upper gastrointestinal tract, whether in rats or in humans, is most unlikely to cause

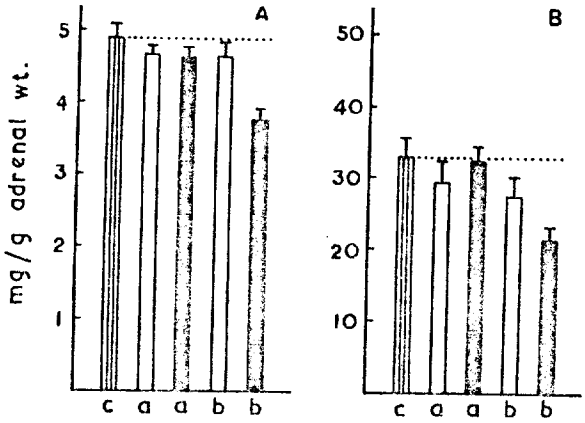


FIG. 5. Changes in adrenal levels of ascorbic acid (A) and cholesterol (B) $4\frac{1}{2}$ hours after alcohol administration; *a*, *b*, *c* as in Fig. 2.

saline and after alcohol, as in the unanaesthetized animals. However, there was still a significant net decrease ($p=0.02$) in the levels following alcohol administration. It is probable that metycaine afforded some protection, although it had no effect on the net response to alcohol.

Figure 5 shows the adrenal levels of ascorbic acid and cholesterol $4\frac{1}{2}$ hours after gastric and intraperitoneal administration of alcohol or control solution. It is apparent that in $4\frac{1}{2}$ hours both the ascorbic acid and cholesterol concentrations were returning to normal. The values in the saline and water control groups, as well as those found after administration of alcohol by stomach tube, were not significantly different from the values in the basic controls. After the intraperitoneal injection of alcohol, the net decrease in ascorbid acid was significantly smaller at $4\frac{1}{2}$ hours than at $1\frac{1}{2}$ hours. Although there was a smaller net decrease in cholesterol also, the difference was not significant. This is expected, since adrenal cholesterol concentrations usually do not change as rapidly as those of ascorbic acid (10).

Discussion

Ethyl alcohol, in a dose of 2 g/kg body weight, produced a significant decrease in adrenal ascorbic acid and cholesterol when administered intraperitoneally but not when given by stomach tube. The insertion of the stomach tube proved to have a greater effect on the adrenal constituents than did the alcohol itself by this route. The alcohol seemed to act as a mild sedative and resulted in smaller decreases in ascorbic acid and cholesterol. Training the rats by giving them water orally or saline intraperitoneally, daily for 1 month, tended to result in smaller decreases in ascorbic acid and cholesterol generally but did not affect the respective responses to alcohol. The maximum adrenal response occurred within $1\frac{1}{2}$ to $4\frac{1}{2}$ hours after administration of alcohol.

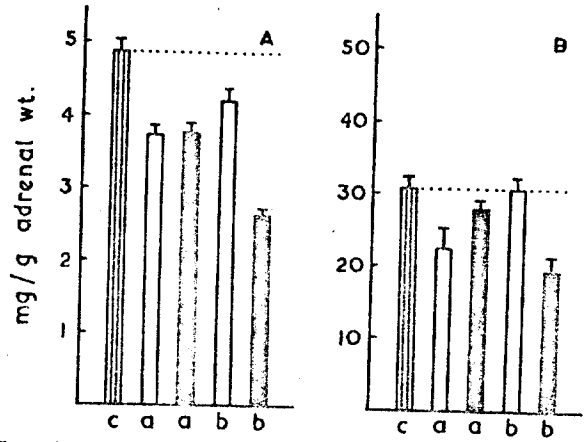


FIG. 3. Effect of training on responses of adrenal ascorbic acid (A) and cholesterol (B) to alcohol; *a*, *b*, *c* as in Fig. 2.

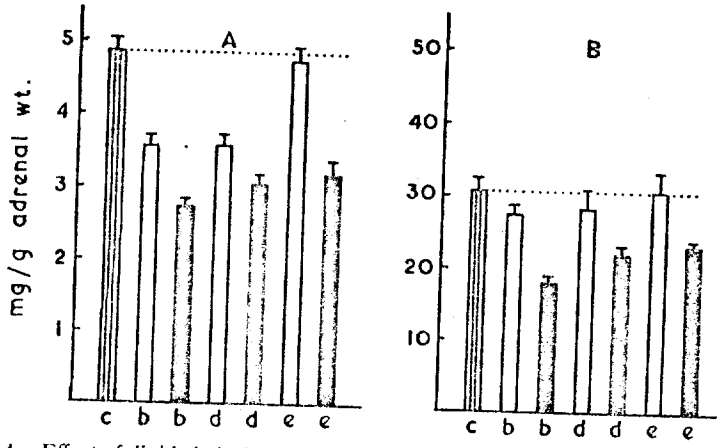


FIG. 4. Effect of divided alcohol dose and of metycaine on changes in adrenal ascorbic acid (A) and cholesterol (B) after injection of alcohol; *c* = basic controls, *b* = single injection, *d* = three injections, *e* = single injection after metycaine.

three spaced injections are shown in Fig. 4. The same saline control values of adrenal ascorbic acid and cholesterol were obtained ($p>0.50$), but the post-alcohol levels were not as low as those found after the single injection. There was a significant difference between the 1-injection and the 3-injection groups with respect to the cholesterol values ($p<0.01$) but not those for ascorbic acid ($p=0.10$), although a trend was evident in the latter also.

The effect of metycaine on the adrenal response to the injection of saline or alcohol is indicated in Fig. 4. When the local anaesthetic was used, the concentrations of ascorbic acid and cholesterol did not decrease as much, both after

The effects of gastric and intraperitoneal administration of alcohol on the adrenal levels of ascorbic acid and cholesterol are shown in Fig. 2. Contrary to published reports, the insertion of the stomach tube had considerable effect on

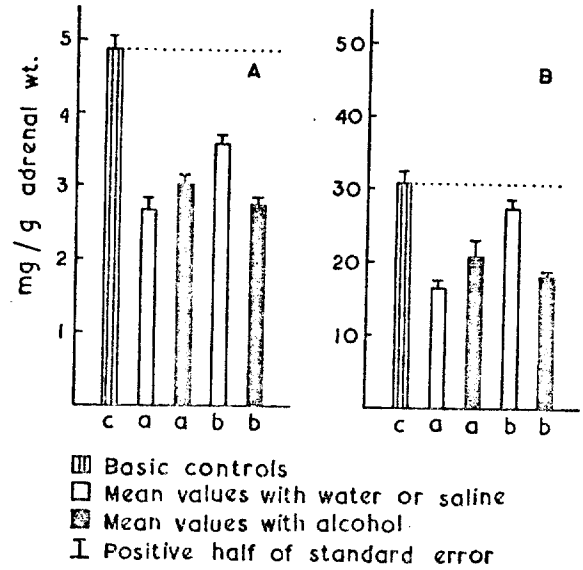


FIG. 2. Changes in adrenal levels of ascorbic acid (A) and cholesterol (B) 1½ hours after alcohol administration; *c* = basic controls, *a* = stomach tube, *b* = intraperitoneal injection.

the adrenals. With the water controls there was a decrease of 45 and 47% in adrenal ascorbic acid and cholesterol, respectively ($p<0.01$). When ethyl alcohol was given, it seemed to act as a mild sedative, causing a *smaller* rather than a greater drop in concentration, though the difference did not attain statistical significance. Intraperitoneal injection of saline resulted in decreases in adrenal ascorbic acid and cholesterol of 27 and 12%, respectively ($p<0.01$). Injection of ethyl alcohol had a much greater effect, causing drops of 44 and 41% ($p<0.01$ when compared to basic controls and to saline-injected animals).

Figure 3 indicates the effect of training on the stress due to the insertion of the stomach tube and to the injection. Training resulted in a marked reduction in adrenal effects due to gavage. There were decreases of only 23 and 27% in ascorbic acid and cholesterol, respectively ($p=0.01$). Again there was no further drop after alcohol administration. After training, the intraperitoneal saline controls were significantly different from the basic controls with respect to adrenal ascorbic acid ($p=0.02$), but not cholesterol ($p>0.05$). On the other hand, after injection of alcohol, the trained rats showed the same percentage decreases in the levels of both substances as did the untrained rats ($p>0.50$).

The results obtained when the same total dose of alcohol was divided into

To investigate the possible effect of alcohol as a pain stimulus on adrenal ascorbic acid and cholesterol, a local anaesthetic (metycaine) was given intraperitoneally 10 minutes before the injection of alcohol or saline. The dose of metycaine was 1 ml of 0.075% solution/100 g body weight.

In another experiment, to make the rate of absorption of the injected alcohol comparable with that following gavage, the dose was divided and given in three injections spaced at 0, 30, and 60 minutes. The rats were decapitated 1½ hours after the first injection.

Since it was not certain that the time of peak blood alcohol level corresponded to the time of peak adrenal response, the effect of alcohol on adrenal ascorbic acid and cholesterol was also determined 4½ hours after its administration. For this experiment, male albino rats weighing 270 to 340 g were used. They had been trained for 2 weeks previously. Alcohol and control solutions were given intraperitoneally and by gavage, as in the experiments described above.

Results

The blood alcohol curves obtained after gastric and intraperitoneal administration of alcohol are shown in Fig. 1. After gavage with alcohol the peak blood alcohol level occurred in approximately 1½ hours. However, when alcohol was

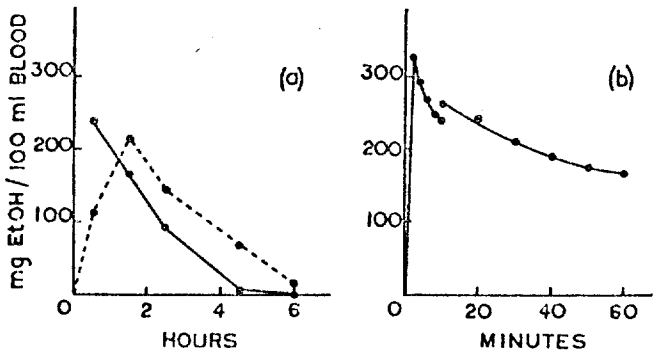


FIG. 1. (*a*) Blood ethanol levels after administration of 20% ethanol in dose equivalent to 2 g/kg; – – – by stomach tube; —— by intraperitoneal injection. (*b*) Blood ethanol levels during first hour after intraperitoneal injection of 2 g/kg. Break in line indicates portions of experiment done on different days.

given intraperitoneally, it was absorbed into the blood almost instantaneously, the peak occurring within 2 minutes of the injection. Furthermore, the peak values were much higher than those found after gastric administration. This is probably the explanation for the observation that the rats given alcohol by injection appeared much more intoxicated than those given the same dose by gavage. They showed almost immediate onset of limpness, stupor, and in-co-ordination, whereas rats receiving alcohol by stomach tube merely became more relaxed and quiet.

saline by intraperitoneal injection, for 1 month prior to the alcohol experiment. This was done to assess the possible adrenal effects of stress due to the insertion of the stomach tube or to the injection. It was felt that this factor might be of greater importance in such experiments than had been recognized previously.

Procedure

Pure (95%) ethyl alcohol was diluted to 20% with water for oral administration or with saline for intraperitoneal injection. This concentration was chosen because it has been shown to result in the most rapid absorption from the gastrointestinal tract (6). It also appeared to be non-irritating to the peritoneum, as judged by the appearance at laparotomy. The alcohol was administered at a dose of 2 g/kg body weight in all cases.

Blood alcohol levels were determined after oral and intraperitoneal administration of alcohol. Female albino rats weighing 170 to 260 g were used. Animals were decapitated at 0, $\frac{1}{2}$, $1\frac{1}{2}$, $2\frac{1}{2}$, $4\frac{1}{2}$, and 6 hours after they had been given the alcohol. Heparin, 200 units in 0.2 ml, was injected prior to decapitation, to prevent the blood from clotting. The ethyl alcohol content of the collected blood was determined by the microdiffusion method of Feldstein and Klendshoj (7). In another series of animals, blood alcohol levels were determined every 2 minutes for the first 10 minutes, and then every 10 minutes for 1 hour, after intraperitoneal injection of alcohol.

To study the adrenal effects of alcohol, male albino rats weighing 220 to 310 g were used. They were decapitated $1\frac{1}{2}$ hours after administration of alcohol or control solution. This time corresponds approximately to the time of peak blood alcohol level after a single oral dose. The adrenals were removed immediately, dissected free of fat, wrapped in parafilm, and weighed to the nearest 0.1 mg. The left adrenal was extracted with 10 ml of 4% trichloroacetic acid for ascorbic acid determination by Geschwind and Williams' modification of the Roe and Kuether method (8). The right one was extracted with 10 ml of glacial acetic for cholesterol determination by the method of Knobil *et al.* (9).

To compare the adrenal effects of alcohol after gastric and intraperitoneal administration, three main groups of 20 rats each were used. Half of one group was given water, the other half alcohol, by stomach tube. The second group was also divided, one half receiving saline, the other half alcohol, by intraperitoneal injection. The third was a basic control group, receiving neither gavage nor injection.

Since the insertion of the stomach tube and the injection might be sufficiently stressful to induce significant changes in the levels of the two adrenal constituents, a group of 40 rats was accustomed to these procedures ("trained") for 1 month prior to the experiment. It was hoped that any stress effect would thus be greatly reduced. Half of the rats were given water by stomach tube daily; the other half received saline daily by intraperitoneal injection. On the day of the experiment, half the rats in each group were given alcohol, the other half the respective control solution.

1752

CANADIAN JOUR. BIOCHEM AND PHYSIOL. 39(2): 327-334. 1961

THE EFFECT OF ACUTE ALCOHOLIC INTOXICATION ON ADRENAL ASCORBIC ACID AND CHOLESTEROL IN THE RAT[1]

CAROLINE CZAJA AND H. KALANT

Abstract

Ethyl alcohol, in a dose of 2 g/kg of body weight, caused a significant decrease in adrenal ascorbic acid and cholesterol when given to rats by intraperitoneal injection, but not when given by stomach tube. Gavage had greater effect on the adrenals than did the alcohol itself. Two possible explanations were considered for the effect of alcohol given intraperitoneally. First, some of the observed effects might be due to pain resulting from the presence of the alcohol in the peritoneal cavity. However, prior administration of a local anaesthetic did not modify the effect of injected alcohol, suggesting that the alcohol, at the concentration used in the experiment (20% v/v), did not act as a pain stimulus. Second, it was thought that the effect of the injected alcohol could be explained largely by its much more rapid absorption into the blood. Blood alcohol measurements showed that intraperitoneal injection of 2 g of alcohol per kg resulted in a peak concentration of over 300 mg% within 2 minutes, in contrast to a peak value of just over 200 mg% at 90 minutes after administration of the same dose by stomach tube. This explanation was confirmed by the finding of smaller decreases in adrenal ascorbic acid and cholesterol after three spaced injections than after a single injection of the same total dose of alcohol. Daily exposure to gavage or to intraperitoneal injection for 1 month reduced the magnitude of adrenocortical response to these manipulations, but did not modify the respective responses to alcohol given by these routes.

Introduction

It has been reported that ethyl alcohol administered either intraperitoneally or by gavage to rats and other experimental animals produces a marked decrease in adrenal ascorbic acid and cholesterol, which is interpreted as evidence of stimulation of adrenocortical hormone release (1, 2, 3). However, most doses of alcohol used in these studies were in the lethal or near-lethal range. The effects of such large doses of alcohol on the adrenals are not necessarily qualitatively the same as those produced by lower doses. In the present experiments a smaller dose was used in an attempt to assess the effects of alcohol per se, rather than effects attributable to circulatory and respiratory collapse that possibly resulted from the near-lethal doses, as suggested by the work of Klingman *et al.* (4, 5).

It seemed possible that some of the effects observed after intraperitoneal administration of alcohol might be due to pain resulting from its presence in the peritoneal cavity. Therefore a comparison was made of the effects of alcohol given intraperitoneally and by gavage, and the modification of the effect of intraperitoneal alcohol by a local anaesthetic was studied. In addition, rats were subjected to daily administration of water by stomach tube or of

[1]Manuscript received September 14, 1960.
Contribution from the Department of Pharmacology, University of Toronto, Toronto, Ontario. This work was generously supported by a grant from the Ontario Alcoholism Research Foundation.

measured 15 seconds after each addition of dye solution. The concentration of the dye was approximately equivalent to 0·4 mg of ascorbic acid per ml and was standardised against 0·005 *N* potassium iodate.

Results and discussion

The modifications described resulted in an increase of precision. The coefficient of variation for quadruplicate determinations on a solution of blackcurrant syrup was 0·6 per cent., compared with 1·4 per cent. by the original method. A similar test on a solution of ascorbic acid gave 0·33 and 1·0 per cent. It is thought that the improvement may be due to the reduction of the current through the system at the end of the titration from 0·4 to 0·05 μA. Three assays on pure ascorbic acid gave 100·0, 99·7 and 100·3 per cent., which indicate good accuracy.

Evans and Simmonds[4] have discussed the mechanism of polarisation end-points with special reference to dilute solutions of iodine and sodium thiosulphate. They state that under certain conditions there may be insufficient anions to keep the cathode depolarised. This effect was shown by a very slow fall in e.m.f. when dye solution was added to 2 per cent. oxalic acid solution in the original method. When the modified circuit was used, a sharp fall in e.m.f. was observed. These observations support the view that, the smaller the size of the electrode, the lower the concentration of anions necessary to depolarise it.

In order to test the effect of anions on the results obtained by the method, a solution of blackcurrant syrup and a dilute solution of ascorbic acid were both assayed with and without the addition of 2 mg of chloride as potassium chloride. The results are shown in Table I.

Table I

Effect of chloride on the determination of ascorbic acid

Sample	Ascorbic acid found in 2 ml of sample, mg	Ascorbic acid found in 2 ml of sample with 2 mg of chloride added, mg
Ascorbic acid solution	0·042 (8), 0·042	0·042, 0·041 (6)
Blackcurrant syrup solution A ..	0·392, 0·387	0·382, 0·383
Blackcurrant syrup solution B ..	0·426, 0·430	0·430, 0·429

In all tests the differences were within the experimental error of the method. The modified method has, therefore, the advantage of increased precision with no loss in accuracy, and it is not affected by concentrations of chloride up to 1 mg per ml of titrating solution.

References

1. Foulk, C. W., and Bawden, A. T., *J. Amer. Chem. Soc.*, 1926, **48**, 2044.
2. Liebmann, H., and Ayres, A. D., *Analyst*, 1945, **70**, 411.
3. Sully, B. D., *Chem. & Ind.*, 1955, 1146.
4. Evans, D. P., and Simmonds, N. T., *J. Soc. Chem. Ind.*, 1944, **63**, 29.

The Analytical Laboratory
H. W. Carter & Co. Ltd.
The Royal Forest Factory
Coleford, Glos.

R. C. Curtis

Received *December 11th*, 1956

Analyst 83, 54-55

THE APPLICATION OF THE POLARISED PLATINUM ELECTRODE TO THE DETERMINATION OF ASCORBIC ACID IN FRUIT PRODUCTS

(Presented at the meeting of the Western Section on Saturday, November 10th, 1956)

THE "dead-stop" method of Foulk and Bawden[1], in which polarised platinum electrodes are used as adapted by Liebmann and Ayres[2] for the determination of ascorbic acid by titration with 2:6-dichlorophenolindophenol, has been used in these laboratories for many years. The end-point is determined graphically from plots of galvanometer deflections read 25 seconds after each 0·02-ml addition of dye solution. The additions of the dye solution are made at 30-second intervals.

It was found that for low concentrations of ascorbic acid the slope of the plots after the end-point was reduced and so a loss of precision resulted. In addition, the galvanometer deflection, on occasions, would be still altering when the time came for its value to be noted. The effect was especially noticeable when the frequency of the additions of dye solution was increased in an effort to improve the precision. The modifications described below were the outcome of attempts to improve the precision of the method.

The method described by Sully[3] was used, in which the e.m.f. is applied to the electrodes through a very high resistance of 2 to 3 megohms, and the potential across them is measured by means of an electronic millivoltmeter. When the system is polarised, a high e.m.f. is observed, but it is reduced when depolarisation takes place.

It was found that the system, when partly depolarised by a small temporary excess of oxidant, became progressively slower to recover as the end-point was approached. By reducing the size of the electrodes to that of pin heads, by cutting them off flush with the glass surface, the sluggish response was largely overcome. This effect was presumed to be due to the smaller surface area of the electrodes, which would require less reductant to achieve polarisation.

The method finally adopted made use of electrodes of small surface area as described above. A Mullard millivoltmeter as supplied with a well known electrometric titration outfit was used for measuring the e.m.f. The circuit of the millivoltmeter was modified so that the existing polarising voltage could be applied through a 2·2-megohm resistor and the voltage was reduced to approximately 260 mV by means of a suitable tapping off the bias resistor network of the first valve in the instrument.

The titration was performed in a small flat-bottomed tube. Two millilitres of extract were taken and mixing was carried out by means of a stream of oxygen-free nitrogen. The dye solution was added in 0·01-ml increments at 20-second intervals near the end-point. The e.m.f. was

(9) MEYNELL, M. J., COOKE, W. T., COX, E. V., AND GADDIE, R., Serum-cyanocobalamin level in chronic intestinal disorders. Lancet, 1957, (i), 901.

(10) MINOT, G. R., Pernicious anæmia and related macrocytic anæmias. The 1943 year book of general medicine, p. 368. Year book publishers. Chicago.

(11) MOHR, W., Bericht über Vitamin-C-Stoffwechseluntersuchungen und pathologisch-anatomische Dünndarmbefunde bei Sprue. Dtsch. Z. Verdauungs-u Stoffwechselkr., 1939, **2**, 142.

(12) MOLLIN, D. L., AND ROSS, G. I. M., Serum vitamin B.12 concentrations of patients with megaloblastic anæmia after treatment with vitamin B.12, folic acid or folinic acid. Brit. med. J., 1953, (ii), 640.

(13) MOLLIN, D. L., AND ROSS, G. I. M., Vitamin B.12 deficiency in the megaloblastic anæmias. Proc. R. Soc. Med., 1954, **47**, 428.

(14) MUELLER, J. F., AND WILL, J. J., Inter-relationship of folic acid, vitamin B.12 and ascorbic acid in patients with megaloblastic anæmia. Amer. J. clin. Nutrit., 1955, **3**, 30.

(15) OWEN, J. A., AND IGGO, B., The use of p-chloromercuribenzoic acid in the determination of ascorbic acid with 2:6-dichlorophenolindophenol. Biochem. J., 1956, **62**, 675.

(16) ROE, J. A., 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., 1943, **147**, 399.

(17) SMITH, E. LESTER, Vitamin B.12. Brit. med. Bull., 1956, **12**, 52.

(18) WILL, J. J., MUELLER, J. F., GLAZER, H. S., FRIEDMAN, B. I., AND VILTER, R. W. The alteration of ascorbic acid oxidation in pernicious anæmia. J. Lab. clin. Med., 1953, **42**, 967.

B.12 corrects these abnormal findings slowly, acting more as a sparing agent than by preventing the immediate destruction of ascorbic acid. Though a similar effect is observed in adult cœliac disease, some further disorder of ascorbic acid metabolism appears to exist even in the B.12 saturated state. The nature of the association of B.12 deficiency and the abnormality of ascorbic acid metabolism is by no means clear though it is evident that the latter plays no fundamental part in the production of a megaloblastic anæmia in B.12 deficiency.

SUMMARY

1. The effect of B.12 administration on the plasma concentrations of ascorbic acid before and after an intravenous injection of 250 mg. sodium ascorbate was studied in patients with pernicious anæmia, adult cœliac disease and regional ileitis.

2. The patients with low serum B.12 levels had significantly lower resting plasma ascorbic acid level than normal controls.

3. The rate of disappearance of the injected sodium ascorbate was increased in the B.12 deficient state (pernicious anæmia 38·7%, adult cœliac disease 39·3% and regional ileitis 36·0% compared with normal of 24·4%).

4. Following B.12 therapy the abnormal ascorbic acid metabolism was corrected in pernicious anæmia and improved in adult cœliac disease. No change was noted in regional ileitis.

5. The effect in pernicious anæmia and in adult cœliac disease was masked in those patients partaking of a plentiful or grossly deficient supply of ascorbic acid in the diet.

6. In regional ileitis, the lack of effect of B.12 was attributed to the activity of the underlying disease. A significant correlation was noted between the levels of seromucoid and resting plasma ascorbic acid (correlation coefficient 0·7211, $p = < 0·001$).

7. The effect of B.12 deficiency on ascorbic acid metabolism was greater when the deficiency was associated with a megaloblastic anæmia in pernicious anæmia and adult cœliac disease, than in similar patients with a normal erythropoiesis.

REFERENCES

(1) ALT, H. L., CHINN, H., AND FARMER, C. J., The blood plasma ascorbic acid in patients with achlorhydria. Amer. J. med. Sci., 1939, **197**, 229.

(2) BOSCOTT, R. J., AND COOKE, W. T., Ascorbic acid requirements and urinary excretion of p-hydroxphenylacetic acid in steatorrhœa and macrocytic anæmia. Quart. J. Med., 1954, **23**, 307.

(3) BROWN, A., Megaloblastic anæmia associated with adult scurvy. Report of a case which responded to synthetic ascorbic acid. Brit. J. Hæmat., 1955, **1**, 345.

(4) COOKE, W. T., FOWLER, D. I., COX, E. V., GADDIE, R., AND MEYNELL, M. J., Seromucoids in regional ileitis and ulcerative colitis. Gastroenterology, 1958, **34**, 910.

(5) COX, E. V., MEYNELL, M. J., COOKE, W. T., AND GADDIE, R., The folic acid excretion test in the steatorrhœa syndrome. Gastroenterology. In Press.

(6) DYKE, S. C., DELLA VIDA, B. L., AND DELIKAT, E., Vitamin C deficiency in "irresponsive" pernicious anæmia. Lancet, 1942, (ii), 278.

(7) EVELYN, K. A., MALLOY, H. T., AND ROSEN, C., Determination of ascorbic acid in urine with the photoelectric colorimeter. J. biol. Chem., 1938, **126**, 645.

(8) MAY, C. D., NELSON, E. N., LOWE, C. U., AND SALMON, R. J., Pathogenesis of megaloblastic anæmia in infancy. An inter-relationship between pteroylglutamic acid and ascorbic acid. Amer. J. Dis. Child., 1950, **80**, 191.

which have been shown to be a good index of the activity of regional ileitis (4). Since similar results were obtained in patients with regional ileitis who had normal serum B.12 levels and in the B.12 deficient patients following B.12 therapy, any sparing action on ascorbic acid that B.12 may have in regional ileitis, is probably obscured by the activity of the underlying disease.

In patients with pernicious anæmia and adult cœliac disease, however, B.12 does appear to have a sparing action on ascorbic acid because their resting plasma ascorbic acid levels and their intravenous ascorbate tests are significantly improved following prolonged B.12 therapy. This apparent sparing action was most marked in patients on moderate diets and not significant in patients with very good or very poor diets. Thus, the B.12 therapy may act simply by reducing anorexia, and though the quality of the diet was unchanged, the increased quantity of the diet could produce this result. While this may account for some of the differences encountered, observations in two patients suggest that B.12 does exert a sparing action on ascorbic acid. At the time of the initial tests, the first patient had been taking approximately 1½ bottles of Ribena* daily (300 mg. ascorbic acid per day) for some weeks and the second had been given 1·5 g ascorbic acid daily for 10 days. Both patients were deficient in B.12, and both still had abnormally rapid rates of disappearance of ascorbic acid from the plasma. Following B.12 therapy, all their findings were normal.

Unlike patients with pernicious anæmia and despite the apparent sparing effect of B.12, the rate of disappearance of sodium ascorbate following its intravenous injection in patients with adult cœliac disease was still abnormally rapid even after B.12 therapy. We have already mentioned that Mohr (11) found difficulty in saturating a patient suffering from sprue with ascorbic acid by the intravenous route, and Boscott and Cooke (2) confirmed this. Thus, it appears that some disorder of the metabolism of ascorbic acid occurs in adult cœliac disease and this disorder is made more apparent in a B.12 deficient state.

From these investigations it seems evident that while the results are influenced by the activity of the underlying disease and the patient's diet, B.12 does have a sparing effect on ascorbic acid. Are there any reciprocal findings? That is, does a tissue depletion of ascorbic acid influence the hæmatological manifestations of vitamin B.12 deficiency? As B.12 deficiency can occur with a normal hæmatopoiesis, it has been suggested that associated changes, such as alterations in the amount of available folic acid (12, 13) may be necessary for the megaloblastosis of pernicious anæmia. Although the levels of plasma ascorbic acid before and after intravenous ascorbate were lower in those patients with megaloblastic anæmia when compared with B.12 deficient patients with a normal erythropoiesis, this alteration in the ascorbic acid findings may be simply part of a greater metabolic disturbance which occurs in the megaloblastic state and not necessarily of fundamental importance. Such a suggestion is supported by the finding of similar low levels of ascorbic acid in 7 patients with B.12 deficiency associated with regional ileitis; only the patient with the highest ascorbate levels in this group had a megaloblastic anæmia. Furthermore, the two patients with only minimal disorders of hæmatopoiesis, but with subacute combined degeneration of the cord, had high resting levels and one of them had an intravenous ascorbate curve within normal limits and significantly higher than the rest of the patients with pernicious anæmia.

Thus it can be concluded that detectable changes in the metabolism of ascorbic acid occur in the B.12 deficient state, but as might be expected these changes are influenced by diet and the activity of the underlying disease. In pernicious anæmia,

* Ribena is a blackcurrant cordial made by H. W. Carter & Co. Ltd., Royal Forest Factory, Coleford, Glos., and containing not less than 20 mg. ascorbic acid per fluid ounce.

with the plasma ascorbic acid. Where duplicate readings were taken from the same patient, they were made at an interval of at least 4 weeks. A definite correlation exists (correlation coefficient — 0·7211, p = < 0·001). As raised seromucoid (upper limit of normality 105 mg./100 ml. (4)) indicates increased disease activity, it would appear that the plasma ascorbic acid is decreased by this activity.

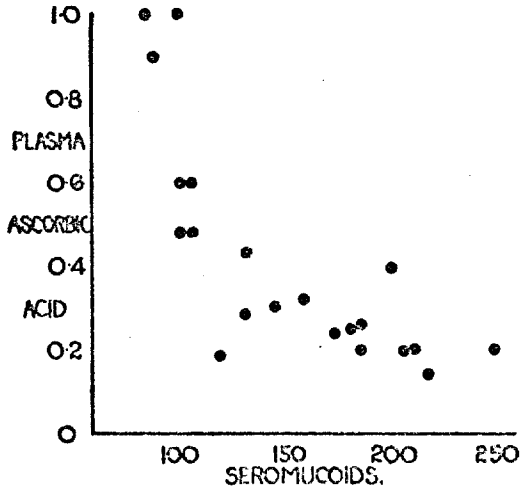


Fig. 4. Relationship between seromucoids (reflecting disease activity) and plasma ascorbic acid levels in regional ileitis (correlation coefficient — 0·7211, p = < 0·001).

Discussion

The plasma ascorbic acid concentration, both resting and at given intervals after an intravenous injection of sodium ascorbate, were lower in patients with reduced serum B.12 levels than in normal controls. Also, despite the lower levels 5 minutes after the injection, the proportion which disappeared from the plasma in the following 10 minutes was significantly greater. As excretion of ascorbic acid in the urine was negligible during the test, the lower levels of ascorbic acid, and the proportionately more rapid rate of disappearance from the plasma after the injection of the sodium ascorbate indicate that the ascorbic acid in the plasma was passing more rapidly into the tissues. This could be explained either by a degree of tissue depletion or by an increased demand or destruction of ascorbic acid. Since at least one property of vitamin B.12 is as a prosthetic group in a reducing enzyme system, Lester Smith (17) suggests that it may be important in maintaining ascorbic acid in a reduced state. In view of the statement that in some cases of pernicious anæmia, ascorbic acid is abnormally rapidly oxidised to dehydroascorbic acid in the blood stream (14, 18), it seemed possible that the rapid disappearance of reduced ascorbic acid from the plasma in the intravenous ascorbate test might be thus explained. In the 18 B.12 deficient patients, who were examined for this, the increase in plasma dehydroascorbic acid was no greater than that in 8 normal subjects. Also the lack of improvement in the response to the ascorbate test in patients with pernicious anæmia 7 to 14 days after B.12 therapy does not suggest a direct protective action of B.12 on ascorbic acid.

In the B.12 deficient patients with regional ileitis, the low levels appear to be the result of the activity of the underlying disease. A definite correlation was observed between the lowering of the plasma ascorbic acid level and the seromucoid

(S.E. ± 2·20)% and after B.12 induced hæmatological remission, this had fallen significantly to 30·3 (S.E. ± 3·42)%. There was a significant difference between the rate of fall in the two groups with B.12 deficiency, classified according to the nature of the hæmatopoiesis. In the normoblastic group, however, though the rate of fall returned to normal, the change was not significantly different to that prior to B.12 therapy. No significant correlation was found between either the hæmoglobin levels or the red cell count with either the plasma ascorbic acid or the rate of disappearance of injected ascorbate in the patients with a megaloblastic erythropoiesis. This was true of the B.12 deficient group as a whole.

Diet

The patients with regional ileitis have not been included. Fig. 3 shows the mean results obtained in the B.12 deficient patients with pernicious anæmia and adult cœliac disease. There were seven patients with good diets, that is adequate

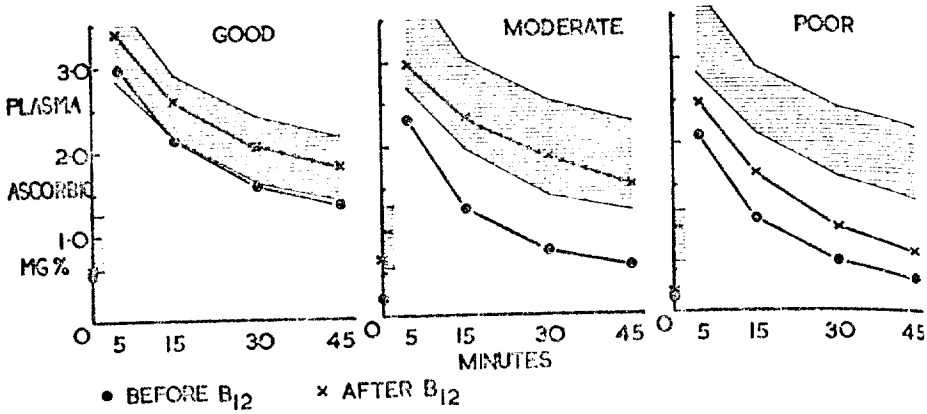


Fig. 3. Intravenous ascorbate tests before and after B.12 therapy in patients with B.12 deficiency and partaking of diets containing plentiful, moderate or poor quantities of ascorbic acid.

quantities of fruit or vegetables and in all but one case both. Four patients had moderate diets in that they rarely ate fruit but took mixed vegetables two to three times weekly. Seven patients had poor diets in that none ate fresh fruit or uncooked vegetables, one took no vegetables apart from potatoes, four ate cooked vegetables only occasionally and the other two took cooked vegetables once and occasionally twice a week.

B.12 therapy raised the ascorbic acid resting level and the curves after the intravenous sodium ascorbate in each group. The increase was significant only in the patients with moderate diets. The three groups in the B.12 deficient state had curves which were significantly lower than the normal subjects, and following B.12 therapy the groups with good and moderate diets had normal tests.

The patients with regional ileitis showed no evidence or suggestion of a correlation between the dietary intake and the resting ascorbic acid levels or the intravenous ascorbate curves. Some of these patients with good diets had high curves and others low and rapidly falling ones.

Activity of the underlying disease

The seromucoid level is a reliable index of the activity of the disease in patients with regional ileitis, where other non-specific means of detecting disease activity are of little value (4). Fig. 4 compares 21 estimations of the seromucoids

TABLE IV

Intravenous ascorbate test in patients with B.12 deficiency

		Megaloblastic				Normoblastic			
umber		15				8			
Time min.		Before B.12		After B.12		Before B.12		After B.12	
		Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
0	Plasma ascorbate mg./100 ml.	0·30	± 0·068	0·54	± 0·067	0·50	± 0·065	0·42	± 0·085
5		2·36	± 0·146	3·05	± 0·190	2·77	± 0·175	3·20	± 0·190
15		1·34	± 0·095	2·10	± 0·130	2·07	± 0·233	2·47	± 0·221
30		0·88	± 0·103	1·60	± 0·142	1·58	± 0·186	1·76	± 0·230
45		0·73	± 0·109	1·35	± 0·129	1·17	± 0·219	1·45	± 0·213
ary lion	mg.	2·0	± 0·28	7·9	± 1·42	6·0	± 2·49	10·3	± 3·5
of ppear-	%	42·6	± 2·20	30·3	± 3·42	27·1	± 4·4	23·3	± 5·34

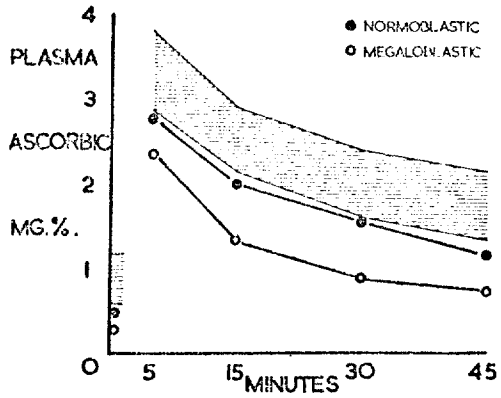


Fig. 2. Mean values of intravenous ascorbate test in patients with B.12 deficiency associated with megaloblastic (○) and with normoblastic (●) bone marrow.

the resting level was significantly increased, unlike the rest of the curve, it was still significantly lower than in the normal group. The B.12 deficient patients with no disorder of erythropoiesis though showing higher mean curves after B.12 therapy did not differ significantly from the pre-treatment group ($F = 0{\cdot}8$, d.f. $= 1/60$, $p = > 0{\cdot}20$).

The calculation of the rate of disappearance of the plasma ascorbic acid led to similar conclusions as the ascorbic acid test as a whole. The 15 patients with B.12 deficient megaloblastic anæmia had a mean rate of disappearance of 42·6

TABLE III

Rate of disappearance of ascorbic acid from the plasma after the intravenous injection of 250 mg. of sodium ascorbate

	Normal	Pernicious anæmia		Adult cœliac disease		Regional ileitis		
Number of subjects	20	12		7		7	7	8
		Relapse	Remission	B.12 Defic.	After B.12	B.12 Defic.	After B.12	B.12 Non
Mean	24·4	38·7	22·7	39·3	34·1	36·0	39·5	34·6
S.E. ±	2·41	4·15	2·96	4·04	2·42	5·87	3·93	5·02

Differences between 5 and 15 min. plasma levels expressed as percentages of levels at 5 minutes.

The rate of disappearance of ascorbic acid was abnormally rapid in patients with regional ileitis and was not influenced by treatment with cyanocobalamin in those with B.12 deficiency.

Following the intravenous injection of sodium ascorbate, the formation of increased amounts of dehydroascorbic acid occurred in only one of 18 B.12 deficient patients when compared with 8 normal subjects. This one patient was suffering from severe pernicious anæmia (R.B.C. $1{\cdot}83 \times 10^6$, HB. 5·5 g., P.C.V. 18%) and had a diet poor in vitamin C. The test was repeated on this patient before starting therapy and only insignificant amounts of dehydroascorbic acid were found. Possibly, the amount of ascorbic acid used in the first test was sufficient to convert the response to normal.

The B.12 deficient patients studied in this way were 5 with pernicious anæmia, 6 with adult cœliac disease, 4 with regional ileitis, 2 with partial gastrectomy and the patient with a B.12 deficiency megaloblastic anæmia for which no satisfactory explanation could be found.

The results were further analysed according to the hæmatological picture, diet and activity of the underlying disease.

Hæmatological picture

In view of the effect of the activity of underlying disease, the patients with regional ileitis were excluded from the analyses of results in the hæmatological groups.

There was a significant difference between the resting plasma ascorbic acid levels in two groups of B.12 deficient patients classified by the presence or absence of a megaloblastic erythropoiesis; the levels in both groups were significantly lower than those in the controls. In comparison, five patients with megaloblastic anæmia but no B.12 deficiency had a higher but not significantly higher mean value. Three of these patients had adult cœliac disease and two were pregnant and had been taking " multivitamin " tablets.

The results of the intravenous sodium ascorbate test are shown in Table IV and Fig. 2. There is a significant difference between the patients with normoblastic and megaloblastic erythropoiesis at 15, 30 and 45 minutes ($F = 39{\cdot}5$, d.f. $= 1/84$, $p = \ll 0{\cdot}001$). B.12 therapy in the patients with dyshæmopoiesis resulted in a significant rise in the concentration of the plasma ascorbic acid after the intravenous injection of sodium ascorbate ($F = 56{\cdot}8$, d.f. $= 1/108$, $p = \ll 0{\cdot}001$) and though

All the plasma concentrations (Table II) of ascorbic acid in the patients with pernicious anæmia in relapse were virtually unaltered 7 to 14 days after B.12 therapy (Ratio of variance (F) = 0·655, d.f. = 1/84, p = > 0·20) and were significantly lower than the normal controls (respectively F = 52·3, d.f. = 1/120, p = ≤ 0·001 F = 16·6, d.f. = 1/116, p = < 0·001). The levels at 15, 30 and 45 minutes in remission were significantly higher than before B.12 and together with the five minute reading did not differ significantly from the normal subjects (Fig. 1). In comparing the

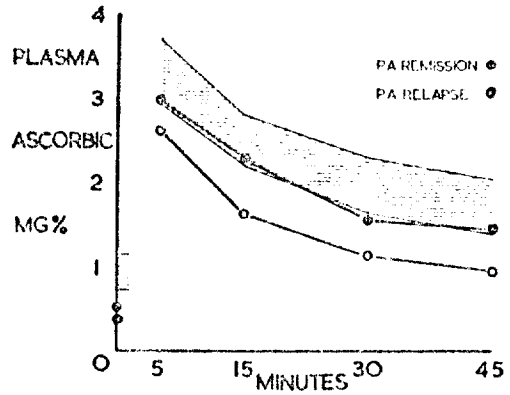


Fig. 1. Mean values of the intravenous ascorbate test in patients with pernicious anæmia in relapse (○) and B.12 induced remission (●). Shaded areas in this and subsequent figures represent the mean value ± twice the corrected standard error of the tests in normal subjects.

patients with pernicious anæmia in relapse and remission, the ratio of variance (F) was 8·75, d.f. = 1/84, p = < 0·01. During the period of the test, the urinary excretion was significantly lower in the patients with pernicious anæmia in relapse than in the same patients in B.12 induced remission and in the normal subjects.

All the plasma concentrations and the urine excretions of ascorbic acid in the patients with adult cœliac disease and B.12 deficiency were significantly lower than those in the normal controls (F = 54·4, d.f. = 1/104, p = > 0·001). After B.12 therapy though the increase in the resting level was not significant, the subsequent levels were (Table II) significantly higher than before (F = 7·2, d.f. = 1/48, p = < 0·01) though still significantly lower than that of the normals at 30 and 45 minutes (F = 8·2, d.f. = 1/104, p = < 0·01).

The patients with B.12 deficiency associated with regional ileitis had curves which were unaffected by B.12 therapy and none of these results differed significantly from 8 similar patients who had never developed a B.12 deficiency.

The rate of disappearance of ascorbic acid from the plasma was represented by the difference between the 5 and 15 minute levels of intravenous ascorbate test expressed as a percentage of the 5 minute level. These results are given in Table III.

Patients with pernicious anæmia in relapse had a significantly more rapid rate of disappearance of ascorbic acid than after B.12 induced remission. The rate of disappearance in pernicious anæmia in remission did not differ significantly from the rate in the normal subjects.

In the patients with adult cœliac disease, though the mean rate of disappearance was reduced by therapy with cyanocobalamin, the reduction was not significant and was still more rapid than the normal controls.

TABLE II

Plasma levels of ascorbic acid after the intravenous injection of 250 mg. of sodium ascorbate

	Group	Normal subjects	Pernicious anæmia			Adult cœliac disease		Regional ileitis		
			Relapse	7–14 day after B.12	Remission	Before B.12	After B.12	Before B.12	After B.12	Non-deficient
No.		20	12	12	11	7	7	7	7	8
Time 0	Mean	0·89	0·38	0·33	0·52	0·31	0·43	0·46	0·27	0·52
	S.E.	0·085	0·073	0·047	0·068	0·052	0·123	0·100	0·038	0·102
5	Mean	3·34	2·62	2·80	2·96	2·25	2·99	2·47	2·61	3·22
	S.E.	0·145	0·181	0·173	0·154	0·168	0·300	0·190	0·410	0·340
15	Mean	2·51	1·63	1·75	2·28	1·36	2·02	1·48	1·45	2·16
	S.E.	0·119	0·191	0·167	0·143	0·159	0·259	0·094	0·123	0·261
30	Mean	1·98	1·12	1·26	1·56	0·86	1·45	0·95	0·95	1·48
	S.E.	0·126	0·172	0·152	0·201	0·175	0·256	0·194	0·135	0·261
45	Mean	1·74	0·95	1·02	1·44	0·57	1·18	0·78	0·75	1·24
	S.E.	0·122	0·186	0·160	0·146	0·106	0·222	0·185	0·127	0·240
Urinary excretion	Mean	23·9	3·9	2·4	22·7	1·6	6·0	6·6	4·4	14·3
	S.E. ±	3·96	3·05	0·70	2·96	0·31	1·80	2·56	2·11	4·84

1694

resting plasma ascorbic acid level were compared with the seromucoid level determined at the same time. One patient with B.12 deficiency and a megaloblastic anæmia for which no satisfactory explanation could be found and three patients with reduced serum B.12 levels without hæmatological disturbance and another with megaloblastic anæmia, each associated with partial gastrectomy were also studied.

Results

The resting plasma levels of ascorbic acid are given in Table I. The patients with low serum B.12 levels had significantly* lower plasma ascorbic acid levels than the controls even though the latter consisted of patients in similar circumstances, resident medical staff whose diets contained very little uncooked vitamin C

TABLE I

"Resting" plasma concentrations of ascorbic acid

Group	B.12 therapy	Number of subjects	Mean plasma ascorbic acid mg./100 ml.	S.E. mg./100 ml.	Range mg./100 ml.	S.E. of difference
Normal		32	0·86	0·06	0·25 to 1·44	
Pernicious anæmia	Before	12	0·38	0·07	0·10 to 0·91	± 0·10
	After		0·52	0·07	0·12 to 0·91	
Adult cœliac disease	Before	7	0·31	0·05	0·18 to 0·54	± 0·10
	After		0·43	0·12	0·10 to 1·01	
Regional ileitis	Before	7	0·46	0·10	0·14 to 0·90	± 0·11
	After		0·27	0·04	0·18 to 0·54	
	Never B.12 deficient	8	0·52	0·10	0·2 to 1·2	

containing foods and medical students on a similar diet. After B.12 therapy, a rise in the resting plasma ascorbic acid level occurred in the patients with pernicious anæmia and adult cœliac disease but was not significant. The resting plasma ascorbic acid concentrations in patients with regional ileitis with and without B.12 deficiency did not significantly differ, nor did B.12 therapy produce a significant change in the B.12 deficient patients.

* A difference between two means greater than twice the standard error of the difference between the means was taken to be significant.

METHODS

The resting level of ascorbic acid was determined, on plasma from heparinized venous blood, from in-patients and out-patients who had received no vitamin C for 12 hours, but had eaten a breakfast of toast and tea preferably without milk.

The intravenous ascorbate test was based on that described by Will *et al.* (18) and was performed on patients in the resting state, 1 to 2 hours after vitamin C free breakfast. After taking a resting blood specimen and a timed urine specimen, 250 mg. sodium ascorbate in 2 ml. water were injected intravenously. Then further venous blood specimens were taken from the other arm at 5, 15, 30 and 45 minutes after the injection. At the end of the test, the urine was again collected.

Ascorbic acid was determined in the plasma by Owen and Iggo's (15) modification of the method of Evelyn, Malloy and Rosen (7). Dehydroascorbic acid was taken as the difference between this figure and the total ascorbic acid determined by the method of Roe and Kuether (16). Urine ascorbic acid was determined on freshly voided acidified urine by direct titration with 2:6: dichlorphenolindophenol.

The serum B.12 levels were assayed with *Lactobacillus leichmannii* using the method of Meynell, Cooke, Cox and Gaddie (9) the normal range being 105 to 450 μμg./ml..

In patients with regional ileitis, the activity of the underlying disease was assessed by estimating the seromucoid content of the patient's sera (4).

A full blood count with absolute values was performed at the same time as the test and, where indicated, an examination of the bone marrow. A dietary history was taken from each patient.

Those patients with low serum B.12 levels were maintained on a diet similar to that which they had been taking but excluding fresh fruit and uncooked vegetables until the intravenous ascorbate test had been repeated after B.12 saturation.

The subjects of the investigation

The resting plasma levels were determined in normal subjects whose ages ranged from 20 to 59. These comprised a number of patients with recent traumatic orthopædic conditions, medical students and hospital staff. The intravenous ascorbic acid test was performed in 20 of these, the age range being the same. Twelve patients with pernicious anæmia in relapse had their resting plasma ascorbic acid level determined together with two patients with subacute combined degeneration of the cord. All these patients fulfilled the criteria for this diagnosis in that they had a macrocytic anæmia, megaloblastic change in the bone marrow, a histamine fast achlorhydria, a low serum B.12 (mean 46·5, range 30–70 μμg. per ml.) level and complete hæmatological remission on B.12 therapy. Twelve of these patients had intravenous ascorbate tests in relapse and in B.12 induced remission. Seven patients with adult cœliac disease who had low serum B.12 levels were studied before and after B.12 therapy. Three of these patients were anæmic and had a megaloblastic erythropoiesis. All patients fulfilled our criteria for the diagnosis of adult cœliac disease in that their average daily excretion of fæcal fat was in excess of 6 g., their folic acid excretion tests were abnormal (5) and no localised small bowel lesion was demonstrable radiologically. Their mean serum B.12 was 69·5, range 12 to 100 μμg./ml..

Seven patients with regional ileitis and an associated vitamin B.12 deficiency (mean serum B.12 50·5, range 25 to 100 μμg. per ml.) none of whom had a megaloblastic erythropoiesis, were also studied. These results were compared with 8 similar patients who were not B.12 deficient. Twenty-one observations of the

Clin. Sci. 17:681-692 1958

Clin. Sci., 1958, 17, 681.

AN INTER-RELATIONSHIP BETWEEN ASCORBIC ACID AND CYANOCOBALAMIN

By E. V. COX, R. GADDIE, D. MATTHEWS, W. T. COOKE and M. J. MEYNELL

(*From the General Hospital, Birmingham*)

Received May 13, 1958

DEFICIENCY of cyanocobalamin (B12) is closely associated with megaloblastic anæmia and a deficiency of ascorbic acid gives rise to scurvy. Megaloblastic anæmia occurred with unexpected frequency in scurvy in infants (8). Recently Brown (3) has observed hæmatological remission with ascorbic acid therapy in a patient with scurvy and megaloblastic erythropoiesis. Alt, Chinn and Farmer (1) observed a significant reduction of the plasma levels of ascorbic acid in pernicious anæmia, and Dyke, Della Vida and Delikat (6) claimed that administration of ascorbic acid was necessary for restoration of normal blood counts, but both these observations have been severely criticised (10). Mohr (11) found difficulty in saturating a patient suffering from sprue by intravenous administration of ascorbic acid. Boscott and Cooke (2) confirmed this observation in a small series of patients with megaloblastic anæmia with idiopathic steatorrhœa and noted the presence of excess amounts of parahydroxyphenylacetic acid in the urine as in scurvy. They considered that the apparent deficiency of ascorbic acid could not be attributed either to dietary or absorptive defects. Will, Mueller, Glazer, Friedman and Vilter (18) found that the rate of disappearance from the plasma of ascorbic acid injected intravenously was increased in five out of 7 patients with pernicious anæmia and that in contrast the levels of the plasma dehydroascorbic rose unduly in these five patients. These abnormalities had disappeared seven days after vitamin B.12 therapy. These selected observations would suggest an association between these two vitamins, but as yet definite evidence of this in an adequate number of patients is lacking. The investigations reported in this paper were undertaken to determine whether there was indeed any relationship between B.12 and ascorbic acid.

Therefore the concentration of ascorbic acid in the plasma of patients with low serum B.12 levels was compared with those found in the same patients following treatment with cyanocobalamin, with those in patients with regional ileitis but without B.12 deficiency and with the values found in normal subjects. The rate of disappearance of an intravenous injection of sodium ascorbate was also studied in some of these subjects. Other factors of possible significance were also considered, such as the degree and type of anæmia, the patients' diet and the activity of the underlying disease.

We wish to thank Dr. J. A. H. Waterhouse, Department of Medical Statistics, University of Birmingham, for his advice on the statistics. We also gratefully acknowledge the Medical Research Endowment Fund of the United Birmingham Hospitals for their financial assistance.

makes them sensitive to control, so that the amount of pigment may be quickly changed. Lack of vitamin C accelerates the production of pigment. Pigment once produced attracts and holds vitamin C.

4. Vitamin C, tyrosine and dopa (levo-di-oxyphenylalanine) absorb selectively the erythema-producing ultraviolet rays, the power of absorption increasing in the given order when the same concentrations and thickness are used. Tyrosine subjected to ultraviolet irradiation and dopa possess greater power to absorb actinic rays than the same non-irradiated substances. The results of the clinical experiments are those which were predictable from the spectrograms alone.

ABSTRACT OF DISCUSSION

Dr. S. W. Becker, Chicago: Dr. Cornbleet has done a large amount of work on this difficult problem. There are two facts which seem to contradict each other: (1) Vitamin C inhibits the dopa reaction, and (2) vitamin C is found in the skin in proportion to the melanin content of the epidermis. It would be interesting to determine whether or not pigment that was not in contact with the epidermis, such as pigment in a blue nevus or in a mongolian spot, would show an increased reaction to silver. This is important because there may be some chemical changes in the epidermis which act independently of the pigment. Ropshaw a few years ago concluded from studies with sulfhydryl that he made on the epithelium that this had something to do with pigment formation.

My co-workers and I repeated his work, using a blue nevus where mesoblast-containing pigment is located deep in the dermis, and we obtained no reaction to sulfhydryl. Failure to demonstrate vitamin C in the vitiliginous areas may be due to the factors which have caused the vitiligo and not to the lack of pigment itself. These factors are not understood at present. The accentuation of the reaction to silver which Dr. Cornbleet has shown so well may be simply a more rapid or a more intense reaction.

Cursory examination of the section presented by Dr. Cornbleet shows the darkening to be confined to the melanin granules; that is, there is no silver deposited in the tissue. The reaction seems to be all in the melanin, but whether a stronger silver solution without the injection of vitamin C would produce the same reaction or whether the same concentration of silver used on the same section for a longer time would produce the same reaction should be determined.

The darkened dopa, which is called "dopa melanin" by Block, may exclude ultraviolet rays by its physical rather than by its chemical properties. I am not well enough versed in chemistry to be able to comment on the action of copper in connection with this phenomenon, although it is known that small amounts of copper do act as catalyzers and give chemists no end of trouble. This study opens up a fruitful field for further research.

Dr. D. M. Pillsbury, Philadelphia: I wish to congratulate Dr. Cornbleet on this experimental amplification of a new concept. Some time ago Dr. Kulchar and I observed two patients with vitiligo treated by the injection of gold sodium thiosulfate who exhibited a gold dermatitis confined to the areas of depigmentation, the normal pigmented skin remaining unchanged.

In this connection we had occasion to review the literature on the varying physiology of pigmented and depigmented skin and found that a considerable amount of experimental work indicating quite marked and, as yet, unexplained

variations has accumulated. Among others Kreibich has shown that the reaction to irritants in pigmented and nonpigmented skin varies considerably. I believe that studies such as that of Dr. Cornbleet may furnish a more logical basis for explanation of these differences, and I shall await reports of his future studies with interest.

Dr. Theodore Cornbleet, Chicago: The discussion has emphasized how much further work must be done in this field before any final conclusions may be drawn.

Dr. Bachem of the University of Illinois, allowed me to use his laboratory and gave suggestions for the carrying out of the spectroscopic work.

absorption band from 289 millimicrons toward shorter wavelengths when vitamin C is present in a concentration of 0.1 per cent and a thickness of 7 mm. The erythema-producing rays are known to occupy the zone between 313 and 240 millimicrons. Vitamin C could therefore be of service in screening out the rays from 289 to 240 millimicrons. Similarly, one may study dopa and tyrosine. Undarkened or unoxidized dopa and tyrosine absorb the ultraviolet rays more selectively than vitamin C. Oxidized dopa and tyrosine, however, absorb actinic rays much more strongly and therefore allow fewer ultraviolet rays to go through than does vitamin C. Oxidized tyrosine irradiated with ultraviolet rays under the conditions which I used, namely, a concentration of 0.1 per cent and a thickness of 7 mm., absorbs all actinic rays shorter than 297 millimicrons. Oxidized dopa under the same conditions as tyrosine and of the same concentration and thickness absorbs all the ultraviolet rays shorter than 313 millimicrons and therefore allows no rays to pass through which may produce an erythematous reaction on the skin.

These facts as elicited by the spectrum were subjected to a clinical check. Flat quartz vessels 7 mm. deep were filled, respectively, with 0.1 per cent darkened dopa, a 0.1 per cent solution of vitamin C, a mixture of 0.05 per cent of each in the total and water. They were placed next to the skin, each at a different place. Through them the rays from a water-cooled quartz mercury vapor arc lamp were applied for two minutes. The skin was examined the next day. A strong erythema developed in the area which had been covered by the water vessel; that which had been covered by the vessel containing vitamin C showed a less intense erythema; that which had been irradiated through a mixture of vitamin C and darkened dopa exhibited a still milder erythema, and that which had been screened by the darkened dopa alone showed no reaction. Thus the expectations deduced from the spectrograms were fulfilled clinically.

SUMMARY AND CONCLUSIONS

1. Pigment is increased in the skin when the depots of vitamin C in the adrenal glands and in the rest of the body are depleted. The excess pigment in the skin in Addison's disease and scurvy is absorbed when vitamin C is administered.

2. Pigment and vitamin C occur together in the skin. Pigment is apparently the anchor that holds vitamin C. If pigment is not present vitamin C is not stored in the skin in demonstrable amounts. Larger amounts of pigment can hold greater amounts of vitamin C.

3. Copper also is associated with vitamin C and melanin in the skin. Copper hastens the darkening and precipitation of dopa by ultraviolet rays. Vitamin C retards this precipitation. The presence of all three

down of pigment from its propigment makes it possible for more propigment to stream into the vicinity. However, because of the low concentration of the propigmentary substance in solution, pigment can be laid down only a little at a time. Hence, it is useful to have a substance like copper to hasten the precipitation and allow the influx of new propigment in the solution to take the place of that which has been precipitated. Particularly significant is the fact that copper hastens the breaking down of vitamin C, since the latter slows the production of pigment from propigment, as judged by the behavior of dopa and vitamin C. When the combination is viewed in this manner the increase

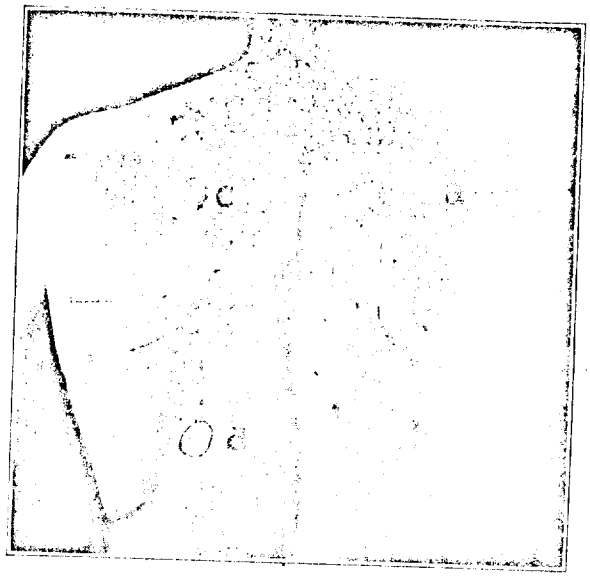


Fig. 3.—Cutaneous reactions from water-cooled quartz mercury vapor arc lamps with filters of: (*a*) water, (*b*) vitamin C, (*d*) dopa and (*c*) vitamin C and dopa combined. Vitamin C reduces the erythematous reaction while dopa prevents it altogether. The cutaneous reactions are predictable from the spectrograms.

in pigment in Addison's disease and scurvy is readily understood. The relative lack of vitamin C gives the copper free rein to produce pigment without the restraint the vitamin exercises by its presence. That the vitamin C content of the skin may be quickly reduced can be shown in the guinea-pig, as a scorbutic diet exhausts the vitamin C content of the skin long before that of the adrenal gland.

Could vitamin C exercise any positive action of its own, other than merely subserving the changes in the rate of pigment production? It may have at least one positive function, as shown by a study of its spectral properties. The ultraviolet absorption spectrum reveals a strong

A trace of copper chloride added to vitamin C is sufficient to show this action, as determined by the amount left by the titration method of Tillmans.

It was pointed out that there is an apparent paradox between the clinical increase in pigment in vitamin C-deficient states and the increase of vitamin C in the skin associated with increased pigmentation. A similar paradox arises with copper, vitamin C and pigment. The metal apparently facilitates both the production of pigment and the destruction of vitamin C. Yet all three are present in proportion, and when one is increased the other two are likewise found in greater concentration. Reflection on this associate trio and its mutual reactions leads one to believe that the check which vitamin C exercises on the oxidation of dopa simply becomes more sensitive to manipulation by the presence

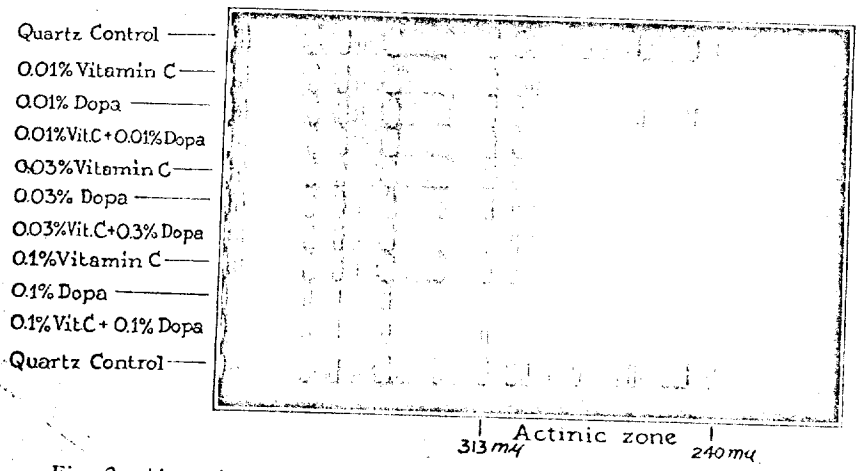


Fig. 2.—Absorption spectra of vitamin C and oxidized dopa together and separately in various concentrations. The amount of actinic rays absorbed depends on concentration of material and on whether it is used singly or in combination.

of the copper. This is shown by exposing dopa and copper in one dish to ultraviolet rays and the same combination with vitamin C in another. Pigmentary precipitates occur more quickly and with greater concentration in the dish without vitamin C. The same reaction takes place when the copper is not present, but it occurs much more slowly than when copper is added.

The solubility of such phenol derivatives as tyrosine and dopa is not great. Even in boiling water it is barely possible to dissolve enough dopa to make a 0.1 per cent solution. If some of the dopa is removed from solution by precipitation through the use of ultraviolet rays, fresh dopa can be added to the same solution. If no precipitation takes place no fresh dopa will be taken up into the solution. By analogy, the laying

nitrate. It was necessary to use the skin from a brunette, because in Negro skin the pigment alone gives too intense a stain and in a fair skin it contains too little vitamin C for a satisfactory comparison by such a crude method.

Of course, the test would be more decisive if the minimal amount of staining of a fair skin could be definitely increased by injecting vitamin C into the skin. The increase in staining is demonstrable, but it is not great unless there is a good deal of pigment present, as in the skin of Negroes. A certain quantity of solution of vitamin C in definite concentration injected into Negro skin increased the intensity of the silver stain of the cut section much more than the same quantity of a solution of like concentration injected into fair skin. Indeed, the increase in the staining intensity varied directly with the amount of pigment present. It appears that greater amounts of pigment bind larger quantities of injected vitamin C.

If the amount of vitamin C influencing the silver stain depends on the amount of pigment present, then there should be no vitamin C in leukodermatous skin. This fact was demonstrated. A 10 per cent concentration of vitamin C was injected intradermally into leukodermatous skin at the border between pigmented and depigmented portions; the skin was excised, and staining showed increased darkening of the pigmented but no change in the depigmented area. Likewise when an area that contained a freckle was similarly dealt with, the hyperpigmented portion became intensely stained but there was little change in the normally pigmented surrounding zone. It is difficult to determine what becomes of the vitamin C which is injected into leukodermatous skin. It does not reduce silver nitrate. Consequently, it may be either rapidly absorbed or chemically changed. It is hardly probable that all of it could be absorbed in the short time elapsing between the injection and the excision. Consequently one must assume that skin without pigment probably changes vitamin C to the oxidized form. At the same time it is difficult to escape the deduction that reduced vitamin C can remain as such in the skin only in the presence of pigment.

Vitamin C and pigment are not the only associate substances in the skin. There is a third—copper. It has been shown that the amount of copper in the skin is proportionate to the amount of pigment present.[10] Especially is this so in the hairs, so that dark hairs contain much more copper than unpigmented hairs. One may surmise that the copper present in the vicinity of pigment is a catalyzer which quickens the production of the pigment. This theory is supported by the fact that copper hastens the oxidation of dopa either with or without skin extracts. Copper, too, has an accelerating action on the oxidation of vitamin C.

10. Sarata, Uichiro: Jap. J. M. Sc., II, Biochem. **3**:79, 1935.

What is known of the relation between vitamin C and pigment seems to me to present a basic conflict. On the one hand, loss of vitamin C by diet or by destruction of the adrenals produces an increase in pigmentation which is removed by the administration of vitamin C; on the other hand, pigment depots in the skin are associated with vitamin C, and, in some cases at least, the areas rich in pigment contain excessive amounts of vitamin C. It occurred to me that pigment may be a means of storing vitamin C and that an increase of the former in cases of scurvy and adrenal destruction may make it possible for the body to retain its vitamin C by increasing the activity of the skin as a storage center.

To test this theory the reducing action of vitamin C on silver nitrate was used. Since both pigment and vitamin C in the skin stain black with silver nitrate, caution was necessary to find some means either to increase or to decrease one component while the other was held sta-

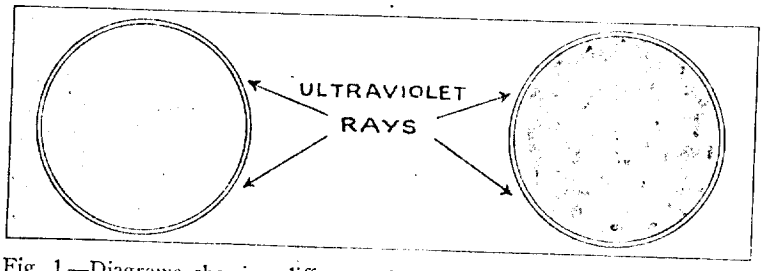


Fig. 1.—Diagrams showing difference in darkening or oxidation of dopa due to vitamin C. The difference is suggestive of the increase of cutaneous pigment in vitamin C deficiency.

tionary. Pigment is fairly inert and does not change much by ordinary manipulation of the skin. If the vitamin C content of the skin is increased the silver stain should be intensified, since its effect is added to that of the pigment and the vitamin C already present. This was found by trial to be the case. When a 10 per cent concentration of vitamin C was injected intradermally and the site of injection immediately removed by biopsy, the specimen stained more intensely than a symmetrical area from the same person into which vitamin C had not been injected.

The conclusion was then reached that if an increase in the amount of vitamin C could be shown by staining, it might also be possible to show a decrease in the amount by the same method. Since ultraviolet rays are an oxidizing agent and vitamin C is a reducing agent, irradiation of the skin might exhaust its vitamin C content. As a test a small area of skin on the back was irradiated for three and one-half minutes and then removed by biopsy together with a nonirradiated symmetrical area. The irradiated area gave a weaker stain with silver

been observed[4] that the skin contains appreciable quantities of vitamin C, the amount being least in the horny layer, slightly larger in the dermis and by far largest in the basal and malpighian layers. Hyperpigmented structures, such as pigmented nevi, are excessively rich in vitamin C. A guinea-pig on a scorbutic diet loses much of the vitamin C contained in its skin. The adrenals too lose vitamin C when the animal is on a scorbutic diet, but they retain a sufficient quantity after the skin loses its store.

The apparent clinical and experimental relationship of vitamin C to pigment has spurred workers to trace the relationship in other directions. The dopa reaction described by Bloch is a popular and accepted method for studying pigment. This reaction takes place when levo-di-oxyphenylalanine comes in contact with the basal layer of the skin, and it indicates the capacity of the tested site to form pigment. It was found[5] that vitamin C interferes with this reaction. It is believed[6] that the agent of the intervention is not the dopa oxidase in the cutaneous tissue but the dopa itself. I believe that the inhibition may be explained by the successful counteraction of the reducing qualities of vitamin C on the oxidation which occurs in the dopa reaction.

Since Vitamin C prevents the dopa reaction it could conceivably prevent formation of pigment if given in adequate amounts. To test this hypothesis castrated guinea-pigs were given injections of an estrogenic substance.[7] This procedure ordinarily causes pigmentation and enlargement of the nipples and areolae. Large quantities of vitamin C were administered before and at the same time as the estrogenic substance. The vitamin C did not prevent the pigmentation of the nipples induced by the estrogenic substance. Thus vitamin C does not reduce the normal amount of pigment present in the skin but affects only the excess quantity which forms when either the intake of vitamin C or the amount stored in the body is decreased.

The question of the increase in amount of pigment due to ultraviolet radiation and its relation to vitamin C has been investigated. The reports are conflicting. Some workers[8] have stated that there is no difference in behavior of the skin in this respect whether vitamin C is given in large amounts or not. Others[9] have reported that the administration of an adequate amount of vitamin C decreases the pigmentation resulting from exposure to ultraviolet rays and that the pigmentation disappears more rapidly when the supply of vitamin C is adequate.

---

4. Giroud, A.; Leblond, C. P.; Ratsimamanga, K., and Rabinowicz, M.: Bull. Soc. franç. de dermat. et syph. **42**:482 (March) 1935.
5. Schroeder, H.: Klin. Wchnschr. **13**:553, 1934.
6. Grueneberg, T., and Schade, H. A.: Klin. Wchnschr. **13**:1353, 1934.
7. Jadassohn, W., and Schaaf, F.: Klin. Wchnschr. **13**:845, 1934.
8. Drigalski, Wolf: Klin. Wchnschr. **13**:1354, 1934.
9. Schade, H. A.: Klin. Wchnschr. **14**:60, 1935.

Summary

The administration of various drugs to rats stimulates L-ascorbic acid synthesis from D-glucose and D-galactose through the glucuronic acid pathway. Increased metabolism of L-ascorbic acid also occurs in drug-treated rats. Although the enzymatic basis for increased ascorbic acid synthesis is not known, drugs appear to act on some step before the formation of D-glucuronic acid. The effect of Chloretone pretreatment to increase the activity of UDPG dehydrogenase in liver has been pointed out. It is of interest that drugs that stimulate ascorbic acid synthesis also induce the synthesis of drug-metabolizing enzymes in liver microsomes. It is possible that the effect of drugs on ascorbic acid synthesis also reflects induced enzyme synthesis.

Results presented here show that vitamin C deficiency makes guinea pigs more sensitive to the muscular-relaxant drug zoxazolamine by decreasing the activity of the liver microsomal enzyme system required for the metabolism of zoxazolamine.

References

1. LONGENECKER, H. E., H. H. FRICKE & C. G. KING. 1940. The effect of organic compounds upon vitamin C synthesis in the rat. J. Biol. Chem. **135:** 497.
2. CONNEY, A. H. & J. J. BURNS. 1959. Effect of foreign compounds on ascorbic acid biosynthesis and on drug-metabolizing enzymes. Nature. **184:** 363.
3. BURNS, J. J., A. H. CONNEY, P. G. DAYTON, C. EVANS, G. R. MARTIN & D. TALLER. 1960. Observations on the drug-induced synthesis of D glucuronic, L-gulonic and L-ascorbic acids in rats. J. Pharmacol. Exptl. Therap. **129:** 132.
4. BURNS, J. J., E. H. MOSBACH & S. SCHULENBERG. 1954. Ascorbic acid synthesis in normal and drug-treated rats, studied with L-ascorbic-1-C^{14} acid. J. Biol. Chem. **207:** 679.
5. GANGULI, N. C., S. C. ROY & B. C. GUHA. 1956. Studies on the biosynthesis of L-ascorbic acid by the rat. I. Effect of adenosine triphosphate and other compounds on the synthesis stimulated by Chloretone. Arch. Biochem. Biophys. **61:** 211.
6. MINESITA, T., K. YAMAGUCHI, K. TAKEDA & K. KOTERA. 1956. Shionogi Kenkyusho Nempo. **6:** 131.
7. TOUSTER, O. 1959. Pentose metabolism in pentosuria. Am. J. Med. **26:** 724.
8. BURNS, J. J. 1959. Biosynthesis of L-ascorbic acid; basic defect in scurvy. Am. J. Med. **26:** 740.
9. STROMINGER, J. L. 1960. Mononucleotide acid anhydrides and related compounds as intermediates in metabolic reactions. Physiol. Revs. **40:** 55.
10. BURNS, J. J. & A. H. CONNEY. 1960. Water-soluble vitamins: Part II. Ann. Rev. Biochem. **29:** 413.
11. BURNS, J. J., C. EVANS & N. TROUSOF. 1957. Stimulatory effect of barbital on urinary excretion of L-ascorbic acid and non-conjugated D-glucuronic acid. J. Biol. Chem. **227:** 785.
12. EVANS, C., A. H. CONNEY, N. TROUSOF & J. J. BURNS. 1960. Metabolism of D-galactose to D-glucuronic acid, L-gulonic acid and L-ascorbic acid in normal and barbital-treated rats. Biochim. et Biophys. Acta. **41:** 9.
13. ENKLEWITZ, M. & M. LASKER. 1935. The origin of 1-xyloketose (urine pentose). J. Biol. Chem. **110:** 443.
14. BURNS, J. J. & C. EVANS. 1956. The synthesis of L-ascorbic acid in the rat from D-glucuronolactone and L-gulonolactone. J. Biol. Chem. **223:** 897.
15. CONNEY, A. H., C. DAVISON, R. GASTEL & J. J. BURNS. 1960. Adaptive increases in drug metabolizing enzymes induced by phenobarbital and other drugs. J. Pharmacol. Exptl. Therap. **130:** 1.
16. CONNEY, A. H., N. TROUSOF & J. J. BURNS. 1960. The metabolic fate of zoxazolamine (Flexin) in man. J. Pharm. Exptl. Therap. **128:** 333.
17. CONNEY, A. H., E. C. MILLER & J. A. MILLER. 1956. The metabolism of methylated aminoazo dyes. V. Evidence for induction of enzyme synthesis in the rat by 3-methyl cholanthrene. Cancer Research. **16:** 450.

18. CONNEY, A. H., E. C. MILLER & J. A. MILLER. 1957. Substrate-induced synthesis and other properties of benzpyrene hydroxylase in rat liver. J. Biol. Chem. **228:** 753.
19. TOUSTER, O., R. W. HEFTER & R. A. SILER. 1960. The inhibition by ethionine of the enhancement of ascorbic acid excretion by barbital and 3-methylcholanthrene in rats. Biochem. Biophys. Research Comm. **3:** 248.
20. ISHIDATE, M. & E. HOSOYA. 1959. Recent advances in the study of glucuronic acid in Japan. Glucuronic Acid Symposium of Europe. Zurich, Switzerland.
21. RICHARDS, R. K., K. KUSTER & T. J. KLATT. 1941. Effect of vitamin C deficiency on action of different types of barbiturates. Proc. Soc. Exptl. Biol. Med. **48:** 403.
22. RICHARDS, R. K. 1947. Effects of vitamin C deficiency and starvation upon the toxicity of procaine. Anesthesia & Analgesia. **26:** 22.
23. AXELROD, J., S. UDENFRIEND & B. B. BRODIE. 1954. Ascorbic acid in aromatic hydroxylation. III. Effect of ascorbic acid on hydroxylation of acetanilide, aniline and antipyrine *in vivo*. J. Pharmacol. Exptl. Therap. **111:** 176.
24. UDENFRIEND, S., C. T. CLARK, J. AXELROD & B. B. BRODIE. 1954. Ascorbic acid in aromatic hydroxylation. I. A model system for aromatic hydroxylation. J. Biol. Chem. **208:** 731.
25. BRODIE, B. B., J. AXELROD, P. A. SHORE & S. UDENFRIEND. 1954. Ascorbic acid in aromatic hydroxylation. II. Products formed by reaction of substrates with ascorbic acid, ferrous ion, and oxygen. J. Biol. Chem. **208:** 741.
26. DALGLIESCH, C. E. 1955. Nonspecific formation of hydroxylated metabolites of the aromatic amino acids. Arch. Biochem. Biophys. **58:** 214.

1653

Dermatol. Syphilol. 35: 471-479 1937

1675

VITAMIN C AND PIGMENT

THEODORE CORNBLEET, M.D.
CHICAGO

In 1928 Szent-Györgyi[1] obtained from the adrenal cortex and from certain fruits an acid (cevitamic acid) which, he showed, had antiscorbutic properties. Shortly afterward the acid was identified as vitamin C.

The specific qualities of vitamin C in preventing scurvy led to intensive investigations of the relation of the adrenal gland to scurvy. Szent-Györgyi[1] observed that the cut surface of the gland reduced silver nitrate. The staining is accomplished through the vitamin C contained in the gland, and the intensity of the stain can generally be used as a rapid indicator of the amount of vitamin C present. It was found that in guinea-pigs receiving a scorbutic diet the original blackening produced by silver nitrate on the cut surface of the adrenal gland begins to turn gray on the first day. When the diet is continued for six days, the intensity of the stain is so diminished as to be barely visible. If vitamin C is then administered to the scorbutic animal, the cut surface of the adrenal gland again stains black with silver nitrate.

Clinically an increase in the pigmentation of the skin may occur in scurvy. This condition as well as other features of the disease is quickly eradicated by the administration of vitamin C. The increase in pigmentation which accompanies Addison's disease is one of the cardinal features of this disorder and is the result of partial or entire destruction of the adrenal glands by tuberculous involvement. Since the association of vitamin C with the adrenal glands has been recognized it has been noted that if sufficient vitamin C is administered to patients with Addison's disease the hyperpigmentation is reduced to normal.[2] The other symptoms and signs are not influenced. Another pigmentary process which has been successfully influenced by the administration of vitamin C is chloasma.[3]

Because of the relationship between these cutaneous disorders and vitamin C, its presence and amount in the skin are of interest. It has

---

From the Department of Dermatology, College of Medicine, University of Illinois, Service of Dr. F. E. Senear.

Read before the Section on Dermatology and Syphilology at the Eighty-Seventh Annual Session of the American Medical Association, Kansas City, Mo., May 14, 1936.

1. Szent-Györgyi, Albert: Biochem. J. **22**:1387, 1928.
2. Szuele, Dionysius: Deutsche med. Wchnschr. **59**:651, 1933.
3. Sellei, J.: Fortschr. d. Therap. **11**:26, 1935.

can be completely overcome by the simultaneous administration of the amino acid methionine. In view of the effect of drugs in inducing the synthesis of drug-metabolizing enzymes, it is possible that drugs may also stimulate ascorbic acid synthesis by inducing the synthesis of enzymes of the glucuronic acid pathway. In this connection it is of interest to note that administration of ethionine to rats has been recently reported to block the ability of barbital and 3-methylcholanthrene to increase ascorbic acid synthesis.[19]

The mechanism by which drugs can both stimulate ascorbic acid biosynthesis and increase the activity of drug-metabolizing enzymes in liver microsomes is not known, but these effects appear to represent adaptive responses to drug administration that occur in the absence of the adrenal gland.[11,17] We have

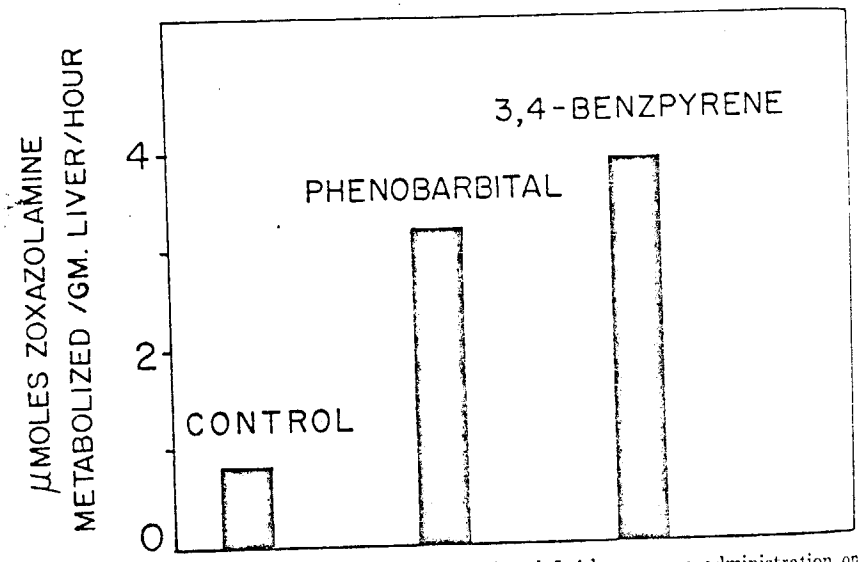


FIGURE 6. Stimulatory effect of phenobarbital and 3,4-benzpyrene administration on the activity of zoxazolamine hydroxylase in rat liver microsomes. Enzyme assays were carried out in the presence of a system that generated reduced triphosphopyridine nucleotide.[15]

been intrigued by the possibility that both of these responses to drugs may represent adaptations that are beneficial to the animal. Certainly, it is apparent that increased activity of drug-metabolizing enzymes in response to drug administration can result in accelerated drug detoxication. The effect of drugs to stimulate the glucuronic acid pathway (FIGURE 3) leads to increased production of UDPGA, D-glucuronic acid, and L-ascorbic acid. UDPGA is necessary for the conjugation of drugs as glucuronides. D-Glucuronic acid, when administered as its lactone, has been reported to protect animals from various toxic agents.[20] The possibility that L-ascorbic acid may be involved in the metabolism of drugs is pointed out in the following section.

Effect of Ascorbic Acid on Drug Metabolism

Reports have appeared in the literature indicating that vitamin C-deficient guinea pigs are unusually sensitive to various drugs. For example, Richards

and his co-workers reported that vitamin C-deficient guinea pigs were more sensitive than normal guinea pigs to pentobarbital and to procaine.[21,22] In other studies Axelrod and his co-workers[23] have shown that the rate of *in vivo* hydroxylation of acetanilid and aniline was decreased in vitamin C-deficient guinea pigs.

Recent studies in our laboratory have indicated that vitamin C-deficient guinea pigs are more sensitive to the muscle-relaxant drug zoxazolamine than are normal guinea pigs. The increased sensitivity toward zoxazolamine can be explained by decreased activity of the enzyme system in liver microsomes that metabolizes this drug (TABLE 6). In these experiments the guinea pigs were placed on a scorbutogenic diet. One half of the animals were given daily doses of 10 mg. of ascorbic acid orally; the other half were given glucose. The animals were maintained on the scorbutogenic diet for 10 to 14 days, and at that time they showed no obvious signs of scurvy. It may be seen that the duration of zoxazolamine paralysis in guinea pigs receiving ascorbic acid supple-

TABLE 6

EFFECT OF ASCORBIC ACID DEFICIENCY IN GUINEA PIGS ON DURATION OF ZOXAZOLAMINE PARALYSIS AND ON THE ZOXAZOLAMINE-METABOLIZING ENZYME SYSTEM IN LIVER MICROSOMES*

Diet	Duration of zoxazolamine paralysis (min.)	*In vitro* metabolism (μg. zoxazolamine metabolized)
Ascorbic acid-supplemented†	156 ± 41 (10)	36 ± 12 (15)
Ascorbic acid deficient	309 ± 27 (20)	12 ± 8 (15)

* The duration of zoxazolamine paralysis was determined after the intraperitoneal injection of 100 mg./kg. of zoxazolamine. The *in vitro* enzyme assays were carried out by incubating liver microsomes from 375 mg. of liver with 100 μg. of zoxazolamine for 15 min. in the presence of a system that generated reduced triphosphopyridine nucleotide.[15] The number of animals used are indicated in parentheses.

† The animals received 10 mg. of ascorbic acid orally each day.

ment was 156 min., whereas the guinea pigs that did not receive the vitamin were paralyzed for 309 min. The increased sensitivity of vitamin C-deficient guinea pigs was paralleled by decreased activity of the liver microsomal enzyme system that metabolizes zoxazolamine. Thus liver microsomes from ascorbic acid-supplemented guinea pigs metabolized an average of 36 μg. of zoxazolamine, while microsomes from the vitamin C-deficient animals metabolized an average of only 12 μg. of zoxazolamine. The addition of ascorbic acid *in vitro* to microsomes obtained from vitamin C-deficient guinea pigs did not increase the activity of this enzyme system. It should be emphasized that the decreased activity of the zoxazolamine-metabolizing enzyme system occurs at an early stage in vitamin C deficiency; this is observed before gross deficiency symptoms such as loss of weight and hair and severe joint manifestations are evident.

A model system consisting of L-ascorbic acid, ferrous ion, ethylenediaminetetraacetic acid, and oxygen has been shown to catalyze the hydroxylation of such aromatic compounds as acetanilid, antipyrine, aniline, anthranilic acid, and kynurenine to yield products identical with those formed in the body.[24-26] The importance of this system for the metabolism of drugs in the animal remains to be established.

1653

per cent. These results are consistent with observations that drugs do not accelerate the *in vivo* conversion of D-glucuronolactone or L-gulonolactone to L-ascorbic acid.[11] It therefore appears that drugs that stimulate ascorbic acid synthesis act at some step prior to D-glucuronic acid. The effects of Chloretone pretreatment on glycolytic enzymes, hexokinase, or glucose-6-phosphatase have not yet been investigated. The levels of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphoglucomutase, and UDPG pyrophosphorylase were not significantly changed by Chloretone treatment. It was found, however, that the level of UDPG dehydrogenase, the system required to convert uridine diphosphoglucose to uridine diphosphoglucuronic acid (UDPGA), was elevated about 100 per cent in Chloretone-treated rats. The activity of the enzyme system in liver particulate that metabolizes UDPGA to D-glucuronic acid-1-phosphate was not stimulated. Only trace amounts of D-glucuronic acid were formed when UDPGA was incubated with livers from either control or Chloretone-treated rats.

TABLE 5
EFFECT OF PRETREATMENT OF RATS WITH CHLORETONE ON THE ACTIVITY OF UDPG DEHYDROGENASE

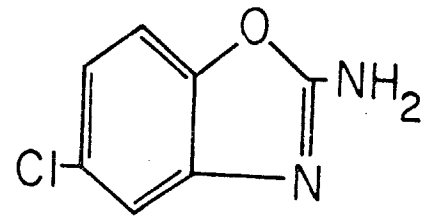
Rats	Relative enzyme activity			
	Fresh	Aged*	Aged* with UDPG	Aged* with UDPGA
Control	100	0	33	77
Chloretone-treated†	185	125	—	168

* The enzyme preparation was allowed to stand at 4° C. for 7 days.
† Rats were pretreated with 40 mg. of Chloretone orally for 7 days prior to the experiment.

Of the enzymes studied, only UDPG dehydrogenase levels were increased by Chloretone administration; therefore, this enzyme system was investigated in more detail. A number of the results are presented in TABLE 5. The interesting observation was made that upon standing at 4° C. the UDPG dehydrogenase activity in the high-speed liver supernatant fraction of control rats decreased in activity. Thus little or no UDPG dehydrogenase activity was observed in enzyme preparations that had been allowed to age at 4° C. for 5 to 7 days. On the other hand, similar enzyme preparations obtained from Chloretone-treated rats decreased only slightly in UDPG dehydrogenase activity when aged. This decrease in UDPG dehydrogenase with aging at 4° C. is prevented by allowing the enzyme to age in the presence of added UDPG, UDPGA, or uridine triphosphate (UTP), but not with added ATP or Chloretone. These studies raise the interesting possibility that Chloretone administration may accelerate *in vivo* the metabolism of UDPG to UDPGA by stabilizing UDPG dehydrogenase. This could possibly occur by increasing the levels of uridine nucleotides in liver. It is not known at present whether the effect of Chloretone in stimulating the synthesis of glucuronic acid may be explained by a stimulation and stabilization of UDPG dehydrogenase. Further studies are

in progress to determine the relevance of this enzymatic step in ascorbic acid synthesis.

Effect of drugs on drug-metabolizing enzymes. The results given here show that various drugs stimulate the metabolism of D-glucose and D-galactose to L-ascorbic acid through the glucuronic acid pathway. Recent studies indicate that drug administration exerts other biochemical effects. One effect studied in our laboratory is the ability of drugs to stimulate the activity of drug-metabolizing enzymes. It was found that drugs potent in stimulating ascorbic acid synthesis in rats are also potent in stimulating the activity of drug-metabolizing enzymes in liver microsomes.[2,15] Examples of drugs that exert these two effects are 3,4-benzpyrene, 3-methylcholanthrene, Chloretone, phenobarbital, barbital, phenylbutazone, aminopyrine, orphenadrine, and chlorcyclizine. Examples of drug-metabolizing enzymes that are increased by administration of these inducer drugs include those enzymes that metabolize hexobarbital, pentobarbital, aminopyrine, phenylbutazone, 3,4-benzpyrene, and zoxazolamine.



ZOXAZOLAMINE

FIGURE 5.

Studies with zoxazolamine (structure shown in FIGURE 5) illustrate the effect of drugs on a drug-metabolizing enzyme. Zoxazolamine, a muscle-relaxant drug, is hydroxylated in the 6-position by liver microsomes to yield a pharmacologically inactive metabolite.[16] In the experiment shown in FIGURE 6, 40-gm. rats were injected intraperitoneally with 75 mg./kg. of phenobarbital daily for 4 days or with 25 mg./kg. of 3,4-benzpyrene 24 hours before killing the animals and isolating their liver microsomes. It was found that pretreatment of the rats with these compounds markedly increased the activity of the liver microsomal enzyme system that metabolizes zoxazolamine. The increased enzyme activity was correlated with a shortened duration of zoxazolamine paralysis. The duration of action of zoxazolamine in control rats was 730 min., while in phenobarbital- or 3,4-benzpyrene-treated rats it was 102 min. or 17 min., respectively. Several lines of evidence indicate that these stimulators of enzyme activity actually induce the synthesis of drug-metabolizing enzymes.[15,17,18] For instance, the increased activity of drug-metabolizing enzymes observed after pretreatment with phenobarbital or 3,4-benzpyrene can be completely blocked by administration of certain amino acid antagonists such as ethionine, an agent that inhibits protein synthesis. The effect of ethionine in blocking enzyme induction

less was noted in the 3-methylcholanthrene-treated rats. Other studies have shown that barbital increased markedly the level of free D-glucuronic acid in urine, whereas little or no increased excretion was observed following 3-methylcholanthrene treatment. The possibility that 3-methylcholanthrene may stimulate the further metabolism of D-glucuronic acid as well as its formation is being investigated.

Evidence for the stimulatory effect of drugs on the glucuronic acid pathway (FIGURE 3) also comes from the earlier studies of Enklewitz and Lasker[13] in human subjects with essential pentosuria. These subjects possess a metabolic defect that prevents the further metabolism of L-xylulose. When aminopyrine or antipyrine, drugs that stimulate ascorbic acid production, were administered to these subjects, the urinary excretion of L-xylulose was markedly elevated.

The exact mechanism by which drugs stimulate the formation of L-ascorbic acid via the glucuronic acid pathway is not known. The possibility that a renal mechanism is involved has been ruled out since drugs can stimulate ascorbic acid formation in nephrectomized rats.[3] The formation of a glucuronide by the drug is not required since barbital, a drug that stimulates markedly the production of free glucuronic acid and also L-gulonic and L-ascorbic acids, is excreted unchanged in the urine.[11] Several compounds such as borneol and α-naphthol, which are extensively conjugated as glucuronides, do not stimulate in rats the synthesis of L-ascorbic acid or its precursor L-gulonic acid.

TABLE 3

EFFECT OF 3-METHYLCHOLANTHRENE ON METABOLISM OF GALACTOSE-1-C^{14} TO GLUCURONIC ACID PATHWAY INTERMEDIATES EXCRETED IN RAT URINE

Pretreatment	Per cent of administered galactose-1-C^{14} in		
	D-Glucuronic acid	L-Gulonic acid	L-Ascorbic acid
None	<0.05	<0.05	<0.05
3-Methylcholanthrene*	0.14	0.61	0.56

* 10 mg. of 3-methylcholanthrene was injected intraperitoneally into rats daily for 4 days. Galactose-1-C^{14} was administered 5 days later.

In vitro studies. The possibility that pretreatment of rats with Chloretone may increase the activity of liver enzymes required for ascorbic acid synthesis was investigated. It was found that increased metabolism of D-galactose-1-C^{14} to free D-glucuronic acid occurred in liver homogenates derived from Chloretone-treated rats (TABLE 4). In this experiment, 300 to 325-gm. rats were given 40 mg. of Chloretone orally each day for 7 days. On the following day liver homogenates were prepared and incubated with D-galactose-1-C^{14} in the presence of adenosine triphosphate (ATP), uridine diphosphoglucose (UDPG), $MgCl_2$, diphosphopyridine nucleotide (DPN), and nicotinamide.[12] Formation of radioactive D-glucuronic acid was determined by a specific carrier dilution procedure. It is seen here that D-galactose is metabolized to D-glucuronic acid 3 to 4 times more readily by liver homogenates obtained from Chloretone-treated rats than by those from control rats.

More detailed studies on the effect of Chloretone on individual enzymatic

steps involved in ascorbic acid synthesis were then carried out. In these experiments 250- to 300-gm. rats were given 40 mg. of Chloretone orally daily for 7 days. They were killed on the following day, and the livers were used to carry out studies on the various enzymatic reactions shown in FIGURE 4. The effect of Chloretone pretreatment on the activities of these liver enzymes is indicated by a plus or minus sign. Chloretone pretreatment did not stimulate the enzyme system that converts D-glucuronolactone to L-gulonolactone or the system that converts L-gulonolactone to L-ascorbic acid. In fact, the enzyme that converts L-gulonolactone to L-ascorbic acid was depressed about 50

TABLE 4

EFFECT OF CHLORETONE ON THE CONVERSION OF GALACTOSE-1-C^{14} TO D-GLUCURONIC ACID IN RAT LIVER HOMOGENATE

Rats	Per cent conversion to D-glucuronic acid
Control	0.21
Control	0.18
Chloretone*	0.68
Chloretone*	0.88
Chloretone*	0.77

* Rats were pretreated with 40 mg. of Chloretone orally for 7 days prior to the experiment.

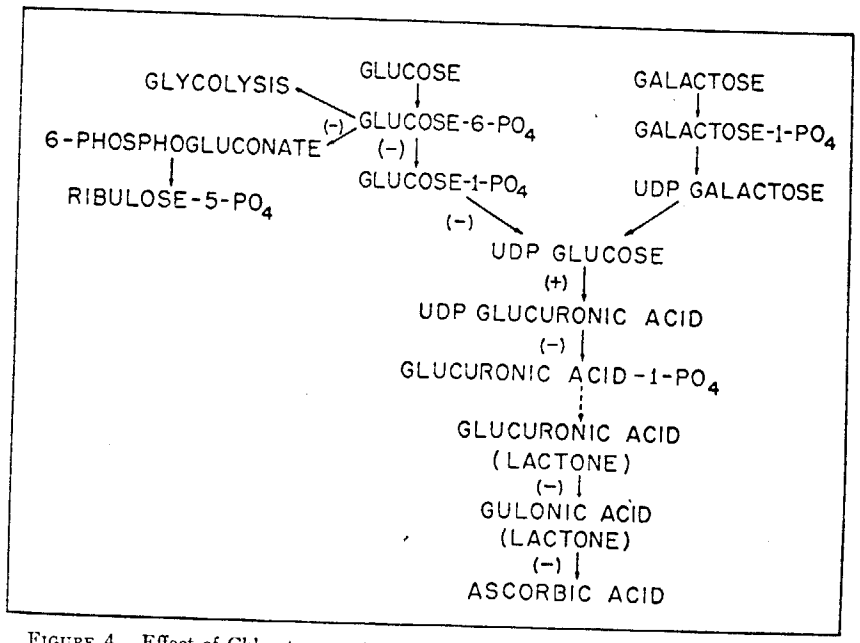


FIGURE 4. Effect of Chloretone pretreatment on liver enzymes involved in ascorbic acid biosynthesis. Chloretone (40 mg.) was administered orally for 7 days prior to the experiment.

1653

(TABLE 1). Thus administration of drugs not only leads to increased urinary excretion of the vitamin, but also to a marked increase in its metabolic breakdown. Examination of the respiratory CO_2 at various intervals after administration of L-ascorbic acid-1-C^{14} showed that significant amounts of C^{14} were excreted by this route. In addition, evidence was found for the excretion of considerable amounts of labeled metabolites of ascorbic acid in the urine of drug-treated rats.

Agents inhibiting ascorbic acid synthesis. Administration to rats of adenosine triphosphate (ATP) has been reported to inhibit the synthesis of ascorbic acid.[5] Experiments were carried out in our laboratory to investigate further this inhibitory effect of ATP. Three rats were given 25 mg. of Chloretone each by stomach tube daily for 7 days. In addition to this dosage of Chloretone, the animals then received by intramuscular injection 120 mg. of ATP daily, divided into 2 equal doses. The excretion of L-ascorbic acid dropped from an average value of 41 ± 2.0 mg. day, while the animals received only the Chloretone, to an average value of 15 ± 2.0 mg. day, when they received both Chloretone and ATP. Increasing the dosage of ATP to 150 mg. day, divided into 3 equal doses, did not decrease further the excretion of L-ascorbic acid.

The alkaloid lycorine has been reported to inhibit the synthesis of L-ascorbic acid in rats.[6] In order to test this, we gave each of 3 rats 20 mg. of Chloretone daily by stomach tube for 3 days. The animals then received 6 mg. of lycorine, divided in two doses by subcutaneous injection, along with the dosage of Chloretone. L-Ascorbic acid excretion dropped from an average control value of 21.6 ± 3.0 mg. to 14.2 ± 1.7 mg. when the animals received both Chloretone and lycorine. The results indicate that lycorine had a distinct but far from complete effect in inhibiting L-ascorbic acid synthesis in Chloretone-treated rats.

Ascorbic acid synthesis via the glucuronic acid pathway. Administration of drugs to rats results in increased metabolism of glucose through the glucuronic acid pathway shown in FIGURE 3. The reactions of this pathway have been reviewed recently by Touster,[7] Burns,[8] Strominger,[9] and Burns and Conney.[10] According to this scheme, D-glucose is oxidized to D-glucuronic acid through the intermediate formation of uridine nucleotides. The D-glucuronic acid undergoes reduction to L-gulonic acid, which serves as the precursor of either L-ascorbic acid or L-xylulose.

Evidence that drugs stimulate the glucuronic acid pathway comes from the finding that administration of barbital or Chloretone to rats stimulates markedly the conversion of D-glucose-1-C^{14} or D-galactose-1-C^{14} to labeled D-glucuronic acid, L-gulonic acid, and L-ascorbic acid.[3,11,12] This effect of barbital to stimulate glucose and galactose metabolism through the glucuronic acid pathway is shown in TABLE 2. Rats were given 100 mg. oral doses of barbital daily for several days and were then injected with D-glucose-1-C^{14} or D-galactose-1-C^{14}. Urine was collected for 24 hours, and the amount of radioactivity in isolated free D-glucuronic acid as well as in L-gulonic acid and L-ascorbic acid was determined. It is seen here that control rats convert less than 0.05 per cent of the D-glucose or D-galactose to D-glucuronic, L-gulonic, or L-ascorbic acids, and that considerable conversion took place in barbital-treated rats.

Additional experiments were carried out on the effect of 3-methylcholanthrene on the synthesis of L-ascorbic acid from D-galactose, and the results are shown in TABLE 3. In these experiments D-galactose-1-C^{14} was administered to rats, and the incorporation of C^{14} into urinary D-glucuronic, L-gulonic, and

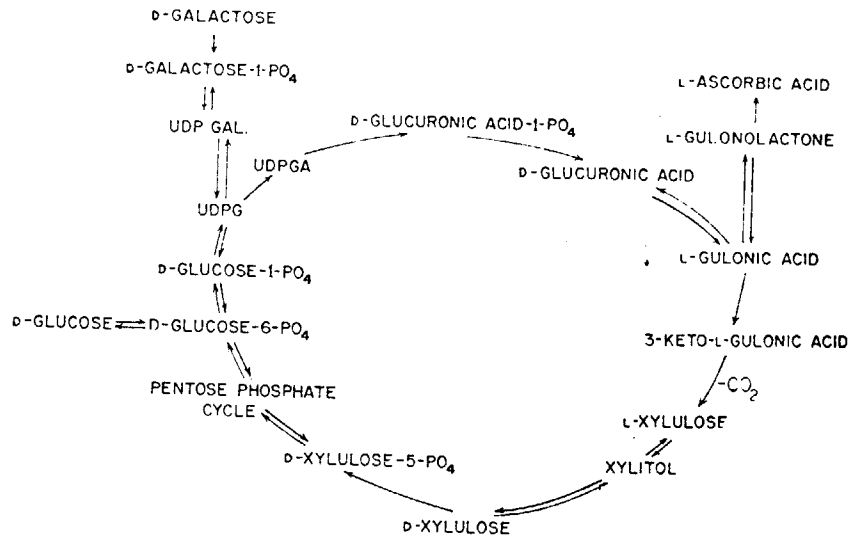


FIGURE 3. Glucuronic acid pathway.

TABLE 2

CONVERSION OF D-GLUCOSE-1-C^{14} AND D-GALACTOSE-1-C^{14} TO GLUCURONIC ACID PATHWAY INTERMEDIATES EXCRETED IN RAT URINE

Precursor	Per cent of administered C^{14} in		
	D-Glucuronic acid	L-Gulonic acid	L-Ascorbic acid
Control rats			
D-Glucose-1-C^{14}	<0.03	<0.03	<0.02
D-Galactose-1-C^{14}	<0.05	<0.05	<0.05
*Barbital-treated rats**			
D-Glucose-1-C^{14}	0.41	0.19	0.19
D-Galactose-1-C^{14}	1.49	0.65	0.77

* Rats were pretreated with 100 mg. of barbital orally for 7 days prior to the experiment.

L-ascorbic acids was measured. It will be noted that there was a considerable increase in the ability of 3-methylcholanthrene-treated rats to metabolize D-galactose-1-C^{14} to labeled L-gulonic and L-ascorbic acids beyond that observed in control rats. These data are similar to those obtained with barbital-treated animals (TABLE 2). However, in contrast to the extensive incorporation of D-galactose-1-C^{14} into D-glucuronic acid in barbital-treated rats, considerably

of drugs on ascorbic acid synthesis the turnover of ascorbic acid was determined in rats treated with 3-methylcholanthrene, Chloretone, and pentobarbital; the data are given in FIGURE 2. In these experiments the drugs were given for several days, and then a tracer dose of L-ascorbic acid-1-C^{14} was administered intraperitoneally to control or drug-treated rats. The specific activity of ascorbic acid in daily urine samples was determined at various times after the dose of the labeled compound.[1] In each case, 24 hours were allowed to lapse before the first specific activity value was determined in order to permit sufficient time for equilibration of the radioactive ascorbic acid with nonlabeled ascorbic acid in the animal. The specific activity values obtained have been

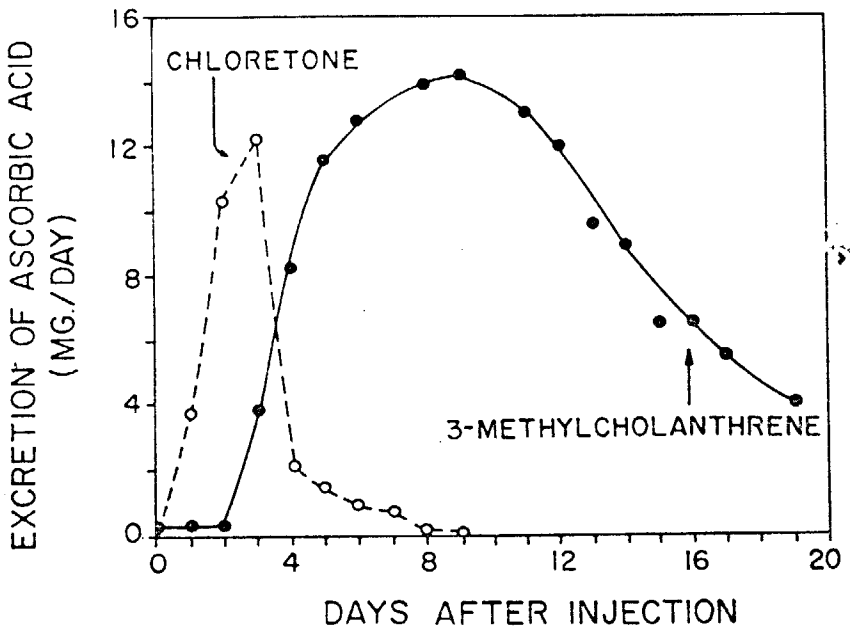


FIGURE 1. Urinary excretion of L-ascorbic acid by rats following a single intraperitoneal injection of 3-methylcholanthrene (10 mg.) or Chloretone (40 mg.).

plotted on a log scale at the mid-point of each urine collection period. The data show that 3-methylcholanthrene, Chloretone, and pentobarbital markedly shorten the half life of ascorbic acid in the rat. For example, in the normal rat the half life is 3 days; in the 3-methylcholanthrene-treated animal it is only 0.8 day.

From these data it is possible to calculate the body pool and turnover rate of ascorbic acid;[4] these values are given in TABLE 1. In untreated rats, the body pool of ascorbic acid is 10.7 mg. per 100 gm. body weight, while in drug-treated animals the body pool of ascorbic acid about doubles. The turnover rate of ascorbic acid (the amount synthesized and metabolized or excreted each day) in control rats was about 2.6 mg. per 100 gm. rat per day. This value was increased about 10-fold by pretreating the rats with Chloretone or 3-

methylcholanthrene, and about 5-fold by pretreating the animals with pentobarbital.

The amount of ascorbic acid excreted daily in the urine did not account for all of the ascorbic acid synthesized each day. For example, in control rats about 1.8 mg. ascorbic acid per 100 gm. body weight was metabolized each day, whereas in the pentobarbital-, 3-methylcholanthrene-, or Chloretone-treated rats, this value increased to about 10 mg. per 100 gm. body weight per day

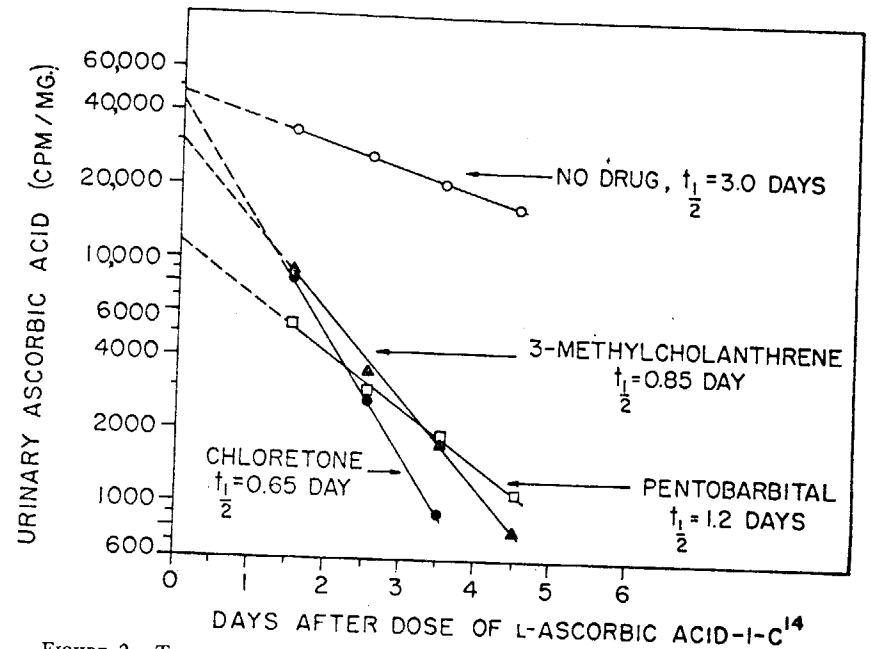


FIGURE 2. Turnover rate studies with L-ascorbic acid in drug-treated rats. Pentobarbital (30 mg.) or Chloretone (45 mg.) was administered orally for several days prior to administration of L-ascorbic acid-1-C^{14}. 3-Methylcholanthrene (10 mg.) was injected intraperitoneally daily for 4 days; the L-ascorbic acid-1-C^{14} 5 days later.

TABLE 1
EFFECT OF DRUGS ON ASCORBIC ACID METABOLISM IN THE RAT*

Drug pretreatment	Body pool of AA (mg./100 gm.)	Turnover rate of AA (mg./100 gm./day)	Excretion of AA (mg./100 gm./day)	Metabolism of AA (mg./100 gm./day)
None	10.7	2.6	0.40	2.2
Chloretone	19.2	21.5	10.2	11.3
3-Methylcholanthrene	22.5	19.0	7.1	11.9
Pentobarbital	19.6	11.6	2.4	9.2

* Pentobarbital (30 mg.) or Chloretone (45 mg.) was administered orally for 4 to 7 days prior to the administration of L-ascorbic acid-1-C^{14}. Methylcholanthrene (10 mg.) was injected intraperitoneally daily for 4 days, and the L-ascorbic acid-1-C^{14} was administered 5 days later. All values are given on a milligram per 100-gm. body weight basis.

1650

Ann. New York Acad. Sci. 92(1):115-127

METABOLIC INTERACTIONS BETWEEN L-ASCORBIC ACID AND DRUGS

A. H. Conney, George A. Bray, Carole Evans, J. J. Burns

Laboratory of Chemical Pharmacology, National Heart Institute, Public Health Service, Bethesda, Md.; New York University Research Service, Goldwater Memorial Hospital, Welfare Island, New York, N.Y.; The Wellcome Research Laboratories, Tuckahoe, N.Y.

Interrelationships between the metabolism of L-ascorbic acid and drugs have been the subject of numerous studies. Two aspects of this problem that will be considered here are as follows: (1) The ability of drugs to stimulate the synthesis and metabolism of ascorbic acid, and (2) the ability of ascorbic acid to influence the metabolism of drugs.

Effect of Drugs on Ascorbic Acid Metabolism

Drug-induced ascorbic acid synthesis. Various drugs possessing completely unrelated chemical and pharmacological properties have been shown to stimulate markedly the urinary excretion of L-ascorbic acid in rats.[1-3] They include the hypnotics: Chloretone and barbital; the analgesics: aminopyrine and antipyrine; the muscle relaxants: orphenadrine and meprobamate; the antirheumatics: phenylbutazone and oxyphenbutazone; the uricosuric agent, sulfinpyrazone; the antihistaminics: diphenhydramine and chlorcyclizine; and the carcinogenic hydrocarbons: 3-methylcholanthrene and 3,4-benzpyrene.

Of considerable interest is the potent stimulatory effect on ascorbic acid synthesis exerted by the carcinogenic hydrocarbons 3-methylcholanthrene, 1,2,5,6-dibenzanthracene, and 3,4-benzpyrene. The striking effect of a single 10-mg. intraperitoneal injection of 3-methylcholanthrene is shown in FIGURE 1. For comparison, the effect of a 40-mg. dose of Chloretone is also given. By 6 days after 3-methylcholanthrene administration, the urinary excretion of ascorbic acid was about 70 times greater than the control value and, in fact, during the 19-day period about 140 mg. of the vitamin was recovered in the urine. It will be noted that Chloretone within the first day exerts an effect on ascorbic acid excretion that reaches a peak by the third day and falls to a low value by the fifth day. In contrast, 3-methylcholanthrene exerts no effect for 2 days, but then urinary ascorbic acid excretion increases rapidly and remains elevated for over 18 days.

In another experiment rats were injected intraperitoneally with 10 mg. of the hydrocarbon daily for 3 to 5 days, and the urinary excretion was measured at various intervals thereafter. The urinary excretion of ascorbic acid increased from control values of about 0.3 mg. per day to values of 17 mg. per day by 6 days after the dose. Even by 50 days after administration of the hydrocarbon, the levels of ascorbic acid in urine were still markedly elevated over the control levels. No definite information is available at the present time on the prolonged effect of 3-methylcholanthrene on ascorbic acid synthesis. The possibility that the hydrocarbon remains in the animal by localization in fat depots is now under investigation.

Turnover rate of ascorbic acid. In order to show further the marked effect

Committee on Specifications. 1972
Food Chemicals Codex, 2nd Edition
Committee on Food Protection, National Academy
of Sciences, National Research Council,
Washington, D.C., pp. 66-67

CHVAPIL, M., AND HOLUŠA, R. (1966). The connection between the dose of quartz dust and the extent of the inflammatory reaction of the lung. (Czech.). *Prac. Lek.* **18**, 145–150.

ČMUCHALOVÁ, B., AND CHVAPIL, M. (1963). Changes in deoxyribonucleic acid, collagen and ascorbic acid during the development of carrageenan granuloma. *Biochim. Biophys. Acta* **69**, 565–567.

ČMUCHALOVÁ, B., AND CHVAPIL, M. (1967). Relationship between the dose of ascorbic acid and its structural analogues and proline hydroxylation in different biological systems. *Biochim. Biophys. Acta* (in press).

FITCH, S. M., HARKNESS, M. L. R., AND HARKNESS, R. D. (1955). Extraction of collagen from tissues. *Nature* **176**, 163.

GOULD, B. S. (1960). Ascorbic acid and collagen fiber formation. *Vitamins Hormones (NY)* **18**, 89–116.

GOULD, B. S. (1961). Ascorbic acid-independent and ascorbic acid-dependent collagen-forming mechanisms. *Ann. NY Acad. Sci.* **92**, 168–174.

HURYCH, J. (1967). Hydroxylation of proline *in vitro* I. *Hoppe Seyler Z. Physiol. Chem.* (in press).

JEFFREY, J. J., AND MARTIN, G. R. (1966). The role of ascorbic acid in the biosynthesis of collagen. I. Ascorbic acid requirement by embryonic chick tibia in tissue culture. II. Site and nature of ascorbic acid participation. *Biochim. Biophys. Acta* **121**, 269–291.

KLOSTERKÖTTER, W. (1960). Discussion to Strecker, F. J., Chemisch veränderte SiO_2-Oberflächen im Tierversuch. *Beitr. Silikoseforsch. S.-Bd Grundfragen Silikoseforsch.* **4**, 121–153.

MARASAS, L. W., AND HARINGTON, J. S. (1960). Some oxidative and hydroxylative actions of quartz: Their possible relationship to the development of silicosis. *Nature* **188**, 1173–1174.

MILSHTEIN, A. JA., AND BARU, A. M. (1959). On the role of vitamin C in the pathogenesis of pneumoconiosis (Russian). Material of the XI Plenary Session of the Republican Commission for Combating Silicosis, *Izdat. Akad. Nauk. USSR* p. 53.

ROBERTSON, W. B., HEWITT, J., AND HERMAN, C. (1959). The relation of ascorbic acid to the conversion of proline to hydroxyproline in the synthesis of collagen in the carageenan granuloma. *J. Biol. Chem.* **234**, 105–108.

RODKINA, B. S. (1963). Effect of corticosteroids on ascorbic acid content of organs in rats with experimental pneumoconiosis (Russian). *Ukr. Biochim. Zh.* **35**, 256–263.

SILGEMANN, H. (1958). Mikrobestimmung von Hydroxyprolin mit Chloramin-T und *p*-Dimethylaminobenzaldehyd. *Hoppe-Seyler Z. Physiol. Chem.* **311**, 41–45.

STONE, N., AND MEISTER, A. (1962). Function of ascorbic acid in the conversion of proline to collagen hydroxyproline. *Nature* **194**, 555–557.

UDENFRIEND, S. (1966). Formation of hydroxyproline in collagen. *Science* **152**, 1335–1340.

WOESSNER, J. F., AND BOUCEK, R. J. (1961). Connective tissue development in subcutaneously implanted polyvinyl sponge. II. Enzymic changes during development. *Arch. Biochem.* **93**, 95–109.

collagen proteins in lungs dusted by aerosil and in lungs of equally old control rats. In both the soluble proportion of collagen proteins forms less than 1% of the total collagen present in lung.

The discrepancy in the histological and biological findings may perhaps be explained by the following considerations: the histologist endeavors to estimate the semiquantitative changes in the relation of collagen fibers to the remaining structures. It is therefore a relative expression of density, i.e., accumulation of collagen fibers in a certain area unit. This corresponds in biochemical nomenclature to collagen concentration expressed in weight units. But this ratio does not visibly change after aerosil, because along with the accumulated collagen, the lung weight also increases. Since with aerosil the changes are more diffuse, the increase is much less evident in the presence of the other noncollagenous lung response.

SUMMARY

We did not confirm the report of Marasas and Harington (1960) on the participation of quartz dust in proline hydroxylation. An explanation of the conflicting results is given.

The increase of collagen in the silicotic lung correlates well with the amount of ascorbic acid but not with other organic acids such as citric, lactic, and pyruvic acid. The significance of this phenomenon is not clear.

There is evidence that the ascorbic acid level has no influence on the extent of collagen synthesis in lung or skeletal muscle, tissues with low collagen metabolism. Only in tissues with a high collagen metabolism (granulation tissue, liver) is it possible to inhibit collagen synthesis by a decrease of the concentration of ascorbic acid.

Collagenous proteins (hydroxyproline) increase markedly in lungs after intratracheal administration of aerosil, although morphologically fibrosis is not evident. The causes of this discrepancy between the chemical and morphological evaluation of the effect are considered to be in the proportional coarsening of the lung stroma of collagenous as well as other lung structures (total lung weight), so that the unit density of the equally distributed collagen remains the same as in controls. It is emphasized that only the total content of collagenous structures in whole lungs should be the measure of the effect of an investigated dust. The fibroproductive capacity of lungs is limited because even repeated administration of aerosil did not increase the collagen content in lungs beyond an initial effect.

REFERENCES

Baru, A. M. (1961). The influence of a varying degree of satiation with vitamin C on the development of experimental silicosis (Russian). Materials of the XIII Plenary Session of the Republican Commission for Combating Silicosis, Kiev. *Izdat. Akad. Nauk USSR* 32–39.

Candlish, J. K., and Tristram, G. R. (1963). The resistance to dispersion of collagen fibres formed in the presence of ascorbic acid. *Biochim. Biophys. Acta* **78**, 289–294.

Catchpole, H. R., Joseph, N. R., and Engel, M. B. (1963). Fixation d'ions, echanges d'ions et autres propriétés colloidales du tissu conjonctiv. *In* "Exposés Annuels de Biochimie Médicale." Masson and Cie, Paris.

Chvapil, M. (1960). Möglichkeiten einer quantitativen Bestimmung des Fibrosengrades bei der Untersuchung experimenteller Silikose. *Beitr. Silikoseforsch.* **64**, 3–71.

weeks of aerosil effect tend to demonstrate significant production of collagen proteins in lung tissue so that hydroxyproline values can then be approximated to the results obtained 3 months after intratracheal administration of a suspension of 50 mg of quartz powder (Fig. 4) (Chvapil and Holuša, 1966). In contrast, histologically evident fibrosis after aerosil administration did not even equal the degree of production of collagenous proteins after administration of much smaller doses of quartz powder. The inconsistence between the results of the chemical and histological methods may be explained by a more diffuse distribution of the administered suspension in lung and by the different qualitative character of the reaction of connective tissue to the administered quantity of aerosil.

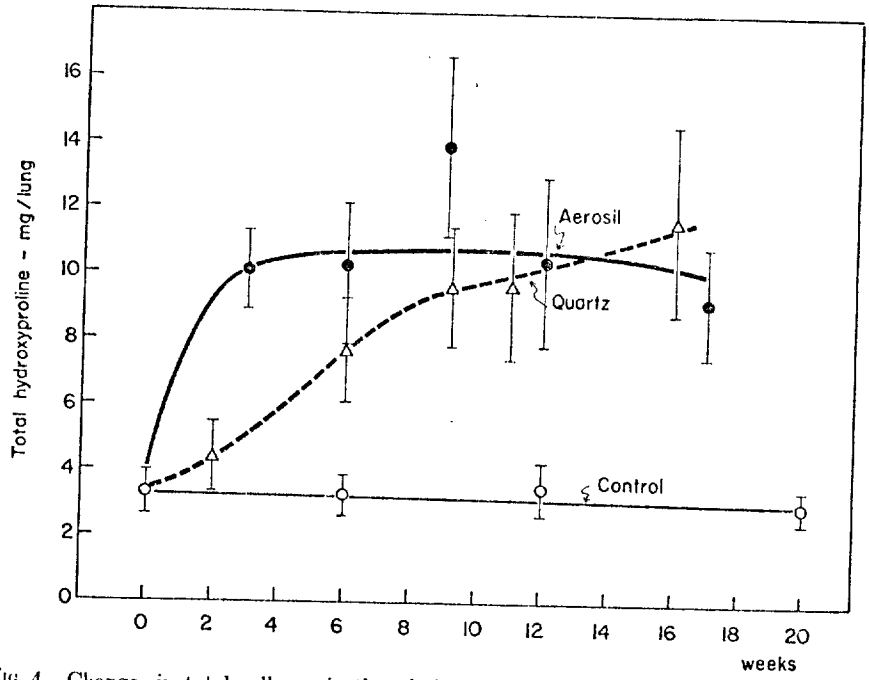


Fig. 4. Changes in total collagen in the whole lung after a single instillation of quartz and repeated application of aerosil to rats. The variability is given by 95% confidence limit. For details see text.

A remarkably similar feature of the reaction to the administration of quartz powder and of aerosil is the increased formation of collagen proteins expressed by the high value of hydroxyproline in lungs. After aerosil administration hydroxyproline increases rapidly even after a smaller dose. The discrepancy between the results of chemical examination and the microscopic picture may suggest that after aerosil administration the change of soluble collagen into insoluble collagen does not take place as readily as after quartz, with fewer collagen fibers demonstrable by classical histological methods. We therefore repeated the same experiments and determined the proportion of collagen proteins extractable by 0.45 M NaCl, representing the young forms of collagen and the residual collagen proteins. To our surprise we found no remarkable difference in the content of soluble

The most significant results for the problem with which we were concerned were in the determination of the total hydroxyproline in the lungs. This indicator reflects the formation of collagen in response to the irritant. Three weeks after the first dusting with a dose of 12 mg aerosil the content of hydroxyproline in lungs practically triples (see Fig. 4): from 3.38 to 10.13 mg. The influence of further intratracheal administrations of aerosil does not produce additional growth of connective tissue structures in the course of the remaining 17 weeks of the experiment. The control rats showed no changes in total collagen content of the lungs during the whole period of the experiment.

TABLE IV

EFFECT OF REPEATED INTRATRACHEAL INSTILLATIONS OF AEROSIL ON SOME LUNG INDICES[a]

No. aerosil instillations	Time (weeks)	Lung wet weight (gm)	Hydroxyproline (mg/lung)	Hydroxyproline (μg/mg dry weight)
Control	0	1.13 ± 0.14	3.38 ± 0.47	16.2 ± 2.9
1	3	2.24 ± 0.46	10.13 ± 1.36	26.0 ± 1.6
2	6	2.51 ± 0.30	10.27 ± 1.96	21.5 ± 1.4
3	9	2.70 ± 0.43	13.98 ± 2.44	25.7 ± 5.5
4	12	2.48 ± 0.48	10.47 ± 4.54	21.8 ± 4.1
5	17	2.61 ± 0.40	9.12 ± 1.29	17.36 ± 2.4
Control	17	1.37 ± 0.18	3.01 ± 0.31	18.7 ± 2.9

[a] The variability is given by the 95% confidence limit.

The histological picture after the intratracheal administration of aerosil is characterized by the development of inflammatory changes with typical cellular granulomas and the formation of fine connective tissue fibers in them. Multiplication of fibers in the remaining parts of lung parenchyma occurs to a far smaller extent. The granulomatous process after administration of quartz dust appears, on the other hand, in the characteristic scattered distribution of typical collagenous nodules in lung tissue. Moreover, there is an earlier development of cellular granulomas after aerosil administration.

Neither hydroxyproline levels in lungs of experimental animals nor histological and weight changes support the concept that gradual accumulation of collagen structures in lungs may be accomplished by repeated aerosil administration. A single aerosil instillation stimulated collagen formation *ad maximum*. It therefore seems that the extent of collagen accumulation is proportional to the total available number of fibroblastic cells present in the organ involved. Highly soluble aerosil exerts a prompt and maximal stimulation of affected tissue elements.

We were unable to confirm Klosterkötter's (1960) report that tissue reaction to aerosil in lungs in the early stages is the same as the reaction to quartz powder. Even by administering 5 consecutive doses of aerosil into the trachea we did not find, as long as 4 months after the first application, collagenous nodules of the type found after intratracheal administration of a single dose of quartz dust. The difference between the results of chemical collagenous hydroxyproline determination and histologically demonstrable collagen fibers in rat lung treated with aerosil is striking. Chemical examination of lungs of experimental rats after 3

INTERRELATION BETWEEN COLLAGEN FORMATION AND LUNG FIBROSIS AFTER AEROSIL

Generalizations based upon observations on the origin and development of fibroproductive inflammation have led to the concept that the inflammation and the resulting fibrosis can be induced by any kind of stimuli, be it physical, chemical, or biological. Decisive for the occurrence of fibrotic changes is the size of the load (dust dose) and duration of action of the stimulus, either by continual irritation, as in the case of quartz dust, or by repeated, interrupted irritation. According to this concept, fibrosis should occur even after repeated application of stimuli which on single application produce only minimal, temporary fibroplastic stimulation. After eliminating the irritating agent from the lungs and after the response recedes, it is postulated that there will be some residuum of several components of connective tissue, mostly collagenous, so that fresh inflammatory irritation of the lung and further development of fibroproductive changes take place against a background of a lung tissue partially modified with regard to structure and reactivity (Fig. 3).

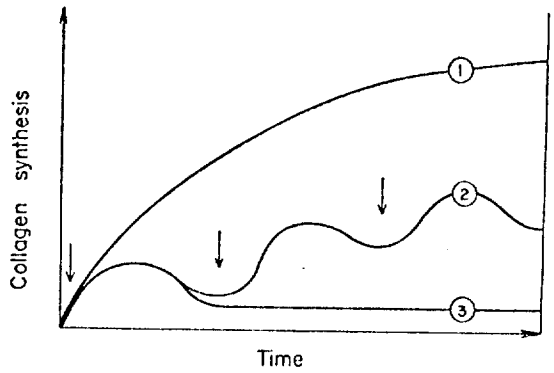


FIG. 3. Schematic representation of the change in collagen synthesis after a single shortlived exposure (3), after repeated shortlived exposure (2), and continuous stimulation of the tissue (1).

To study this hypothesis, we repeatedly dusted a group of rats, at approximately 3-week intervals, with aerosil Degussa. Five dustings were done, with doses of 8–12 mg in each rat. Simultaneously, at intervals lungs of rats dusted with quartz dust and control rats were biologically, histologically, and biochemically examined.

It was found that after the first intratracheal aerosil application, lung weight (wet and dry) doubles in the course of 3 weeks (Table IV). As the percentage of dry lung substance in all experimental groups remained approximately the same as in the control group, the increase did not consist of an increase of the fluid content in the lungs. With considerable variability of values, no statistically significant differences were found in the weight of lungs (dry and wet) and in the percentage of lung dry substance, between the groups which were dusted 1–5 times with aerosil. The control group showed no increase in lung weight after 14 days.

the other hand, it is known that ascorbic acid is essential for numerous protective and defensive biologic reactions of the organism and is involved in the maintenance of a broad range of reactions in the cell (Jeffrey and Martin, 1966). In silicosis its level is decreased. There is thus no evidence to support the recommendation that the supply of vitamin C to miners should be limited to prevent or retard silicotic fibrosis (Baru, 1961).

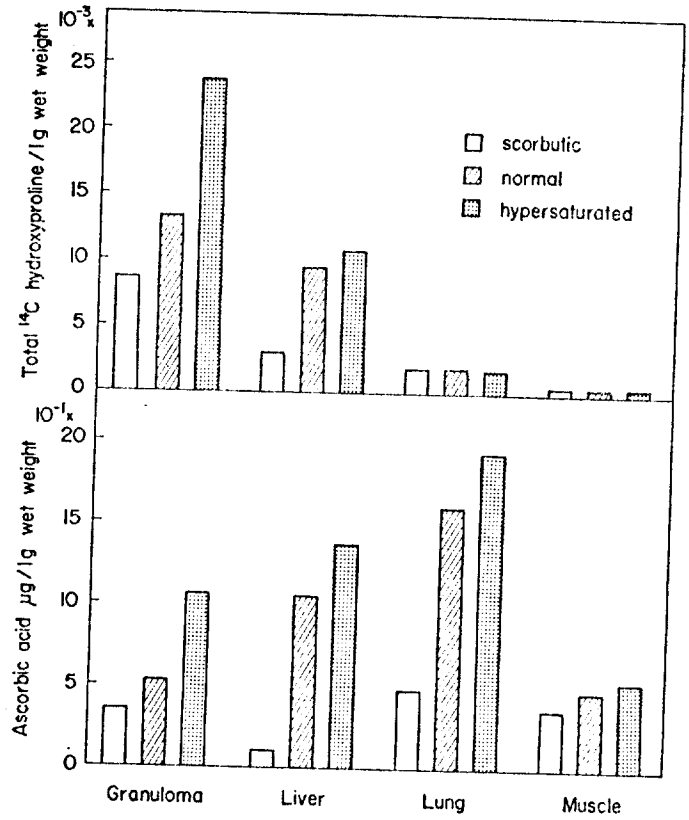


FIG. 2. The relation between the level of ascorbic acid in various tissues and collagen synthesizing capacity. Carrageenan granuloma was induced on the eleventh day after stopping the ascorbic acid supply by subcutaneously injecting 5 ml of 1% carrageenan solution in three groups of guinea pigs: (a) depleted of ascorbic acid, (b) resaturated with 25 mg ascorbic acid daily (normal), and (c) resaturated with 100 mg ascorbic acid (hypersaturated). The granuloma was isolated on the seventh day of development and after slicing was incubated in a Krebs-Ringer bicarbonate buffer with an amino acid mixture (3 mM), ATP (2 mM), and ^{14}C-proline. The incubation lasted for 2 hours at 37° in an atmosphere of 95% O_2 and 5% CO_2 with slight shaking. After the end of incubation, trichloroacetic acid was added to a final 0.3 M concentration. The collagen was extracted according to Fitch *et al.* (1955). The extracts were dialyzed against water and hydrolyzed (6 N HCl for 16 hours at 105°), and the α-amino acids were removed by nitrosation. After desalting the samples, hydroxyproline was separated on Dowex 50-W X8 (H^+) column. The isolated ^{14}C-hydroxyproline was rechromatographed on Whatman No. 4 paper; after elution, its specific activity was determined.

collagen biosynthesis exist, as Gould (1961) assumes, i.e., a mechanism dependent on ascorbic acid, such as might be present in tissues with high turnover of collagen metabolism (for example, in situations with artificially increased fibroplasia) and an ascorbic acid independent mechanism as, for example, slow formation of collagen proteins in the majority of organs. Gould's findings (1961) suggest that tissues with high metabolic collagen protein turnover should be more susceptible to the presence of ascorbic acid than tissues with low collagen metabolism. We therefore studied the influence of ascorbic acid on collagen synthesis in organs with various metabolic collagen turnover.

The experiments were carried out by incubation of tissue slices of various tissues from scorbutic guinea pigs, normal guinea pigs, and others hypersaturated by ascorbic acid. Granulation tissue and liver were studied as structures with high collagen metabolism and lung and skeletal muscle as tissues with the lowest collagen metabolism.

Results presented in Fig. 2 show that the extent of collagen synthesis depends on the level of ascorbic acid in granulation tissue and liver, i.e., in tissues with high collagen turnover. Among these tissues too a certain difference is evident. In granulation tissue collagen synthesis is proportional to the content of ascorbic acid present in tissue. In liver, on the other hand, collagen formation differs only between the scorbutic group and the group with normal vitamin C content. Further increase of ascorbic acid levels in the liver does not effect collagen synthesis. In both organs with low turnover of collagenous proteins, i.e., lung and muscle, synthesis of collagen takes place without relation to the level of ascorbic acid in the organ studied.

The relation of collagen formation to ascorbic acid in these tissues with varying collagen metabolism suggests that ascorbic acid stimulates collagen synthesis to some extent, but that the extent of the tissue reaction to the presence of ascorbic acid depends on the type of tissue and its collagen turnover. In tissues with high collagen metabolism, such as liver, and in carrageenan granuloma, the presence of ascorbic acid is necessary for the development of maximum collagen synthesis. On the other hand, collagen synthesis in tissues with low collagen metabolism, including the lung, was not influenced by a low level of ascorbic acid.

Although it may be expected that, consequent upon the effect of the inflammatory process, collagen synthesis in silicotic lungs of the rats will be stimulated, we found (Chvapil and Huryeh, unpublished results) that the increase due to the influence of quartz depends on the stage of the fibroproductive inflammation in the lung and, moreover, is relatively small so that the specific activity and therefore metabolic turnover of collagen in silicotic lungs is substantially less than that in normal liver tissue.

The following conclusions relative to silicosis suggest themselves at this point. In the course of fibroproductive inflammation, ascorbic acid accumulates in the lung (see Milshtein and Baru, 1959; Rodkina, 1963). However, there is no proof that ascorbic acid influences the low rate of synthesis of collagen proteins in the lung; this occurs at a rate independent of the ascorbic acid level. There also is no proof that ascorbic acid interferes with the maturation of collagen molecules. On

lish and Tristram (1963) found, however, that collagen fibers, reconstituted in the presence of ascorbic acid, manifested higher structural stability.

Another explanation may be offered, i.e., that accumulation of ascorbic acid in granulation tissue is a nonspecific phenomenon, associated with the binding ability of newly formed collagen or other extracellular colloids (Catchpole *et al.*, 1963). In this case we might expect that collagen would have the same ability to also interact with other organic acids which occur in the tissue as metabolites. In order to study this possibility we investigated the development of carrageneen granuloma where, besides previously studied substances, citric, pyruvic, and lactic acid were also studied. None of these acids was found to accumulate in the granuloma during development (Table III), and their concentrations did not demonstrate any relation to collagen.

TABLE III

RELATION BETWEEN THE ACCUMULATION OF ORGANIC ACIDS AND COLLAGEN PROTEINS IN CARRAGEENAN GRANULOMA DURING DEVELOPMENT

Days after induction of granuloma[a]	Citric acid (μg/gm wet weight)	Lactic acid (mg/gm wet weight)	Pyruvic acid (μg/gm wet weight)	Ascorbic acid (μg/gm wet weight)	Collagenous hydroxyproline (μg/gm wet weight)[b]
3	46.1	1.51	15.4	26	22
4	43.5	1.76	10.5	—	—
5	54.5	1.97	10.9	20	20
6	39.0	2.17	10.8	26	24
7	42.5	2.45	11.1	21	29
9	49.5	2.07	10.8	700	38
10	45.6	1.68	11.4	820	40
12	29.6	2.44	13.7	840	46
14	37.0	1.26	7.3	640	46
16	—	1.56	14.9	56	54
17	46.5	1.84	11.5	—	—

[a] Carrageenan granuloma was induced by subcutaneously injecting 5 ml 1% carrageenan solution into the subcutis of guinea pigs weighing 250 gm.

[b] Corresponds to all substances containing hydroxyproline with the exception of substances soluble in 0.14 M NaCl.

INTERRELATION BETWEEN ASCORBIC ACID LEVELS AND COLLAGEN SYNTHESIS IN LUNG TISSUE

Hitherto obtained data favor the hypothesis that the extent of proline hydroxylation, which reflects collagen biosynthesis, depends on the amount of ascorbic acid in the tissue (for review see Gould, 1960). Nevertheless, not even in the case of scorbutic granuloma is the collagen formation entirely blocked, although it is considerably decreased (Cmuchalová and Chvapil, 1967). Robertson *et al.* (1959) and Stone and Meister (1962) state that in scorbutic granuloma the conversion of labelled proline to hydroxyproline is decreased; still, some collagen hydroxyproline activity was present. This raises the question of whether collagen biosynthesis is entirely dependent on ascorbic acid or whether several mechanisms of

In rats dusted intratracheally with a single dose of quartz (1 μ size, 50 mg in 1 ml saline solution) within a period of 1–70 days, the total collagen proteins in lungs [determined as total hydroxyproline (Chvapil, 1960)] and the content of ascorbic and dehydroascorbic acid were determined. All values of ascorbic acid during development of silicotic fibrosis were classified in two groups (Table II). In the first group were those observations measured within one week after dusting, while all remaining results, measured 14–70 days after dusting, are included in the second group. The mean values of two repeated determinations for each group of animals studied are reported. The mean values of ascorbic acid concentrations in the first group were 147.8 and 214 μg per gram wet weight in the second group. The difference between these values is statistically significant ($p < .01$). A similar picture is obtained if we recalculate ascorbic acid to dry weight (mean value in the first group, 77.4 μg/100 mg; in the second, 108.4 μg/100 mg dry weight).

TABLE II
CHANGES IN THE CONTENT OF ASCORBIC ACID AND COLLAGEN IN LUNGS DURING DEVELOPMENT OF EXPERIMENTAL SILICOSIS

Ascorbic and dehydroascorbic acid were determined according to Roe and Kuther (1963).

Weeks after dusting	Collagenous hydroxyproline (μg/mg dry substance)	Ascorbic acid (μg/gm wet weight)	Dehydroascorbic acid (μg/gm wet weight)
0	6.0	150	75
1	6.5	156	64
2	7.4	195	60
4	8.5	216	60
5	9.2	254	74
7½	10.0	260	64
8½	10.0	—	70
10	10.5[a]	240[a]	—

[a] $r = 0.9455$; $p < .01$.

Changes of total hydroxyproline in the lungs during development of silicosis were similarly evaluated. Here, too, differences were significant. The relation of ascorbic acid to collagen was very much the same in various phases of silicosis development; the correlation coefficient r amounted to 0.94 and was highly significant.

It may be concluded that the increase of ascorbic acid concentration during the period 14–70 days after dusting is on the average statistically highly significant and correlates well with changes in collagen protein content in lungs. At the outset of the inflammatory process, when no changes in lung collagen can be measured, there are no changes in ascorbic acid concentrations. Similarly, no change takes place in the content of dehydroascorbic acid during the formation of silicotic fibrosis.

Time relationships in the changes of ascorbic acid and collagen suggest that ascorbic acid accumulation in the granuloma coincides with increased synthesis of collagen structures. Ascorbic acid therefore may participate in the control of structural stability of collagen fibers. *In vitro* experiments did not demonstrate the influence of ascorbic acid upon aggregation of tropocollagen molecules. Cand-

free proline hydroxylation in tissues bears no relation to collagen synthesis, since free hydroxyproline is not incorporated into collagen proteins.

BIOLOGICAL ROLE OF ASCORBIC ACID LEVELS IN FIBROTIC LUNGS

Several investigators have studied the relation between the changes in collagen protein content and the ascorbic acid content, predominantly in the course of development of granulation tissue (Woessner and Boucek, 1961; Čmuchalová and Chvapil, 1963). Good correlation was found between ascorbic acid levels and

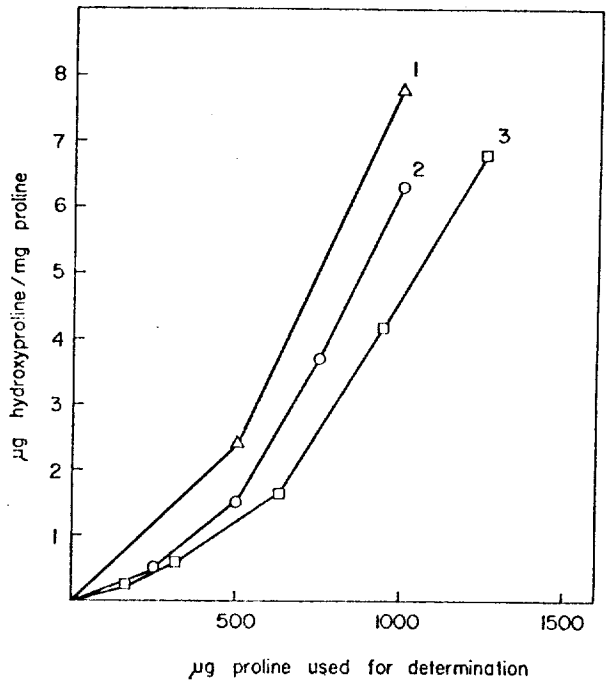


FIG. 1. Determination of hydroxyproline in proline preparations without separation. Hydroxyproline was determined in different amounts of proline by Stegemann's method (1958). Hydroxyproline found was calculated per 1 mg of proline. By paper chromatography (500 μg of each preparation respectively) hydroxyproline was not detected, even when highly a sensitive spot test (sensitivity less than 1 μg) was used. 1, Proline (NRC grade), Calbiochem., Los Angeles; 2, proline synthetically prepared by Dr. E. Wünsch, Max-Planck-Institut für Eiweiss- und Lederforschung, Munich; 3, proline (hydroxyproline-free), Sanabo, Vienna.

collagen proteins. Woessner and Boucek found an equally good correlation with cell content (DNA). On the contrary Rodkina (1963) did not find any relation between the lung ascorbic acid content and the rate or degree of development of the fibrotic process.

In order to study whether the observed relationship between ascorbic acid changes and collagen structures is a general one, we investigated another model of fibroproductive inflammation, i.e., the formation of connective tissue structures in lungs in silicosis.

containing large quantities of proline (250–1250 μg), without previous separation, a color characteristic for hydroxyproline is formed, although hydroxyproline-free proline (purity verified by paper chromatography) is used for the determination. The color does not increase proportionally to the increasing quantity of proline used for the determination. This indicates that if a larger quantity of proline is used, the greater part undergoes unusual oxidation to pyrrol, which with *p*-dimethylaminobenzaldehyde forms the characteristic color (Fig. 1). When we deducted this nonspecific hydroxyproline value in the control sample from the hydroxyproline content found in the medium incubated with variously modified quartz powder, there was no evidence for proline hydroxylation.

TABLE 1

HYDROXYLATION OF ^{14}C-PROLINE IN OXYGEN ATMOSPHERE

Incubated 4 hours at 37° under slight shaking in oxygen atmosphere. Total volume of 6 ml contained 0.76 μC ^{14}C-proline/ml incubation medium (phosphate buffer, pH 6.0). One μC ^{14}C-proline used for incubation contained 5100 dpm ^{14}C-hydroxyproline. Values in the table were corrected for this contamination. For washing hydroxyproline from the Dowex 50 × 8 column, 1.5 N HCl was used. As carrier 200 μg hydroxyproline was added. Hydroxyproline was determined according to Stegemann (1958). Efficiency of radioactive assay was 63%.

Addition to medium		Disintegrations	
Substance	Amount (conc.)	^{14}C-Hypro-min/ μC Pro	Conversion (%)
Quartz[a]	800 mg	0	0
Quartz washed with HCl[b]	800	0	0
Quartz activated by X-ray[c]	800	1250	0.06
$Fe_3(PO_4)_2$	4 mM	53900	2.42
$Fe_3(PO_4)_2 + SiO_2$	4 mM, 800 mg	55300	2.42
Ascorbic acid	5 mM	33300	1.50
Fe^{2+} (EDTA)	4 mM	25100	1.13
Fe^{2+} (EDTA) + ascorbic acid	4 mM, 5 mM	75400	3.40
CuCl	3 mM	31800	1.43
$CuCl + SiO_2$	3 mM, 800 mg	32400	1.46
CuCl + ascorbic acid	3 mM, 5 mM	109000	4.91

[a] The quartz used (Quartex) contains 93.84% particles less than 1 μ; 4.95% particles, 1–2 μ. Before incubation the sample was striated for 1 hour at 400°.

[b] One gm quartz boiled with 40 ml 6 N HCl for 2 hours; then washed into neutral reaction. Before incubation sample striated for 1 hour at 400°.

[c] Immediately before incubation irradiated by dose of 1000 r (60 kV; 20 mA; filter, 2.5 mm Al).

In addition to the experimental demonstration of the ineffectiveness of quartz in proline hydroxylation, there are theoretical reasons which speak against the conception of Marasas and Harington. It has already been mentioned that proline hydroxylation occurs after it is built into the collagen polypeptide. This process is enzymically controlled by hydroxylase. It is a highly specific process, because only those prolines destined for hydroxylation, whose positions are determined by an appropriate amino acid sequence in polypeptide chain, are acted upon. With free radicals, on the other hand, whose formation must be assumed as a possible effect of quartz dust, nonspecific proline hydroxylation occurs in positions 4 and 3 under *cis* and *trans* formation of hydroxyproline (Hurych, 1967). Such possible

formation may be expected. In this area we studied changes in collagen proteins in lungs after application of a substance with toxic properties (aerosil) in comparison to fibrogenic quartz dust. Lastly, changes in collagen structures were studied by chemical and morphological methods in order to provide data on another question, i.e., the reliability of evaluating the degree of fibrosis by such techniques.

PROLINE HYDROXYLATION BY QUARTZ DUST

Hydroxyproline as well as hydroxylysine are amino acids, quite unique for collagen. After exogenous administration they are not utilized in collagen biosynthesis. They can therefore only be used for the building of collagen molecules if they are formed endogenously by proline and lysine hydroxylation. It is now accepted that such hydroxylation only takes place after the incorporation of proline and lysine into the peptide chain (for review see Udenfriend, 1966).

It seemed logical to therefore assume, with Marasas and Harington (1960), that fibrogenic quartz participates in proline hydroxylation by its oxidative activity, thus stimulating collagen protein formation, since they found that the oxidative activity of various dusts correlates with their fibrogenic capacity. They reported experimental data indicating that quartz produces several oxidative and hydroxylative conversions of organic compounds including, among others, the catalyses of proline conversion to hydroxyproline. The maximum yield of hydroxyproline obtained by them with quartz, under optimum conditions, was 0.1% in the presence of ascorbic acid.

Research in proline and lysine hydroxylation and control of collagen biosynthesis are basic tasks in our laboratory. A review of the studies of Marasas and Harington has therefore been of considerable interest to us.

With regard to methods it should be mentioned that we used purified ^{14}C-proline (U) which we added to phosphate buffer, pH 6.0. Other substances were added in the quantities noted in Table I, and the mixture was aerated by a constant volume of 95% O_2 and 5% CO_2. After 4 hours of incubation hydroxyproline was separated by ion-exchange column chromatography. For further methodologic details, see Table I.

Typical results are summarized in Table I. It appears that quartz, unmodified or modified by hydrochloric acid or activated by X-ray irradiation (1000 r), added to the medium immediately after irradiation, did not influence hydroxylation of ^{14}C-proline incubated in the medium. Proline hydroxylation, on the other hand, can be demonstrated but must be evaluated in the knowledge that the separate presence of the individual components of the hydroxylating system, in its complete composition, leads to 3–5% proline conversion to hydroxyproline. If in addition quartz is added to the individual components of the hydroxylating mixture, their hydroxylating effectiveness is not enhanced.

In order to clarify the causes of these differing results we attempted to reproduce the technique used by Marasas and Harington, i.e., the nonradioactive determination of the amount of hydroxyproline and proline. But here the first difficulties appeared.

If hydroxyproline determination is carried out (Stegemann, 1958) in solutions

ENVIRONMENTAL RESEARCH 1, 89–101 (1967)

Conflicting Hypotheses on Experimental Silicotic Fibrogenesis: New Experimental Data

M. CHVAPIL

In collaboration with J. HURYCH, B. ČMUCHALOVÁ, R. HOLUŠA, AND E. EHRLICHOVÁ

Department of Experimental Biology, Institute of Industrial Hygiene and Occupational Diseases, Prague, Czechoslovakia

Received February 23, 1967

Experimental evidence is presented that quartz dust, native or modified, does not participate in proline hydroxylation. Close correlation exists between collagen and ascorbic acid accumulation in fibrotic lesions. Nevertheless, the level of ascorbic acid is without effect on the synthesis of collagenous proteins in lung, muscle, i.e., in tissues with low metabolic turnover of collagen. In tissues with high collagen metabolism its extent is affected by the ascorbic acid level. Implications of these results are discussed.

Contradictory results are doubtlessly an incentive of research. It often happens, however, that results which were never really reproduced are quoted in scientific papers or text-books.

It is the aim of this report to submit results of our own experiments in the field of experimental silicosis in which our data suggest conclusions either contradictory to some existing concepts or which appear to clarify others.

Our studies were concerned with basic problems of the development of fibroproductive changes in lungs, characterized by collagen formation. Typical for this protein is the presence of hydroxyproline, due to proline hydroxylation in collagen polypeptide chain. Marasas and Harington (1960) reported that the quartz surface has oxidative properties and participates in proline hydroxylation, thus influencing collagen protein synthesis. This is the first problem which we wish to consider.

It is commonly accepted that ascorbic acid is essential for collagen synthesis. Many experimental observations point to the suppression of wound healing by ascorbic acid deficiency and the accumulation of ascorbic acid in the site of the fibrotic lesion. On the other hand, it is known that in some tissues collagen biosynthesis occurs even with lack of ascorbic acid. Second, therefore, we will discuss the important questions of ascorbic acid and collagen synthesis.

Another generally valid observation is the connection between the solubility of quartz dust and its toxic or fibrogenic properties. In our studies over a number of years of connective tissue reactivity during inflammation, we arrived at the conclusion that any stimulus evoked in affected tissues the same sequence of reactions, these differing only in the extent of response and duration of the individual phase of the inflammation. Thus, exposure to either even a relatively inert substance or to an active toxic substance, means increased collagen

2. There does not appear to be any appreciable variation in the level of plasma vitamin C with advancing age.

3. Sex cannot be said to have any marked effect on the level of plasma vitamin C either in patients with pyorrhoea alveolaris or in normal persons.

REFERENCES.

BLAKE, G.C., and TROTT, J.R. (1962) ... 'Periodontology' published by Butterworth and Co. Ltd., London.

BLOCKLY, C.H., and BAENZIGER, P.E. (1934) An investigation into the connection between the vitamin C content of the blood and periodontal disturbances. *Brit. Dent. Jour.*, 75, 57.

KING, E.J., and WOOTON, L.P. (1956) ... 'Micro Analysis in Medical Biochemistry', 3rd ed.

MARSHALL-DAY, C.D. (1943) ... ... Effect of vitamin C in gingival and periodontal diseases in Punjab. *Ind. Jour. Med. Res.*, 31, 153-160.

PUNAKAR, B.D. (1961)... ... Blood ascorbic acid levels of mental patients in different age groups. Clinical categories and economic status. *Ibid.*, 49, 828-833.

VAISHWANAR, P.S. (1959) ... ... Blood ascorbic acid levels of Indians in different age groups. *Ibid.*, 47, 158.

in the patient is 0·39. The difference between the means of patient and control is 0·475 which is more than three times greater than standard error and thus the difference of vitamin C in their blood is highly significant. It is, therefore, evident that the plasma content of vitamin C among patients is significantly lower than that in normal persons. The above finding in this study is in collaboration with those of Blockly and Benziger (1934), Marshall-Day (1943) and Blake and Trott (1962).

TABLE II.

Showing vitamin C content in blood of patients and controls of various age groups.

Age groups in years.	PATIENT (102) :		CONTROL (102) :	
	Number of patients.	Average plasma vit. C mg./100 ml.	Number of patients.	Average plasma vit. C mg./100 ml.
25 – 30	3	0·29	3	0·7
30 – 35	10	0·42	5	0·74
35 – 40	11	0·34	9	1·10
40 – 45	14	0·34	16	0·90
45 – 50	24	0·36	26	0·85
50 – 55	28	0·46	24	0·78
55 and onwards.	12	0·37	19	0·83

TABLE III.

Showing vitamin C content of blood in different patients and controls according to sex.

Sex.	Number of patients.	Average plasma vitamin C mg./100 ml. P.C.	Number of controls.	Average plasma vitamin C mg./100 ml. P.C.
Male	81	0·39	81	0·86
Female	21	0·39	21	0·84
Total	102	0·39	102	0·85

The level of vitamin C was studied in different age groups of the subjects with pyorrhoea alveolaris and normal controls (Table II). There does not appear to be any appreciable variation in the level of plasma vitamin C with the change in age.

The variation of plasma vitamin C according to sex has been shown in the (Table III). The average levels of male subjects as well as of the female in both the patients and controls are practically the same. Thus, sex cannot be said to have any appreciable effect on the level of plasma vitamin C either in the patients with pyorrhoea alveolaris or in normal persons.

SUMMARY.

1. The average plasma content of vitamin C of the 102 patients investigated in the present study was 0·37 mg. per cent and that of control group was 0·85 mg. per cent. This difference is highly significant statistically.

Indian J. Med. Res 57: 2250-2 Dec 69

1471

Ind. Jour. Med. Res., 57, 12, December, 1969.

STUDY OF ROLE OF PLASMA ASCORBIC ACID IN PYORRHOEA ALVEOLARIS.

B. K. CHAUDHRY.

(*From the Dental College and Hospital, Patna-4, Bihar.*)

INTRODUCTION.

THE role of vitamin C in relation to pyorrhoea alveolaris has been a subject of extensive study during recent years. The present study was undertaken to find out whether there is any association between vitamin C deficiency in the plasma and pyorrhoea alveolaris.

MATERIALS AND METHODS.

A retrospective investigation with 102 patients suffering from pyorrhoea alveolaris attending the Patna Dental College Hospital was undertaken. Control was selected for each case for the purpose of comparison. The controls selected were patients not suffering from pyorrhoea alveolaris, and as far as possible of the same age, sex and socio-economic conditions.

Plasma ascorbic acid was determined by the method of Roe and Kuchher (2·4 dinitrophenyl hydrazine) as described by King and Wooton (1956).

RESULTS.

TABLE I.

Showing the statistical indices of vitamin C content of blood in patient of pyorrhoea alveolaris and controls.

	Patient.	Control.
(1) Average vitamin C, per 100 mg. of blood.	0·378	0·854
(2) Variance vitamin C, per 100 mg. of blood.	0·0627	0·0903
(3) Standard error vitamin C, per 100 mg. of blood:	0·039	
(4) $t = 12·20 > 1·96$		

DISCUSSION.

It will be evident from Table I that average vitamin C in the blood of the controls was found to be 0·854 mg. per cent. The normal average level of vitamin C in human plasma has been found about 0·80 mg. per cent by Vaishwanar (1959) and Punakar (1961). Thus, the average level of vitamin C of the controls in this study tallies with the average of the above workers. The average plasma ascorbic acid level in the patients was found to be 0·37 mg. per cent (Table I). The standard error of vitamin C per mg. of blood

Table 1

Adrenal weights of normal, CCl_4-treated and CCl_4 with ascorbic acid-treated rats

Treatment	Body weight (g)	Adrenal weight	
		Actual wt. (mg)	Relative wt. (mg 100g)
None (Normal)	134.2 ± 47.3 (6)	30.3 ± 0.69	22.6 ± 0.61
CCl_4	124.5 ± 58.8 (6)	43.1 ± 2.39	34.6 ± 1.05
CCl_4 with Ascorbic Acid	125.5 ± 48.2 (6)	30.6 ± 1.16	24.4 ± 0.76

Averages ± s. e. Number of animals between brackets.

reduce the 17-ketosteroid excretion due to hyperplasia of adrenal cortex (9), adrenal hypertrophy in cold shock (7), alloxan diabetes (5), scurvy (12) and in stilbestrol-induced stress (6) possibly by depressing the augmented release of the adenohypophysial corticotrophin (1). Therefore, from the present experimental observations and the available evidence, it is tempting to conclude that the adrenocortical hypertrophy and its prevention by an ascorbic acid therapy in the stressful situation as developed by the administration of CCl_4 is due to an augmented release of adenohypophysial corticotrophin.

Summary

Carbon tetrachloride — a nonspecific stressor — was found to induce adrenocortical hypertrophy in rat. But ascorbic acid was able to prevent the effect of carbon tetrachloride on the adrenocortical physiology.

Acknowledgments

The technical assistance of Mr. Bimal Shankar Paul and the encouraging co-operation of Principal K. C. Gupta and Prof. Suvendu Gupta are gratefully acknowledged. The vitamin used in this experiment was kindly furnished through Dr. M. C. Watsa of Glaxo Laboratories (India) Private Ltd., Bombay.

References

1. Bacchus, H., and N. Altszuler: Endocrinology **51**, 1 (1952). — 2. Brody, T. M., and D. N. Calvert: Am. J. Physiol. **198**, 682 (1960). — 3. Calvert, D. N., and T. M. Brody: Am. J. Physiol. **198**, 669 (1960). — 4. Chatterjee, A.: Experientia **22**, 395 (1966). — 5. Ders.: Endokrinologie **47**, 19 (1964). — 6. Chatterjee, A., and N. R. Bardhan: Naturwissenschaften **53**, 110 (1966). — 7. Dugal, L. P., and M. Therien: Endocrinology **44**, 420 (1949). — 8. Greep, R. O., and H. W. Deane: Ann. N. Y. Acad. Sci. **50**, 596 (1949). — 9. Kayhan, S.: J. Endocrinol. **8**, 211 (1952). — 10. Long, C. N. H.: Recent. Progr. in Hormone Res. **7**, 75 (1952). — 11. Miller, R. A.: Amer. J. Anat. **86**, 405 (1950). — 12. Nadel, E. M., and J. J. Schneider: Endocrinology **51**, 5 (1952). — 13. Sayers, G., and M. A. Sayers: Recent. Prog. Hormone Research **2**, 81 (1948).

(Eingang bei der Redaktion 13. 9. 1966)

Anschr. d. Verff.: Dr. Amar Chatterjee, Dr. N. R. Bardhan, Department of Physiology, Raja Peary Mohan College, Uttarpara, Hooghly, West Bengal India.

306 Endokrinologie Bd. 51 Heft 5/6, 1967

From the Department of Physiology, Raja Peary Mohan College, Uttarpara, Hooghly, West Bengal, India

Prevention of Carbon Tetrachloride Induced Adrenal Hypertrophy by the Use of Ascorbic Acid in Rats

By A. Chatterjee and N. R. Bardhan

Introduction

Carbon tetrachloride (CCl_4) — a nonspecific stressor, has been reported to produce a prolonged stimulatory action upon the central sympathetic areas (3) and causes an augmented release of catechol amines from the adrenal medulla (2). An induction of gonadal inhibition by CCl_4 has recently been documented (4). Epinephrine is capable of evoking the release of ACTH from the adenohypophysis (10). Chatterjee (5) and Chatterjee et al. (6) have noted that the ascorbic acid therapy is able to counteract the hypertrophy and hyperfunctioning states of the adrenal cortex in stressful situations. Interest has therefore been stimulated to study the adrenocortical response to administration of CCl_4 and the influence of ascorbic acid in such instance.

Materials and Methods

Adult female albino rats were used in this experiment, provided with balanced vitaminized diet and water ad lib. 12 of the animals received CCl_4 and cocoanut oil mixture (1 : 1) at a dose level of 0.3 ml/100 g rat body weight through an intraperitoneal route after 24 hours of fasting. The remaining 6 received cocoanut oil as controls. Ascorbic acid (Celin, Glaxo) at a dose level of 1 g/kg was injected twice a day to 6 of the CCl_4-intoxicated rats through an intramuscular route. After 72 hours of experimental period, the animals were sacrificed, adrenal glands were taken for gravimetric and histologic comparison. Blood films were drawn for counting eosinophils and lymphocytes.

Results

CCl_4 produced a significant increase in adrenal weight with depletion of sudanophilic lipid droplets, cholesterol esters and ascorbic acid specially from the zona fasciculata as compared to the oil-treated controls and ascorbic acid-treated CCl_4-intoxicated animals (Table 1). Similarly, a marked eosinopenia and lymphopenia were also noted in the CCl_4-treated animals compared to the oil-treated controls and the ascorbic acid-treated CCl_4-intoxicated rats.

Discussion

Hypertrophy of the adrenal cortex with depletion of its lipid content is associated with markedly increased cortical function (8, 11). Moreover, according to Sayers et al. (13), the decrease in concentration of cholesterol, stainable lipid and ascorbic acid is closely related with the corticotrophic response. The reduction in the number of circulating eosinophils and lymphocytes has also been used as bioassay parameter of ACTH. Ascorbic acid has been reported to

TABLE III. Urinary Excretion of Ascorbic Acid in Adult Male Rats Fed 10% Casein Diet Containing All Vitamins and 2 and 4% Sulfaguanidine.
6 Rats in each group. Period of feeding: 4 wk.

Group	Diet	Urinary total ascorbic acid (mg/rat/day), avg
1.	10% casein diet containing vitamins A, D, E, K, B_1, B_2, B_6, pantothenate, niacin, folic acid, biotin, B_{12}, choline and inositol	2.99 ± .28 ‡
2.	*Idem* with 2% sulfaguanidine	1.02 ± .19 †
3.	Same as 1 with 4% sulfaguanidine	.47 ± .13 *

Differences significant at: * 5%, † 1% and ‡ 0.1% level. See footnotes, Table I.

guanidine was ascertained. Two groups of rats whose excretion of urinary ascorbic acid had been depressed to very low values by maintaining for 3-4 weeks on the 10% protein diet containing 2% sulfaguanidine subsequently received supplements of biotin (4 μg/rat/day orally) and Vit. B_{12} (5 μg/rat/day intraperitoneally) respectively for 12 days with no enhancement in urinary ascorbic acid. In view of the reported involvement of Vit. E in biosynthesis of ascorbic acid in the rat(3, 4) the influence of Vit. E was also examined similarly with negative results. Finally rats distributed into comparable groups by simple randomization were fed for 4 weeks a 10% casein diet containing all the vitamins and 2% and 4% sulfaguanidine. Total Vit. C excretion in the urine of these rats is presented in Table III. Supplementation with all the vitamins simultaneously also failed to counteract the effect of the sulfadrug.

Discussion. The adverse effects of sulfadrugs in animals maintained on low protein diets confirm some of the observations of Schwartz and Williams(5) and their coworkers(6), during their studies on the interrelationship between Vit. C and folic acid. They recorded decreased excretion and liver levels of the vitamin in aminopterin-fed rats and also reported lower liver levels in sufasuccidine-fed rats. It is noteworthy that we have obtained similar results on diets adequately provided with folic acid. The important additional finding is the annulment of the depressing effect of sulfaguanidine on urinary and liver ascorbic acid in rats by high levels of protein in the diet. This protective effect may be ascribed to the effect of excess protein *per se* on the biosynthetic mechanisms or on the precursors, or indirectly on the microflora.

The effect of dietary lactose in reversing the unfavorable effects of sulfaguanidine appears to indicate the possible intervention of intestinal microflora in the biogenesis of the vitamin. In this connection, Evans, Conney, Trousof and Burns(7) reported that galactose is a better precursor for Vit. C than even glucose. Whether lactose serves such a purpose, through the galactose component, has to be critically examined.

The results obtained with animals receiving the vitamin supplements preclude that any deficiency of these vitamins, caused either directly by their absence from the diet, or indirectly, through inhibiting the proliferation of microflora producing these vitamins, is responsible for the effects observed with sulfadrugs.

In addition to the influence of sulfadrugs on the intestinal microflora, their direct effects have also to be considered. Whether these drugs, even in the small concentrations in which they may reach the blood, would exert appreciable influence on liver enzymes directly concerned with Vit. C synthesis is not clear. Another possible effect of these drugs is their influence *per se* on the intestine. Morphological changes in the intestine induced by feeding of such drugs have been reported(8). The counteracting influence of high dietary protein levels and of lactose would then have to be understood in terms of preventing such damage. If the effects of these drugs on Vit. C biogenesis arise through such changes in the intestinal wall, the intestinal tissue would itself be importantly implicated in synthesis of either the vitamin or its precursors.

TABLE I. Effect of Sulfadrugs on Urinary and Liver Ascorbic Acid in Adult Male Rats Fed Diets of Different Protein Content.

Set*	Dietary Casein	Dietary Sulfadrug (%)	Period of feeding (wk)	Urinary total ascorbic acid (mg/rat/day), avg		Liver ascorbic acid: Total (mg), avg		Liver ascorbic acid: mg/g fresh tissue, avg	
1.	0	0	2–3	.46 ±.11	†	2.40 ±.18	†	.35 ±.015	‡
	0	SS—2	"	.23 ±.025		1.60 ±.10		.27 ±.017	
2.	10	0	6–7	1.30 ±.27	‡	3.22 ±.33	NS	.37 ±.026	NS
	10	SG—2.5	"	.49 ±.056		2.57 ±.21		.37 ±.007	
3.	10	0	8	2.91 ±.40	‡	3.74 ±.32	NS	.42 ±.008	NS
	10	SS—2	"	1.04 ±.55		3.69 ±.26		.39 ±.020	
4.	10	0	6	2.25 ±.38	‡				
	10	SS—4	"	.64 ±.11					
5.	20	0	4	3.11 ±.21	‡				
	20	SG—2	"	1.78 ±.27	NS				
	20	SG—4	"	1.42 ±.22	§				
6.	50	0	6–7	1.19 ±.098	NS	3.07 ±.22	NS	.37 ±.018	NS
	50	SG—2.5	"	1.06 ±.11		2.83 ±.18		.34 ±.002	
7.	50	0	6	1.63 ±.30	NS				
	50	SS—4	"	1.48 ±.39					

* The rats in each set were distributed into groups of 6 each by simple randomization.

SS—Sulfasuccidine
SG—Sulfaguanidine

Differences significant at: † 5%, ‡ 1%, § 0.1% level. Since variances are not homogeneous the t-test has been done by approximate method suggested by G. W. Snedecor in Statistical Methods of Analysis, 1950, (Iowa State Coll. Press, Ames, Iowa), p. 83.

observed between animals receiving high and low protein diets.

2. *Influence of lactose.* The effect of the sulfadrugs suggested possible involvement of intestinal microflora in biosynthesis of ascorbic acid in the rat. The influence of inclusion of lactose, known to promote high proliferation of intestinal microflora, in the different diets was therefore examined. In these experiments the sucrose moiety (10%) of the diet was replaced by an equal amount of lactose. From the results (Table II) it is evident that in both high and low protein diets lactose inclusion did not in itself elevate either urinary excretion or liver levels. However, 10% lactose in the diet served to abolish the adverse effects of 2% sulfaguanidine which could again be reversed by increasing the sulfadrug level to 4%.

3. *Influence of vitamins.* Since biotin and Vit. B_{12}, 2 of the important vitamins known to be synthesized by intestinal microflora in the rat, had not been provided in the basal diet, their influence on the effect of sulfa-

TABLE II. Urinary Excretion and Liver Content of Ascorbic Acid in Rats Fed Diets Containing 10% Lactose and Different Levels of Protein and Sulfaguanidine. 6 Rats in each group. Period of feeding: 2 wk.

Set	Dietary Casein (%)	Dietary Sulfaguanidine (%)	Urinary total ascorbic acid (mg/day), avg		Liver ascorbic acid (mg/g tissue), avg	
1.	10	—	1.66 ± .23	NS	.38 ± .04	NS
	10	2	1.30 ± .05		.37 ± .02	
2.	10	—	2.03 ± .33	†		
	10	4	.74 ± .08			
3.	30	—	1.88 ± .18	NS	.41 ± .04	NS
	30	2	1.84 ± .11		.35 ± .028	

Difference significant at: * 5%, † 1%, ‡ 0.1% level. NS = Not significant. See footnotes, Table I.

Proc Soc Exptl Biol. 112(4): 817-830, 1963

Dietary Protein Levels, Vitamins, and Lactose on Depressant Effect of Sulfadrugs on Urinary and Liver Ascorbic Acid in the Rat. (28180)

N. Chandrasekhara, M. V. L. Rao and M. Srinivasan
(Introduced by E. L. Severinghaus)
Central Food Technological Research Institute, Mysore

It has been reported(1) that increase in dietary protein level enhances urinary excretion and liver ascorbic acid content in the rat. Preliminary observations on the depressant effect of sulfaguanidine in low protein diets and its counteraction on 50% protein diets were also recorded. This report concerns an investigation of the effects of sulfaguanidine and sulfasuccidine in relation to dietary protein levels and supplementation with lactose and certain vitamins.

Experimental materials and methods. As in the earlier investigations, these observations were made on adult male albino rats in the body-weight range 160-200 g. Composition of the diets and details of collection of urine were as described previously. The basal diet provided Vit. A and D and thiamine, riboflavin, pyridoxine, pantothenate, niacin and folic acid. In testing the effect of some of the other vitamins they were administered either orally or intraperitoneally. In the final test to ascertain the influence of supplements of all the vitamins omitted from the basal diet the vitaminized starch used at 1% level in the diet contained: 0.5 g menadione (sodium bisulfite), 0.5 g thiamine, 1.0 g riboflavin, 0.4 g pyridoxine, 4.0 g calcium pantothenate, 4.0 g niacin, 0.05 g folic acid, 0.02 g biotin, 0.002 g Vit. B_{12}, 200.0 g choline chloride and 25.0 g inositol per kilo, and the oil soluble vitamins were provided by feeding orally one drop of a suitably diluted stock solution of Vit. A acetate, calciferol and tocopheryl acetate in refined peanut oil so as to provide 20, 10 and 1 I.U. of Vit. A, D and E respectively to each rat daily. In this experiment the salt mixture originally used was also supplemented with 40 mg zinc carbonate per kilo to make up any deficiency of the diet in zinc.

Total ascorbic acid in urine and tissue was estimated by the Roe and Kuether method (2).

Results. 1. *Influence of dietary protein levels.* Data in Table I show results of ingestion of sulfaguanidine and sulfasuccidine at 2-2.5 and 4% levels in diets containing different amounts of protein. The findings fully confirm previous observations concerning the depressant effect of the sulfadrugs on urinary and liver ascorbic acid levels with low protein diets and the counteracting influence of higher levels of protein in the diet, the effect being completely nullified by 50% protein in the diet. The results obtained with the 2 sulfadrugs and at the 2 levels were similar. In the present series of experiments the effect of the sulfadrugs on liver ascorbic acid was not as pronounced as in the previous study(1), except in animals receiving protein-free diets.

Urinary excretions observed in these experiments corroborate the earlier finding(1) that prolonged ingestion, in contrast to short-term feeding, tends to equalize the differences

Experiments are now in progress to ascertain the influence of antibiotics and the direct correlation, if any, between intestinal microflora, as measured by fecal and cecal counts, and the ascorbic acid status of the rat on diets containing different levels of protein, sulfadrugs and also lactose.

Summary. 1. The adverse effect of sulfadrugs on urinary excretion of ascorbic acid in the rat and its counteraction by high levels of dietary protein have been substantiated. 2. Lactose, known to enhance proliferation of intestinal microflora, did not in itself raise ascorbic acid levels in the liver or urine when added to high and low protein diets at 10% level. However, it abolished the adverse effects of the sulfadrugs at 2% level which again was reversed by increasing the sulfadrug level to 4%. 3. Supplementation of the diet with all the known vitamins failed to reverse the effect of the sulfadrug.

We are thankful to Dr. V. Subrahmanyan, for his interest in the work and to Mr. A. N. Sankaran for statistical examination of the data.

1. Srinivasan, M., Rao, M. V. L., Bhagavan, H. N., PROC. SOC. EXP. BIOL. AND MED., 1960, v105, 615.

2. Roe, J. H., Kuether, C. A., *J. Biol. Chem.*, 1943, v147, 399.

3. Chatterjee, I. B., Kar, N. C., Ghosh, N. C., Guha, B. C., *Arch. Biochem. Biophys.*, 1960, v86, 154.

4. Caputto, R., McCay, P. B., Carpenter, M. P., *Am. J. Clin. Nutr.*, 1961, v9, No. 4, Part II, p61.

5. Schwartz, M. A., Williams, J. N., Jr., *J. Biol. Chem.*, 1952, v194, 711; v197, 481; v198, 271.

6. Schwartz, M. A., de Salegui, Miriam, Williams, J. N., Jr., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 858.

7. Evans, C., Conney, A. H., Trousof, N., Burns, J. J., *Biochim. et Biophys. Acta*, 1960, v41, 9.

8. Asenjo, C. F., Quintana, M. L., Lebron, A. P., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 561.

Received September 13, 1962. P.S.E.B.M., 1963, v112.

Lancet 1:1401-1402 1972

ASCORBIC ACID AND THE COMMON COLD

SIR,—Most of the earlier reports [1,2] claiming that supplementary ascorbic acid has little or no effect on the incidence and severity of the common cold have been based on dosages of the order of 200 mg. per day. Pauling [3] lately publicised other work which indicated that higher intakes of ascorbic acid might be beneficial in cold prevention. We wish to report a survey carried out between November, 1971, and March, 1972 (climatically, not a severe winter in Glasgow), amongst some of the staff and students of the University of Strathclyde.

The test group (47 out of the original 50 people completed the experiment) were supplied with 4 × 250 mg. ascorbic-acid tablets to be swallowed every day after breakfast, and the control group (43 out of the original 45 completed the experiment) were given a placebo similar in appearance but containing lactose and 5% citric acid. Every week any symptoms of cold, and their duration, were recorded; only the operator of the survey (S. S. C.) knew the identity of the subjects in the two groups.

INCIDENCE AND DURATION OF COLDS DURING 15 WEEKS IN WINTER

	Ascorbic acid 1 g./day	Placebo
No. of persons in group	47	43
No. of persons having: 0 cold(s)	16	6
1 "	19	11
2 "	11	14
3 "	1	7
4 "	0	5
Total no. of colds	44	80
Average no. of colds per person	0·94	1·86
No. of colds of: 2 days' duration ..	12	2
3 " " ..	13	11
4 " " ..	12	38
5 " " ..	5	26
6 " " ..	1	3
14 " " ..	1	0
Average duration of cold (days)	3·5	4·2

Symptoms during the first week of the survey were negligible and are not included in the accompanying table, which is based on 15 weeks of observations; it was known that up to 7 days might be required for the test group to approach saturation. The tabulated results show that the incidence of colds was reduced by 49% in the group receiving 1 g. ascorbic acid supplement a day compared with the control group. On the basis of a one-tailed statistical test (it was not necessary to consider the possibility of the placebo influencing cold symptoms) it can be shown that 1 g. ascorbic acid a day is effective in reducing the incidence of colds at the 0·002 level of significance. For the subjects taking ascorbic acid, statistical analysis showed that the duration of colds was less at the 0·05 level of significance, and narrowly missed the 0·01 significance level because of one prolonged 14-day cold.

An interesting observation from the present survey is that the reduction in the incidence of colds when taking prolonged supplementary doses of vitamin C was more significant than the reduction in duration of cold symptoms. Some of the previous findings [4] in favour of ascorbic acid as an ameliorator for the common cold have tended to emphasise the reduced severity of the cold rather than the number of colds during the experimental period.

We are aware that criticism can be levelled at this survey (in common with most surveys) on the grounds that it should have been of a double-blind design, should have run for longer, should have had larger groups, and should have investigated even higher levels of ascorbic acid, but most of these factors were outwith our resources and facilities. However, the results obtained are so clearcut in favour of vitamin C as a positive agent in reducing the incidence and duration of the common cold when ingested in higher amounts than could be achieved on a normal dietary regimen or with low supplementation, that we suggest further investigations are needed.

Department of Food Science,
University of Strathclyde,
131 Albion Street,
Glasgow G1 1SD.

SHEILA S. CHARLESTON
K. MARY CLEGG.

1. Cowan, D. W., Diehl, H. S., Baker, A. B. *J. Am. med. Ass.* 1942, **120**, 1267.
2. Glazebrook, A. J., Thomson, S. *J. Hyg., Camb.* 1942, **42**, 1.
3. Pauling, L. Vitamin C and the Common Cold. San Francisco, 1970.

globin. The saline cathartic taken did not have, to our knowledge, any methemoglobinemia-producing substances. Argyria is not known to be associated with methemoglobinemia, nor was methemoglobin found in another patient with argyria. The nitrate ingested with the silver might cause cyanosis, but this medication had been stopped over six months prior to the patient's admission to the hospital: This case is presumed, then, to be a case of idiopathic methemoglobinemia or enterogenous cyanosis.

In the second patient "nitrite syncope" apparently developed from overdosage or from sensitivity to the drug. Although the exact amount of sodium nitrite given is unknown, the rapid development of cyanosis, which became pronounced eight hours later, shows clearly the relation of the drug therapy to the production of methemoglobin. Without doubt the presence of 35 per cent of the hemoglobin in an unusable form gave this woman a functional hypoxia which in the presence of a poor cardiovascular system was of serious consequence. Because of the severe clinical picture the intravenous use of ascorbic acid was preferred to oral therapy. The clinical evidence plus the results of the laboratory studies furnishes conclusive proof that the methemoglobin in the blood was reduced to hemoglobin. We wish to stress that in the second case the reduction was accomplished in a matter of hours rather than days by the intravenous use of ascorbic acid.

SUMMARY

Administration of ascorbic acid was followed by a rapid decrease in the concentration of methemoglobin in the blood of 2 patients showing cyanosis due to methemoglobinemia. At the same time there was prompt alleviation of other symptoms due to the methemoglobin. Ascorbic acid is far more desirable for this purpose than is methylthionine chloride, which may further increase the concentration of methemoglobin in the blood.

According to Goodman and Gilman,[7] "methylene blue [methylthionine chloride] has two actions on hemoglobin. In high concentrations it converts the ferrous ion of the reduced hemoglobin to the ferric form, and thus methemoglobin is produced. Low concentrations of methylene blue or the chemically related thionine are capable in vivo of hastening the conversion of methemoglobin back to the form which once more can carry oxygen." It may be postulated that the action of methylthionine chloride in the reduction of methemoglobinemia is the result of the stimulation of tissue respiration, as indicated by the experiments of Warburg,[9] with the reduction of the methemoglobin accomplished by virtue of an increased enzymatic action of the dehydrogenases.

In contrast to methylthionine chloride, ascorbic acid has been shown to reduce methemoglobin in vitro.[10] The reducing properties of ascorbic acid are well known and have been investigated to a considerable extent. Considering the fact that sodium ascorbate has been administered intravenously in large doses without any deleterious effect and that it is a normal metabolite, it was suggested by one of us (B.D.P.) that the powerful reducing properties exhibited in vitro might be successful in vivo.

At present there is no evidence that methemoglobinemia occurs in subclinical ascorbic acid deficiency or frank scurvy. King and associates,[2f] who studied a patient with chronic methemoglobinemia, were able to demonstrate conclusively that as the ascorbic acid level of the plasma rose there was a concomitant drop in the methemoglobin of 50 per cent in five days with the oral administration of 300 mg. daily of ascorbic acid. The methemoglobin disappeared completely in twenty days under therapy. They then withheld ascorbic acid and were able to demonstrate that as the ascorbic acid fell to low levels the methemoglobin rose to its previous high levels. Denny [4a] and Graybiel [4b] were also able to demonstrate a lowering of the methemoglobin level by oral administration of ascorbic acid. We believe that the low ascorbic acid level encountered in the first case is more fortuitous than etiologic. It is to be emphasized that the use of ascorbic acid in the treatment of methemoglobinemia merely takes advantage of its reducing properties. The possibility that increased susceptibility to methemoglobinemia results from low intake of ascorbic acid requires investigation.

In the first patient the cause of the methemoglobinemia is unknown. So small a quantity as 0.7 mg. of glyceryl trinitrate is probably insufficient to cause the oxidation of approximately 30 per cent of the hemo-

9. Warburg, O.; Kubowitz, F., and Christian, W.: Ueber die katalytische Wirkung von Methylenblau in lebenden Zellen, Biochem. Ztschr. **227**:245, 1930.

10. Keise, M.: Biochem. Ztschr. **316**:264, 1944.

depressed, and slight cyanosis was noted that night. The next morning, March 24, the blood pressure had returned to 180 systolic and 100 diastolic. However, there was an extreme cyanosis of the body, most pronounced in the face and extremities. A sample of blood taken at this time showed 3.9 Gm. of methemoglobin per hundred cubic centimeters when examined spectroscopically. At 12:30 p. m., twenty-four hours after the original medication, 500 mg. of ascorbic acid was given intravenously. Within four hours there was a noticeable decrease in the amount of cyanosis. A further dose of 200 mg. of ascorbic acid was given intravenously. At 6.30 p. m. the cyanosis had disappeared clinically, and the methemoglobin level was 1.9 Gm. A total of 300 mg. was given in two doses intravenously during the remainder of the day. Fifty-six hours after initial therapy the methemoglobin level was 0.4 Gm. per hundred cubic centimeters.

COMMENT

Methemoglobin is the derivative formed by the oxidation of hemoglobin iron to the ferric state, with resultant loss of oxygen combining capacity. By the action of strong reducing agents methemoglobin iron is reduced to the ferrous state and the ability to form oxyhemoglobin restored.

In man, methemoglobinemia may be produced by drugs such as acetanilid and acetophenetidin, by oxidizing agents such as chlorates (used in gargles, matches and explosives), by nitrites, glyceryl-trinitrate and bismuth subnitrate (used in control of diarrhea) and by aniline dyes and their various derivatives. The formation of methemoglobin, in the absence of drug therapy, has been speculatively attributed to absorption of toxic products from the intestinal tract. There is, moreover, increasing evidence that the equilibrium hemoglobin $\leftrightarrows$ methemoglobin is shifted to the normal state by virtue of the glycolytic systems resident in the erythrocyte.[6] Any toxic product tending to inactivate or slow this glycolysis would bring about increasing concentrations of methemoglobin.

Cyanosis due to methemoglobinemia becomes clinically evident, according to Goodman and Gilman,[7] when the methemoglobin attains a level of 3 Gm. per hundred cubic centimeters of blood. Vigness and associates[8] found that cyanosis resulting from the administration of sulfanilamide became evident with a methemoglobin level of 12.3 per cent of the total blood pigment.

Treatment of methemoglobinemia has been undertaken in the past by the use of intravenously administered methylthionine chloride.

6. Drabkin, D. L.: Hemoglobin, Glucose, Oxygen and Water in the Erythrocyte. Science **101**:445, 1945.

7. Goodman, L., and Gilman, A.: The Pharmacological Basis of Therapeutics, New York, The Macmillan Company, 1941.

8. Vigness, I.; Watson, C. J., and Spink, W. W.: Relation of Methemoglobin to the Cyanosis Observed After Sulfanilamide Administration, J. Clin. Investigation **19**:83, 1940.

the patient was symptom free, she was discharged on July 26 and placed on a fat-free diet, with the diagnoses of acute cholecystitis, argyria and methemoglobinemia. Follow-up studies at monthly intervals have shown no return of the methemoglobin nor complaints referable to the gastrointestinal tract. An attempt was made to place the patient on a low ascorbic acid diet to reproduce the syndrome, but the patient was uncooperative.

CASE 2.—A. R., a 69 year old white woman, was admitted to the ophthalmologic ward of the Philadelphia General Hospital, service of Dr. Diechler, because of severe pain and a loss of vision in the right eye of three weeks' duration and a loss of vision in the left eye of thirty-six hours. The patient complained of

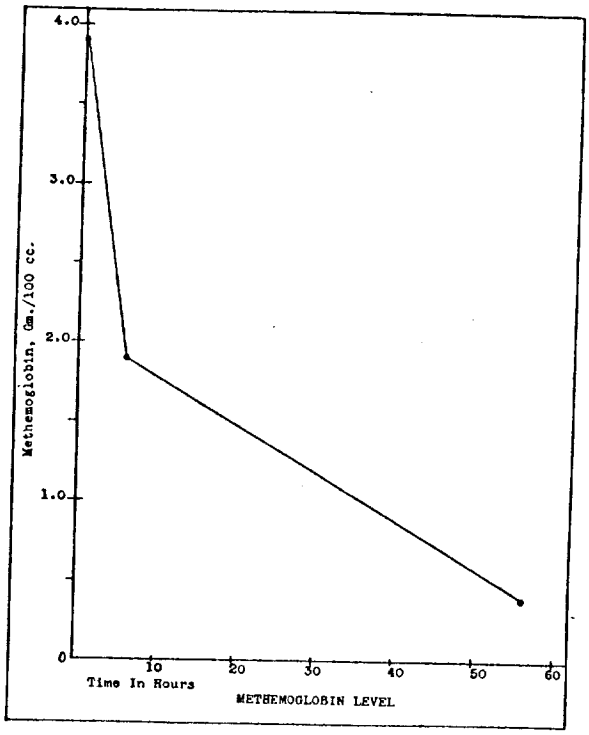


Chart 2 (case 2).—Methemoglobin level of the blood after intravenous administration of a total of 1,000 mg. of ascorbic acid in the first nine hours of treatment. No additional ascorbic acid was administered after the initial nine hour period.

rings around lights, loss of vision and vomiting. There was also a history of mild exertional dyspnea for the past three years. On physical examination the blood pressure was 200 systolic and 100 diastolic. The eyes showed signs of acute congestive glaucoma. Aside from a slight enlargement of the heart the physical examination failed to reveal other significant abnormalities.

On March 23 the patient received 6 cc. of an old solution of sodium nitrite. The exact sodium nitrite content is not known but it was estimated to be between 0.5 to 2.0 Gm. The patient immediately vomited. In one-half hour the patient's blood pressure had fallen to 90 systolic and 50 diastolic. The respirations were

trinitrate and fluids intravenously administered. The temperature became normal in six hours, but the patient still complained of pain in the abdomen and continued to vomit. It was noticed that, although the argyria was prominent, the nail beds appeared cyanotic; the diagnosis of enterogenous cyanosis was suggested. On July 10 a specimen of blood was examined spectroscopically and showed the presence of 3.6 Gm. of methemoglobin.[5] The plasma ascorbic acid level was 0.2 mg. per hundred cubic centimeters. Ascorbic acid therapy was instituted; the patient was given 500 mg. daily by mouth in divided doses for three days. There was noticeable improvement in symptoms within twenty-four hours. By the end of forty-eight hours the patient was able to tolerate food without pain

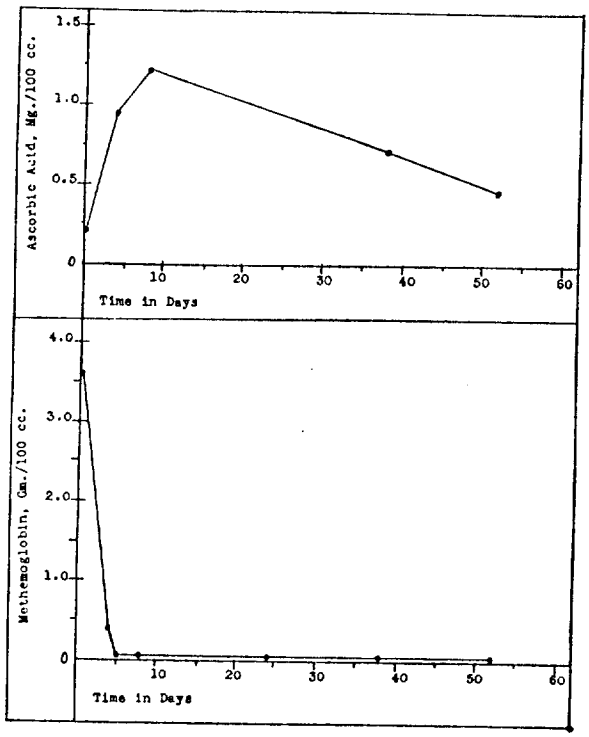


Chart 1 (case 1).—The upper curve portrays the ascorbic acid level of the blood after oral administration of a total of 4,500 mg. in the first six days of treatment. The lower curve illustrates the reduction in the methemoglobin content of the blood concomitant with the administration of ascorbic acid.

or vomiting. In seventy-two hours the methemoglobin level was merely a trace and the ascorbic acid level was 0.95 mg. per hundred cubic centimeters. The patient was then given 1,000 mg. of ascorbic acid daily. On July 15, five days after beginning therapy, there was no methemoglobin demonstrable in the blood. Since

5. Methemoglobin was determined spectroscopically by the procedure of Austin and Drabkin (Austin, J. H., and Drabkin, D. L.: Spectrophotometry of Methemoglobin, J. Biol. Chem. **112**:67, 1935) with a Bausch and Lomb visual spectrophotometer.

Of particular importance in both cases was the striking response of the cyanosis to treatment with ascorbic acid, with concomitant alleviation of all clinical symptoms. We were not aware that ascorbic acid had been previously used at the time it was employed in the treatment of our first patient; however, perusal of the literature has since revealed that Lian and associates[3] were the first to use ascorbic acid in the treatment of methemoglobinemia. Subsequently other reports appeared.[4] Descriptions of the clinical observations in our patients and of their response to treatment with ascorbic acid follow:

CASE 1.—M. K., a 54 year old obese white woman, was admitted to the surgical service of Dr. William Erb in the Philadelphia General Hospital on July 8, 1944, because of a pain of six hours' duration in the right lower abdominal quadrant. From 1940 to 1943, during which time friends began to notice a change in the color of her skin, the patient had been taking silver nitrate pills for a "stomach ulcer." The patient took a teaspoonful of a saline cathartic a day for five or six months preceding admission, until she began to feel "sick to her stomach." A week before admission transient nonradiating pain in the right upper abdominal quadrant developed, which she stated was treated with mild mercurous chloride by her physician. This pain became generalized on the day before admission, and it was associated with several loose, watery, greenish stools. On the day of admission the patient was vomiting.

Physical examination on admission showed a temperature (oral) of 100 F., pulse rate 100 beats per minute and respiration rate 28 per minute. The patient, who on gross examination was retching and appeared acutely ill, was well developed and well nourished. The skin and mucous membranes showed the characteristic changes of argyria. The blood pressure was 130 systolic and 80 diastolic, but examination of the heart revealed no other abnormalities. The lungs were normal to auscultation and percussion. The abdomen showed poorly localized tenderness throughout but was soft, and peristalsis was normal.

Laboratory Examination.—Laboratory studies showed hemoglobin content 14 Gm. per hundred cubic centimeters and leukocytes 7,800 per cubic millimeter, with a normal differential count. The serum protein level was 6.9 Gm. per hundred cubic centimeters and chlorides as sodium chloride 584 mg. Sugar, urea and carbon dioxide-combining power were normal. The reaction to the Kline test was negative. On July 10 the icterus index was 22, but by July 19 it had fallen to 5. The serum cholesterol level was 272 mg. per hundred cubic centimeters, of which 159 mg. was esterified. Roentgenologic examination of the gallbladder showed poor function, but no stones or gross abnormalities were visible.

On admission a tentative diagnosis of acute cholecystitis was made. The patient was treated with morphine, atropine, 1/100 grain (0.6 mg.) of glyceryl

3. Lian, C.; Frumusan, P., and Sassier: Méthémoglobinémie congénitale et familiale: action favorable de l'acid ascorbique, Bull. et mém. Soc. méd. d. hôp. de Paris **55**:1194, 1939.

4. (*a*) Denny, J.; Murdock, E. T., and Rogan, J. J.: Familial Idiopathic Methemoglobinemia with a Note on the Treatment of Two Cases with Ascorbic Acid, Brit. M. J. **1**:721, 1943. (*b*) Graybiel, A.; Lilienthal, J. L., and Riley, R.: The Report of a Case of Idiopathic Congenital (and Probably Familial) Methemoglobinemia, Bull. Johns Hopkins Hosp. **76**:155, 1945. (*c*) King, Gilchrist and White.[2f]

1342

Arch. Internal Med. 78 (3): 296-302. 1946.

METHEMOGLOBINEMIA

Treatment with Ascorbic Acid

LIEUTENANT MILLARD CARNRICK
MEDICAL CORPS, ARMY OF THE UNITED STATES
B. D. POLIS
Senior Biochemist, Division of Laboratories, Philadelphia General Hospital
AND
THOMAS KLEIN, M.D.
Visiting Physician, Department of Medicine, Philadelphia General Hospital
PHILADELPHIA

CYANOSIS is caused most frequently by cardiac or pulmonary disease, but it may be the result of an increased concentration of methemoglobin, among other etiologic factors. In the absence of cardiac or pulmonary disease further study of the blood to establish or exclude the presence of this hemoglobin derivative is of considerable importance. We have recently encountered 2 patients presenting cyanosis as an important symptom. In 1 the disease is of unusual interest in that a spontaneous formation of methemoglobin was apparently the cause of the cyanosis. A similar syndrome was described by Stokvis in 1902 and named enterogenous cyanosis.[1] Since that time a few additional reports of spontaneously occurring methemoglobinemia in the absence of drug therapy have been described.[2] Unfortunately, laboratory examinations of the blood pigments were not always made. In the other patient sodium nitrite therapy was the cause of the cyanosis.

Methemoglobinemia is commonly accompanied not only with cyanosis but also with disturbances in gastrointestinal function; frequently diarrhea, usually of long duration, has been noted. In 1 of our patients the combination of cyanosis and gastrointestinal symptoms was prominent. In both patients methemoglobinemia was suspected and confirmed by laboratory studies.

---

1. Stokvis, B. J.: Casuistic Contributions to the Autotoxic Enterogenous Cyanosis, Nederl. tijdschr. v. geneesk. **2**:678, 1902.

2. (*a*) Talma, S.: Intraglobuläre Methaemoglobinaemie bei den Mensch, translated, Berl. klin. Wchnschr. **39**:865, 1902. (*b*) Berron, G. A.: Enterogenous Cyanosis (Stokvis Syndrome), Rev. méd. Yucatan **13**:15, 1924. (*c*) Innes, F. R.: Case of Enterogenous Cyanosis, Indian M. Gaz. **66**:262, 1931. (*d*) Dunaevskiy, M. L. and Kozlovskaya: A Case of Autotoxic Methemoglobinemia, Vrach. delo **22**:367, 1940. (*e*) Traxler, P. S.: Methemoglobinaemia or Enterogenous Cyanosis, M. Bull. Vet. Admin. **20**:227, 1943. (*f*) King, E. J.; Gilchrist, M., and White, J. C.: A Case of Methemoglobinemia, Biochem. J. **38**:8, 1944.

Treatment and Prevention of Scurvy

Ascorbic acid is the specific therapy for scurvy. The vitamin is usually administered by the oral route but it may also be given by intramuscular or intravenous injection. The tablets are available in 25, 50 or 100 mg. of the vitamin; the solution usually comes in 100 mg. per 5 ml. In the majority of cases of scurvy, 100 mg. of ascorbic acid daily and a well balanced diet are enough to produce rapid cure. The dose should be increased in the presence of tuberculosis, pregnancy, lactation, malignancies or hyperthyroidism, conditions where the daily requirements for vitamin C are increased. One gram of ascorbic acid may be administered daily until the symptoms are under control. There is no evidence of toxic effects from large doses of vitamin C.

The estimated daily requirements of vitamin C vary according to different sources. The National Research Council of the U. S. recommends a daily allowance of 70 mg. The British Medical Association has stated that a daily intake of 30 mg. of vitamin C would provide a good margin of safety. Their statement is supported by the experiment conducted in 1948 with 20 volunteers where seven of them were kept fit on a daily intake of only 10 mg. of ascorbic acid; moreover, six individuals in whom scurvy was induced in this experiment were relieved of the disease by the same daily dose. The question remains though as to whether this daily intake which obviously prevents scurvy is enough. It is, in any case, insufficient to maintain the body in a state of full saturation. In animals which are capable of synthesizing vitamin C the vitamin content in the different organs has a constant value, i.e., they are saturated.[2] It would appear then that the state of saturation is the most favorable for man as well.

References may be secured from the Grand Rounds Editor.

Diagnosis

The clinical diagnosis of scurvy is based upon the combination of poor dietary history, presence of hyperkeratosis of the hair follicles, perifollicular hemorrhages, ecchymosis and typical gum changes. The differential diagnosis should include the *purpuras* (low platelet count, prolonged bleeding time and clot retraction, drugs, fever and blood cultures for subacute bacterial endocarditis, lupus erythematosus preparations, etc.); *dilantin toxicity* may have similar gum lesions but gingivitis due to ascorbic acid deficiency seldom occurs without other florid manifestations of scurvy; *rheumatoid arthritis* also gives swollen joints but not bloody joint effusions; moreover the scorbutic patient will not have deformities such as are common to rheumatoid arthritis.

The following is an outline of laboratory diagnostic aids that should be used to confirm the clinical diagnosis:

Ascorbic Acid Levels in Blood and Urine

The plasma level of ascorbic acid falls too rapidly after the vitamin is removed from the diet and is of no diagnostic value. According to Crandon,[9] ascorbic acid levels in plasma fell to zero after 41 days of ascorbic acid deprivation, and signs of scurvy did not appear until day 134. Therefore, the plasma ascorbic acid level indicates the state of ascorbic acid nutrition for the preceding few weeks. A plasma level of 0 mg.% is compatible with but not diagnostic of scurvy. If ascorbic acid has not been administered previously, any amount of ascorbic acid found in plasma is incompatible with the diagnosis of scurvy.

The ascorbic acid level in the white cell-platelet layer (buffy coat) has diagnostic significance. Normal values are 25-40 mg. per 100 Gm. The buffy-coat level reaches zero after 125 days of ascorbic acid deprivation, paralleling the onset of scorbutic lesions. As Vilter[26] has stated, a value of 0 mg.% in the buffy-coat is the only laboratory evidence of scurvy.

Ascorbic acid almost disappears from the urine even before the level in the plasma is zero. Therefore, a low level of ascorbic acid in urine has the same significance as a low plasma level: it will make a diagnosis compatible but no more. This applies to a single collection as well as a 24-hour collection.

Saturation Test

This test depends upon changes in the urine and plasma ascorbic acid levels after an appropriate test dose. The usual technique is: (1) Administration of 100 mg. of ascorbic acid by intravenous injection; and (2) Determination of urinary excretion of ascorbic acid during the next three hours. A normal, well saturated person will excrete 50% of the test dose; a depleted person will excrete 15% or less, and a scorbutic person will excrete less than 5% of the test dose.[26] The test indicates only that a person's tissues are saturated or unsaturated. The results can be compatible but never diagnostic of scurvy. This is due to the fact that the kidneys will reabsorb most of the vitamin when the tissues are at all unsaturated.

Other Tests

Test for capillary fragility, x-ray of the bones, increased tyrosil derivatives, etc., have been used but are not diagnostic. It is of importance to mention that in infantile scurvy x-rays are the most helpful tool for the diagnosis.

tion; and (3) increased blood destruction. It is quite clear now, however, that the first factor is of little importance. Cutforth[21] found no correlation between the severity of the anemia and the degree of extravasation of blood into the tissues. Goldberg and Cox[24] have reported consistently negative tests for occult blood in feces in all their patients with scurvy, and the same was reported by Chazan and Mistilis. The last two factors (lack of blood formation and increased blood destruction) have been supported by clinical and experimental observations. As was mentioned, ascorbic acid is related to folic acid metabolism and to the intestinal absorption of iron and the transfer of iron to the ferritin form. It has been shown that blood transfused into scorbutic patients survived poorly. By using 51-Cr, Goldberg[24] demonstrated increased hemolysis in four of six scorbutic patients with anemia. The hemolytic process was corrected by the administration of ascorbic acid.

In summary, the anemia of scurvy is multifactoral in origin; and in the adult it conforms to no particular type: it may be normocytic or macrocytic, normochromic or hypochromic. In the majority of cases that we have reviewed, the anemia was of the macrocytic normochromic type and there was a high reticulocyte count initially. WBC and platelets are usually normal. The bone marrow is generally hypercellular. The serum Fe and IBC are normal or low.

In most cases the anemia is corrected by the administration of ascorbic acid alone. Folic acid sometimes provides benefit, the same as iron. It is evident that chronic general malnutrition, chronic liver disease, chronic infection and external blood losses complicate the type of anemia seen in scurvy and also complicate the therapy. In conclusion: ascorbic acid deficiency has a deleterious effect on blood formation. Whether this is a direct or an indirect effect through interference with folic acid metabolism has not been determined.

Mental Changes

Finally, striking mental changes have been reported in scurvy: confusion, depression and lethargy. They were noted by Cutforth[21] in more than half of his patients. As a rule, all mental changes clear up rapidly with the administration of ascorbic acid.

TABLE 5
Biochemical Data. Chazan and Mistilis[22]

Case No.	Plasma Ascorbic Acid (mg. %)*	Serum Folic Acid (mμg./ml.)**	Serum Vitamin B_{12} Levels (μμg./ml.)†	Serum Iron Level (μg. %)††	Serum Bilirubin (mg. %)	Urine Urobilinogen	Stool Guaiac Test for Occult Blood
I	0.03	5.0	539	64	3.3	1:32	0
II	0.00	....	....	29	2.2	....	0
III	0.18	3.6	400	66	....	....	0
IV	0.12	1.5	160	75	1.6	1:16	0
V	0.05	1.5	2,027	174	....	....	0
VI	0.00	....	....	67	....	....	0
VII	0.05	2.7	170	81	2.4	1:64	0

experimental scurvy was not due to blood loss or reduction in blood volume. It was easily corrected by administration of ascorbic acid. The only explanation we have so far for the hypotension encountered in human scurvy is that the peripheral vascular system becomes less responsive to epinephrine and more prone to vascular collapse and shock. There are experiments in scorbutic animals[1] suggesting that relative adrenal insufficiency may be responsible for the hypotension; but this observation has not been substantiated in man.

Anemia

The incidence of anemia in scurvy has been the subject of numerous papers ever since Mettier, Minot and Townsend[23] gave the first detailed account in 1930. According to recent reviews, anemia is quite common in adult scurvy. Goldberg,[24] reviewing 55 cases of scurvy seen at the Western Infirmary in Glasgow over a 23-year period, found that 49 (89%) of them had hemoglobin values below 12 Gm. on admission. Cutforth[21] found anemia in nine out of eleven patients with scurvy; Shafar[25] has reported severe anemia in two of the three cases seen over eight months at a public hospital. Chazan and Mistilis[22] found anemia in all seven patients reported, the condition being severe (hematocrit values below 23%) in four of them. Recently Cox et al.[13] and Asquith[27] have reported several cases of anemia in patients with scurvy.

There are three factors to which the anemia of scurvy has been attributed: (1) loss of blood (from the gut or into the tissues); (2) lack of blood forma-

TABLE 3

Summary of the Presenting Symptoms. Data from Chazan and Mistilis[22]

Case No.	Weakness and Lassitude	Weight Loss	Anorexia	"Red Spots" on Arms and Legs	Swelling and Ecchymosis of Extremities	Syncope Dizzy Spells	Painful Gums
I	+	+	+	+	+	0	0
II	+	+	+	+	+	0	+
III	+	0	+	0	+	0	0
IV	+	+	0	+	+	+	0
V	+	+	0	+	0	0	0
VI	+	+	+	+	0	0	0
VII	+	+	+	+	+	+	0

Note: + = present; 0 = absent.

TABLE 4

Summary of the Physical Findings on Admission. Data from Chazan and Mistilis[22]

Case No.	Pulse (beats/min.)	Blood Pressure (mm. Hg.)	"Cork-screw Hairs"	Peri-follic-ular Hemor-rhages	Hyper-keratosis of Hair Follicles	Gum Changes	Ecchy-moses	Capil-lary Fragility Test	Infection
I	84	100/60	+	+	+	+	+	0	0
II	110	110/55	+	+	+	+	+	+	Tuberculosis
III	84	130/70	0	0	0	0	+	0	0
IV	80	110/60	+	+	+	+	+	0	0
V	80	104/70	+	+	+	+	0	0	0
VI	78	110/60	+	+	+	Edentia	0	0	Pneumonia
VII	88	115/70	+	+	+	Edentia	+	+	0

Cutforth[21] has reported 11 cases of adult scurvy seen in a teaching hospital in London over a six-year period. There were ten men and one female; the average age was 65 years; and all but one were living alone, on diets of tea, toast, bread and butter, meat and fish-paste sandwiches. Three of the patients had a previous history of duodenal ulcer and were purposely avoiding fruits and vegetables. All of them had been eating the above diet for a period longer than six months prior to admission. The characteristic manifestations are recorded in Table 2.

TABLE 2
Symptoms Found in 11 Patients with Scurvy[21]

Symptom	Number of Patients
Bruising	11
Extensive spontaneous extravasation present in six of them	
Anemia	9
Normocytic normch. 5	
Macrocytic 4	
Pain in limbs	8
Anorexia	8
Lethargy	6
Purpura	6
Hyperkeratotic hair follicles	6
Swollen gums	2
(Seven of the cases were edentulous)	

Ascorbic acid in doses of 600 mg. daily was given to all the patients. Five received a standard ward diet, ascorbic acid and no other medication; the remaining six were given ferrous sulphate and vitamin B complex as well as ascorbic acid. All cases responded immediately to therapy; the purpura and smaller bruises were gone within one week and the hemoglobin levels became normal within a month. There was no difference in the response of the two groups.

Chazan and Mistilis[22] reported seven cases of adult scurvy seen at Boston City Hospital during a one-year period. The average age was 65 years; six were single men living alone. All gave a history of poor dietary intake; none could recall having eaten fresh fruits or vegetables for at least six months prior to admission.

Symptoms

Ecchymosis

Tables 3 to 5 show the most important symptoms, physical findings and biochemical data. It is worth noting that a huge ecchymotic area on a lower limb was the outstanding complaint in three instances. Five of the patients had a relatively low blood pressure. In fact, two of the patients had sudden, profound hypotension with no response to therapy and died. Post-mortem examination failed to reveal an immediate cause of death.

Low Blood Pressure

The work by Crandon[9] in 1940 showed that low blood pressure in human

of ascorbic acid. This leads to a topic that has received particular attention from Russian investigators: the effects of vitamin C on serum lipids. According to Miasnikova and Tiapina[17] high doses of vitamin C (500 mg. I.V. daily) would reduce serum cholesterol and improve the clinical status of patients with myocardial ischemia. However, the recent work of Samuel and Salchi[18] has failed to confirm this.

Other Possible Functions

Ascorbic acid has been implicated in many different body processes: carbohydrate metabolism, formation of antibodies, activity of serum complement, phagocytosis, detoxicating function, etc. However, we lack good experimental evidence to support any such theory.

Clinical Manifestations of Scurvy

Under Experimental Conditions

There are three stages in the development of experimental scurvy in humans:[9,19] (1) initial stage, with changes in the vitamin content of the blood and no clinical signs; (2) clinical scurvy, with changes in the skin, gums and impaired wound healing; (3) final stage with hypotension and danger of death. Our present knowledge is based mainly on the work of Crandon[9] who induced scurvy in himself and on the Medical Research Council experiment[19] in 1948.

Symptoms of scurvy appeared after about 150 days on a vitamin C-free diet. They consisted of keratosis of the hair follicles in the skin and perifollicular hemorrhages. After six months, swelling and hemorrhages of the gums appeared, and wound-healing processes were affected. Later on, signs of cardiac ischemia and peripheral collapse may appear, together with loss of neuromuscular coordination and marked asthenia, forcing the end of the experiment. No anemia, bleeding, weight loss or susceptibility to infection were noted.

Naturally Occurring in Adults

Most cases are encountered among old men living alone ("bachelor scurvy") whose diet is grossly imbalanced and devoid of sources of ascorbic acid. Ignorance, apathy, alcoholism and poverty are the usual basis for its development. In some cases scurvy is only one of the many manifestations of malnutrition. A fair number of cases have been reported among people with gastrointestinal disturbances (peptic ulcer, diverticulosis, chronic cholecystitis) who were advised to avoid fruits and vegetables and followed the advice too drastically. It is worth noting that the Sippy diet affords a daily intake of vitamin C of only 0.5 mg.,[20] obviously not enough to cover the daily requirements.

Adult scurvy tends to appear more commonly in the spring, reflecting the relative lack of fresh fruits and vegetables during the winter months. The most frequent clinical manifestations are:

1. Malaise, weakness, lassitude
2. Perifollicular hemorrhages (abdomen, thighs and forearms)
3. Petechiae and ecchymoses
4. Swollen and bleeding gums with loss of teeth
5. Anemia, leukopenia
6. Mental confusion
7. Postural hypotension and syncope
8. In fatal cases, vomiting and "addisonian crisis" precede death.

inhibition by its substrate, but does not act as a cofactor for that enzymatic step. Moreover, other reducing agents have been shown to be equally effective in "protecting" the same enzyme.

Bone and Connective Tissue Metabolism

The synthesis of collagen requires ascorbic acid.[12] It has also been shown that scorbutic guinea pigs not only fail to synthesize new collagen fibers but show a rapid collagen degradation. Although the synthesis of collagen is a very complex matter and not totally understood as yet, present evidence suggests that ascorbic acid may intervene in the conversion of proline to hydroxyproline, before the synthesis of the peptide chain. Hydroxyproline is not synthesized in the absence of ascorbic acid, and it is important to remember that exogenous hydroxyproline is not incorporated into collagen.

Ascorbic acid is also essential for the formation of bone and cartilage; the proper functioning of osteoblasts and odontoblasts depends on it. In scurvy, bone formation ceases; hence the typical low serum alkaline phosphatase values. The osteoblasts are unable to synthesize osteoid and become surrounded by liquid with a region of edematous connective tissue appearing at the end of the diaphysis. In growing bones, subperiosteal hemorrhages frequently occur. Fractures heal slowly, as do wounds.

Adrenal Glands

No other organ in the body contains as much ascorbic acid as the adrenal cortex (about 5 mg. of ascorbic acid per gram of dried tissue). However, we simply do not know what ascorbic acid does in the adrenal. There is no evidence that it is involved in the synthesis of cortical hormones. Patients with scurvy have normal adrenal function, and patients with Addison's disease do not develop scurvy.[10]

*Erythropoiesis**

Anemia (of no particular type) is frequently seen in cases of scurvy and is usually corrected by the administration of ascorbic acid alone. Although this is still a controversial problem, most investigators now agree: (a) *Ascorbic acid plays some role in the metabolism of folic acid;* it seems to be essential for the conversion of folic acid into its active form, folinic acid (citrovorum factor). This has recently been questioned by Cox et al.[13] who found no evidence for that theory but concluded that ascorbic acid is indeed involved in the protection of folic acid reductase, maintaining a higher concentration of folic acid coenzymes. (b) *The transfer of plasma-bound iron into the liver and its incorporation into ferritin in this tissue requires the presence of ascorbic acid.* Since ascorbic acid is rather specific for this reaction and cannot be replaced by other reducing agents this could well represent a unique biochemical function for vitamin C.[14]

Cardiovascular System

It has been shown[15] that the peripheral vascular system requires vitamin C to maintain its responsiveness to stimulation with epinephrine. Ascorbic acid is involved in the maintenance of good capillary tone and resistance. It is also known[16] that patients with scurvy may develop electrocardiogram abnormalities (prolonged PR, elevation of ST) that are corrected by the administration

*See Test Your Word Score Page 759.

100 ml.; RBCs contain about twice as much, and WBC and platelets contain 20 to 40 times more than plasma.[8] The adrenal cortex and the anterior pituitary contain more than any other organ or tissue in the body.

Metabolism

The vitamin exists in the body largely in the reduced form (ascorbic acid) in reversible equilibrium with a relatively small amount of dehydroascorbic acid (oxidized form). Ascorbic acid is partially destroyed and partially excreted by the body. It is believed that on a daily intake of 100 mg. about 50 percent undergoes metabolic conversion to CO_2 (excreted by the lungs) and oxalic acid (excreted in the urine). It is also excreted as ascorbic acid in the urine, the total amount being some 20 mg. in 24 hours.

There is no evidence that any particular organ or tissue serves as a storage reservoir for vitamin C. However, the body probably does have mobilizable reserves of ascorbic acid since clinical manifestations of scurvy develop in man only after several months of being on an ascorbic acid-free diet. It has been estimated[9] that the total vitamin C content of the human body when fully saturated ranges between 4 to 6 Gm., of which perhaps 200 mg. is present in the plasma and extracellular fluid, and the rest within the cells.

There is a renal threshold for vitamin C excretion. The vitamin is excreted by the kidneys in large amounts only when the plasma levels exceed this threshold, which is approximately 1.4 mg.%. Therefore, when the body is saturated with ascorbic acid, the plasma level will be approximately that of the renal threshold; when further amounts of vitamin C are administered most of it escapes into the urine. When the tissues are not saturated and plasma level is low, the ingestion of ascorbic acid results in little or no renal excretion. This is the basis for the so-called "saturation test."

Function

The precise functions of ascorbic acid in tissue metabolism are totally unknown; so also are the biochemical lesions which cause scurvy. Vitamin C stimulated more inquiry than perhaps any other vitamin, but we have not yet gotten an answer as to what it does in the body. Following is a short list of the metabolic functions vitamin C is probably related to.

Tissue Oxidation and Reduction

Shortly after its discovery it was thought that the main role of ascorbic acid was to act as hydrogen acceptor in tissue oxidation mechanisms. However, we lack any good evidence of this, and most investigators now agree that ascorbic acid acts probably as an anti-oxidant by providing a supply of readily available hydrogen. This goes along with the fact that vitamin C is present largely in the body in the reduced state (ascorbic acid) and only a small part in the oxidized form (dehydroascorbic acid). Thus, ascorbic acid may play an important role in keeping SH-activated enzyme systems in their reduced state.

Tryosine Metabolism

It is well known that scurvy patients metabolize tyrosine incompletely when given extra amounts of this amino acid.[10,11] This led to the discovery that ascorbic acid protects the enzyme p-hydroxyphenylpyruvic acid oxidase from

Scurvy was a subject of interest for several scientific investigators of the 19th century. Hirsch (1860),[2] in his "Handbuch der historisch-geographischen Pathologie," concluded: "Scurvy appears when a special ingredient is lacking in nourishment and the disease disappears as soon as this substance is administered." This concept may well have instigated the theory of nutritional deficiencies. It was also around this time (1883) that Barlow published his classic observations on infantile scurvy.

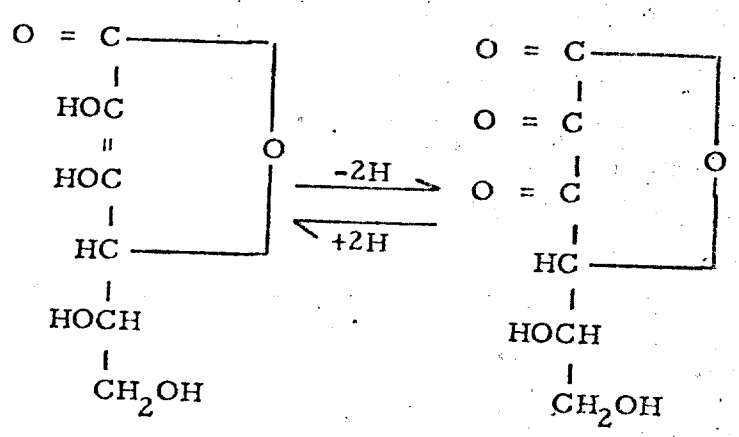
The era of experiments on nutrition began in 1881, when Lunin started animal experiments on the influence of diet on nutrition.[3] This lead to the discovery of polyneuritis gallinarum by Eykman in 1896, which founded the new theory of vitamins. In 1912, Holst and Fröhlich[4] fed guinea pigs exclusively on cereals and induced a disease essentially similar to scurvy, which could be cured by adding fresh vegetables and their extracts to the diet. The concept of vitamins was introduced by Casimir Funk in 1913. Diseases like scurvy, beri-beri and pellagra were called avitaminosis. The vitamin lacking in scurvy was called vitamin C.

In 1928 Szent-Gyorgyi[5] isolated from adrenal glands and cabbage leaves a crystalline substance which had an acid reaction and possessed strongly reducing properties. He called this substance hexuronic acid; but it was not until 1932 that hexuronic acid was identified with vitamin C and renamed ascorbic acid.

Chemistry

The structural formula of vitamin C was established in 1933 as a result of the work done by Haworth, Hirst and co-workers.[6]

Ascorbic acid forms colorless crystals which are freely soluble in water. It is a strong reducing agent and is therefore readily oxidized. The first oxidation product is dehydroascorbic acid, which can be reduced to ascorbic acid by hydrogen sulphide, cysteine and glutathione. Dehydroascorbic acid is as potent an antiscorbutic as ascorbic acid itself (Figure).



L-Ascorbic Acid L-Dehydroascorbic Acid

Figure

or at the latest twelve hours, after this dose. The urine is tested for ascorbic acid by the dichlorophenol-indophenol indicator (Roche). This method follows the saturation test of Harris and Ray (1935). It readily enables one to determine the ascorbic acid saturation of the patient. The presence of 5 mg. or more of ascorbic acid per 100 ml. of urine is taken to indicate adequate saturation.

The patient returns for inspection the following day, when a further 300 mg. of ascorbic acid is given. The urine passed after this dose is then tested. Even after one such dose a great difference in the gum tissue can be observed. The pain has largely disappeared, and the red and congested area of gum has shrunk to half the original size. The gums still bleed on pressure. During the first and second days of treatment some patients experience a tingling sensation in the gums. This is probably associated with the reparative process. The treatment is continued for a further four days, or for two days longer than the time it takes to increase the amount of ascorbic acid in the urine to a value of 5 mg. per 100 ml. The amount of ascorbic acid found necessary to saturate the patients has varied from 900 to 4,200 mg. The average dose has been 2,000 mg.

In most cases there is no bleeding from the gums on pressure after four days' treatment. The long-standing cases appear to be more refractory. One patient who had suffered from marginal gingivitis for four years required 4,200 mg. before his gums responded satisfactorily. The statement that no bleeding occurs on pressure must be qualified to some extent, however, by remarking that even after the gum has returned to its normal healthy consistency firm pressure will still provoke some bleeding from the interdental papilla between teeth 2 and 3 (lateral incisors and canines). For some unexplained reason this area heals slowly. It may be that this persistent bleeding is associated with lack of a factor that is capable of restoring capillary permeability to normal. Szent-Györgyi claims that the haemorrhagic lesions in scurvy are in part due to absence of vitamin P (hesperidin). Patients have all volunteered the information that they feel much better in general health, and their appearance certainly bears this out. When the patients are fully saturated and their gums are in good condition they are given a maintenance dose of 100 mg. a day. They return for inspection each week.

During the course of treatment by ascorbic acid no dental treatment whatever is given. We did not adopt the customary procedure of scaling or application of mouth-washes, etc., as we wished to confine our observations to the action of ascorbic acid alone. It is interesting to remark that in 7 cases the state of oral hygiene and of the teeth was very bad indeed.

The results would apparently indicate that lack of ascorbic acid is one important factor in the causation of such cases of gingivitis. The large amounts necessary to saturate the patients are very striking. The disappearance of the lesion with continued administration of the vitamin must be more than purely fortuitous. As long ago as 1930 Hanke advocated the use of massive doses of orange juice in the treatment of gingivitis. He recommended one pint of orange juice daily for long periods—up to a year at least. This amount of orange juice contains approximately 300 mg. of ascorbic acid and possibly other compounds. He attributed his excellent results to the presence of vitamin C. Roff and Glazebrook (1940) have also obtained successful results. They state, however, that marginal gingivitis can be cured by better oral hygiene. Other workers who have reported favourable results of ascorbic acid in the treatment of gingivitis are Kramer (1937), Demoulin (1938), and Herlitz (1939).

Summary

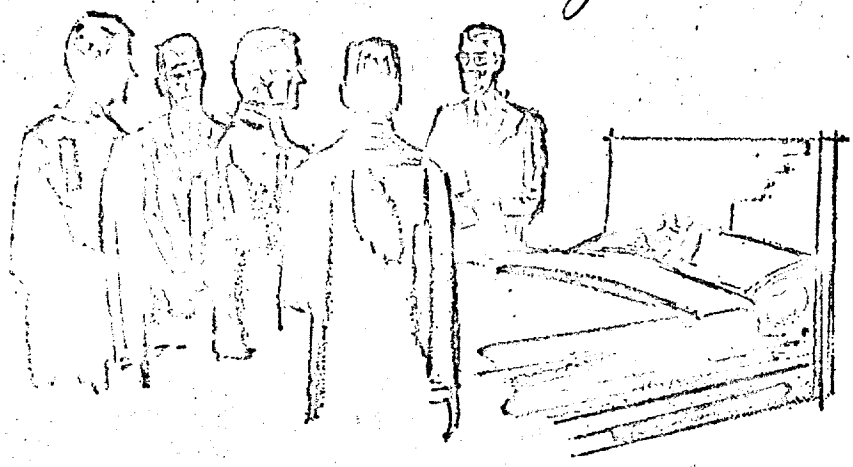
Fourteen cases of gingivitis have been treated with massive doses of ascorbic acid without any other dental treatment such as scaling or mouth-washes. 300 mg. of ascorbic acid was given daily until a urine test showed that the patient was saturated with the vitamin. On an average a total of 2,000 mg. was needed. The previously sore and inflamed gums became normal after approximately four days' treatment. The treatment should be followed by a maintenance dose.

We are indebted to Roche Products Ltd. for our supply of ascorbic acid and indicator tablets.

REFERENCES

Demoulin, P. (1938). *Rev. belge Stomatol.*, **35**, 164.
Hanke, M. T. (1930). *J. Amer. dent. Ass.*, **17**, 957.
Harris, L. J., and Ray, S. N. (1935). *Lancet*, **1**, 71.
Herlitz, C. W. (1939). *Acta paediatr.*, Stockh., **24**, 341.
Kramer, J. (1937). *Dtsch. Militärarzte*, **12**, 489. Cited by Roff and Glazebrook.
Roff, F. S., and Glazebrook, A. J. (1940). *Brit. dental J.*, **68**, 135.

Minn Med 52:817-27 May 69



Grand Rounds

Adult Scurvy*

RAFAEL CARMENA, M.D.
Minneapolis, Minnesota

THE FIRST RECORDED accounts of scurvy are to be found among the writings of the physicians who accompanied the Crusades in the XIII century. Vasco de Gama, who sailed round the cape of Good Hope in 1498, described how a hundred of his crew of 160 perished from scurvy at sea. Scurvy ran an extremely high incidence among the crews of most maritime expeditions during the 16th and 17th centuries, and it was also seen on land, particularly during wars and in prolonged sieges. The main manifestations were "putrid gums, lassitude, pain in the knees, and skin hemorrhages," and it was soon realized that they could be prevented and treated by eating fresh fruits and vegetables. Sir Richard Hawkins' "Observations in the Voyage to the South Sea," published in 1593,[1] contains an account of the prevention and treatment of the disease with lemon juice. Jacques Cartier (1535), during his exploration to Canada and what is now northern Minnesota, found that the native Indians prevented the disease by drinking a decoction of pine needles, which we now know contain ascorbic acid.

In 1734 Bachstrom[2] wrote that scurvy should be attributed solely to a total abstaining from fresh vegetables, foods and greens, and not to cold climates, sea air, eating salted meat, etc. A few years later James Lind (1753),[3] an English ship's surgeon, published his "Treatise on the Scurvy," wherein, by means of dietetic experiments with 12 scurvy patients, he demonstrated that oranges and lemons were the most effectual remedies. By 1804 regulations were introduced into the British Navy, making compulsory a daily ration of lemon or lime juice to all British sailors. This led to the almost complete eradication of scurvy in the British Navy, and to this day the nickname "limies" is applied to British sailors.

*Presented as a Medical Grand Rounds at Hennepin County General Hospital, March 5, 1968.

Brit. Med. J. (4183); 360-361

TREATMENT OF GINGIVITIS WITH ASCORBIC ACID

BY

H. GORDON CAMPBELL, L.R.C.P., L.R.C.S., L.D.S.
Professor of Dental Surgery

AND

R. P. COOK, Ph.D.
Lecturer in Biochemistry

(From the Dundee Dental Hospital and the Department of Physiology, University College, Dundee, University of St. Andrews)

At the Dundee Dental Hospital, during ten weeks recently, 18 out of a total of 505 new patients have been found to be suffering from gingivitis: 14 of these have been treated by us with massive doses of ascorbic acid with favourable results; 4 failed to co-operate.

The clinical features in a typical case are as follows. The patient complains of painful and bleeding gums. The bleeding is usually spontaneous, but is worse when the teeth are brushed. In 10 cases there was marked lassitude. On examination the gums are found to be red and congested. The area involved varies, but in most cases it extends from the gum margin upwards or downwards for a distance of 8 to 10 mm. On pressure the gums bleed readily, the blood flowing freely from the gum margin. In 2 cases severe sloughing of the gum was observed. In spite of the varied types of gingivitis described in the text-books we have taken as our main criterion of this condition the prompt bleeding of the gum on pressure. This sign is of great value, as it provides a ready means of following the reparative process. In no case could the gingivitis be attributed to trauma, calculus, or the administration of drugs. There were no other gross signs of a pre-scorbutic state.

Treatment

No dental treatment, such as scaling or the use of mouth-washes, was undertaken in any of our cases. On admission the patient is given 300 mg. of ascorbic acid, and a sample of urine is collected approximately six hours,

levels is being investigated by means of a controlled trial of vitamin C supplementation.

References

1. KATARIA, M. S., D. B. RAO AND R. C. CURTIS. Vitamin C levels in the elderly. Gerontol. Clin. 7: 189, 1965.
2. TAYLOR, G. Diet of elderly women. Lancet 1: 926, 1966.
3. LOH, H. S., AND C. W. M. WILSON. The relationship between leucocyte ascorbic-acid and haemoglobin levels at different ages. Intern. J. Vitamin Nutr. Res. 41: 259, 1971.
4. GRIFFITHS, L. L., J. C. BROCKLEHURST, D. L. SCOTT, J. MARKS AND J. BLACKLEY. Thiamine and ascorbic-acid levels in the elderly. Gerontol. Clin. 9: 1, 1967.
5. BOWERS, E. F., AND M. M. KUBIK. Vitamin C levels in old people and the response to ascorbic-acid and to the juice of the acerola. Brit. J. Clin. Pract. 19: 141, 1965.
6. BRIN, M., M. V. DIBBLE, A. PEEL, E. McMULLEN, A. BOURQUIN AND N. CHEN. Some preliminary findings on the nutritonal status of the aged in Onondaga County, New York. Am. J. Clin. Nutr. 17: 240, 1965.
7. MILNE, J. S., M. E. LONERGAN, J. WILLIAMSON, F. M. L. MOORE, R. McMASTER AND N. PERCY. Leucocyte ascorbic-acid levels and vitamin C intake of older people. Brit. Med. J. 4: 383, 1971.
8. MILNE, J. S., M. M. MAULE AND J. WILLIAMSON. Method of sampling in a study of older people with a comparison of respondents and non-respondents. Brit. J. Prevent. Social Med. 25: 37, 1971.
9. HUGHES, R. E. The use of homocysteine in the estimation of dehydroascorbic acid. Biochem. J. 64: 203, 1956.
10. DENSON, K. W., AND E. F. BOWERS. The determination of ascorbic-acid in white blood cells. Clin. Sci. 21: 157, 1961.
11. BARTLEY, W., H. A. KREBS AND J. R. P. O'BRIEN. Vitamin C requirement of human adults. Med. Res. Council Special Rept. Ser. No. 280. London: H. M. Stationery Office, 1953.
12. ELWOOD, P. C., R. E. HUGHES AND R. J. HURLEY. Ascorbic-acid and serum cholesterol. Lancet 2: 1197, 1970.
13. CALDER, J. H., R. C. CURTIS AND H. FORE. Comparison of vitamin C in plasma and leucocytes of smokers and non-smokers. Lancet 1: 556, 1963.
14. BROOK, M., AND J. J. GRIMSHAW. Vitamin C concentration of plasma and leukocytes as related to smoking habit, age, and sex of humans. Am. J. Clin. Nutr. 21: 1254, 1968.
15. PELLETIER, O. Smoking and vitamin C levels in humans. Am. J. Clin. Nutr. 21: 1259, 1968.
16. WOODHILL, J. M. Australian dietary surveys with special reference to vitamins. Intern. J. Vitamin Res. 40: 520, 1970.
17. DODDS, M. L. Sex as a factor in blood levels of ascorbic acid. J. Am. Dietet. Assoc. 54: 32, 1969.
18. GOLDBERG, A. The anaemia of scurvy. Quart. J. Med. 32: 51, 1963.

vegetables seem to be important, the influence of eating fruit being stronger than that of any other variate studied. Despite possible inaccuracies in the dietary information due to defective memory, a consistently high level of statistical significance was obtained between eating fruit and blood concentrations.

Fresh fruit was eaten more frequently by the women than by the men, but green vegetable consumption was similar in the two sexes. Women in all the age groups had markedly higher mean PAA and LAA values than men. When men and women with similar fruit scores were compared, the sex difference in ascorbic acid levels decreased but did not disappear. Thus, the higher fruit consumption of the women contributed to but probably did not entirely account for their higher ascorbic acid levels.

The effect of smoking was considered because it was so uncommon among the women that it could have made a contribution to the sex difference in blood levels. Smokers are known to have lower ascorbic acid levels than nonsmokers (13–15) and it has been considered that diet is not a confounding factor in this relationship (13, 14). In this survey, however, heavy smokers were found to eat fresh fruit significantly less often than nonsmokers. The total intake of ascorbic acid is not known so that the full importance of this interaction cannot be assessed, but some difference in dietary pattern exists and will have to be considered when smokers are compared with nonsmokers. When allowance was made for the available dietary information, smoking was still associated with a downward trend in PAA and LAA levels, which did not achieve statistical significance. The ascorbic acid levels in nonsmoking men were still below those in women of similar fruit eating habits, so that it seems unlikely that the sex difference in blood levels could be wholly explained by fruit consumption and smoking combined.

Other surveys have similarly shown higher blood ascorbic acid levels in women than in men despite a similar dietary intake (7, 16, 17). It appears that there may be a physiological difference in the metabolism or retention of the vitamin in the two sexes.

Assessment of the clinical importance of ascorbic acid blood levels is not easy. In this population, the largest groups had the lowest levels, and the question arises as to whether the lower values should be regarded as pathological. Ultimately this can be answered only by randomized controlled trials of vitamin C supplementation, and one such trial is in progress.

The present data supply only a little indirect evidence about the clinical relevance of PAA and LAA levels. It has been suggested that much ill health among the elderly is caused or aggravated by undiagnosed vitamin C deficiency (1, 2). If this were so, we could expect that old people receiving medical treatment (other than vitamins) would have lower mean ascorbic acid levels than those not receiving treatment. In fact, ascorbic acid levels are comparable in the treated and nontreated groups. It therefore seems that unrecognized vitamin C deficiency is not a major factor in those chronic conditions for which old people commonly receive treatment. This is only a crude and indirect index of ill health, however, and as such it would reveal only a major association with ascorbic acid deficiency. As acutely ill patients were not seen in the survey, evidence about acute disease is unavailable.

Goldberg (18) reported that scorbutic patients are usually anemic, although an inverse relationship between LAA and hemoglobin has also been described (3). In this survey, ascorbic acid concentrations were negatively correlated with hemoglobin and hematocrit in women aged 65 to 69 years; otherwise vitamin C levels did not appear to be related to anemia in any way. No relationship could be found between ascorbic acid levels and two simple indices of weight and skin-fold thickness.

Summary

Plasma and leukocyte ascorbic acid levels (PAA and LAA) were measured in the elderly inhabitants of a town in South Wales. PAA and LAA values were lower than those reported from studies of younger people, and the men had lower levels than the women. The factors most strongly associated with the PAA and LAA concentrations appeared to be dietary, and showed some differences between men and women, and between smokers and nonsmokers. The possible clinical importance of the lower

TABLE 6
PAA and LAA levels in cigarette smokers and nonsmokers with high and low fruit consumption[a]

	Fruit score 0–3			Fruit score 4–7		
	Male smokers	Male nonsmokers	Women	Male smokers	Male nonsmokers	Women
Number	40	39	154	9	21	102
Mean PAA, mg/100 ml	0.16	0.20	0.24	0.27	0.37	0.45
Mean LAA, $\mu g/10^8$ cells	14.1	14.8	19.8	20.0	20.8	23.1

[a] Subjects aged 75 years and over and not taking vitamins.

PAA, LAA, and medical treatment

Table 7 shows mean PAA and LAA levels in subjects over 75 years of age, grouped according to whether or not they had taken any treatment prescribed by a doctor (other than vitamins and oral hematinics) during the 48 hr prior to the interview. No significant differences in PAA or LAA levels between treated or nontreated groups were detected. A similar analysis of data for subjects aged 65 to 74 years also showed no significant difference between the two groups.

TABLE 7
Mean PAA and LAA levels in subjects with and without treatment[a]

	No.	Mean age, years	PAA, mg/100 ml		LAA, $\mu g/10^8$ cells	
			Mean	SE	Mean	SE
Males						
With treatment	95	79.4	0.21	0.02	15.6	0.7
Without treatment	82	80.0	0.21	0.02	16.2	0.9
Females						
With treatment	195	79.7	0.33	0.02	21.3	0.6
Without treatment	141	80.9	0.33	0.03	20.9	0.7

[a] Subjects aged 75 years and over and not taking vitamins.

Discussion

In an observational study of this kind, the possibility must be considered that the persons who were seen were not representative of the elderly population as a whole. Response rates of 88% for the men and 84% for the women were achieved, so that serious bias due to selection is unlikely to have occurred.

It is also possible that fluctuations in ascorbic acid levels due to variations in intake may render meaningless a measurement made on a single occasion. Some evidence on the stability of these levels may be obtained by comparing PAA and LAA concentrations. A diet deficient in vitamin C produces minimal PAA levels in 4 weeks, whereas LAA levels take about 12 weeks to reach a minimum (11). Variations in intake within this period would therefore tend to weaken the correlation between PAA and LAA values. In this survey, however, PAA and LAA concentrations were quite well correlated (r = 0.77 for males and 0.69 for females), and correlated consistently with other variates, suggesting that ascorbic acid levels were in a fairly steady state in the population studied.

The concentration of a nutrient in the blood is determined partly by the intake of that nutrient and partly by various physiological and pathological factors acting on it. This study provides some limited information on the relative importance of age, diet, sex, and smoking in relation to PAA and LAA levels.

The findings confirm several previous reports of low ascorbic acid blood levels in elderly people compared with those commonly occurring in the general population (1, 5, 7, 16). We found a mean PAA concentration of 0.58 mg/100 ml in a random sample of 254 women of all ages in a similar area (12), whereas in this survey, women aged 75 years and over had a mean PAA level of 0.37 mg/100 ml. This decline appears to continue throughout old age, being especially marked in the women.

The diet of these elderly people appears to be the most prominent factor relative to their ascorbic acid levels. Both fresh fruit and green

TABLE 4
Vegetable intake and mean ascorbic acid levels (subjects aged 75 years and over and not taking vitamins)

	Vegetable score					
	0–1	2–3	4–5	6–7	Total 0–7	Not known
Males						
Number	13	63	25	25	126	49
Percent of total with known vegetable score	10	50	20	20	100	
Mean age, years	79.4	79.1	78.7	81.0	79.4	70.2
Mean PAA, mg/100 ml	0.10	0.20	0.27	0.26	0.22	0.20
Mean LAA, $\mu g/10^8$ cells	13.8	15.6	17.6	15.2	15.7	16.2
Females						
Number	27	108	50	57	242	93
Percent of total with known vegetable score	11	45	21	24	100	
Mean age, years	81.1	79.7	79.6	80.0	79.9	81.1
Mean PAA, mg/100 ml	0.26	0.30	0.31	0.41	0.32	0.34
Mean LAA, $\mu g/10^8$ cells	18.8	20.6	23.1	22.0	21.2	20.7

TABLE 5
LAA levels and diet scores in various smoking categories[a]

	Not smoking	<15 cigarettes/day	≥15 cigarettes/day	Pipe smoker
Number	60	29	20	27
Mean fruit score	2.6	1.7	1.1	1.8
Mean vegetable score	3.5	3.3	3.2	3.1
Mean LAA, $\mu g/10^8$ cells	17.5	15.7	13.8	15.5
Adjusted mean LAA[b]	16.8	16.1	15.1	15.8

[a] Men aged 75 years and over and not taking vitamins. [b] Adjusted to common mean fruit and vegetable scores (see text).

tically significant ($P < 0.05$). In order to distinguish between the effects of diet and smoking on LAA levels, a covariance analysis technique was used. The resulting mean LAA levels, adjusted for diet scores, still appear to be lower in smokers than in nonsmokers, but the differences are not statistically significant. Age does not appear to be a confounding factor and has been ignored in this procedure. PAA values are not shown in this table because their distribution was unsuitable for covariance analysis.

Table 6 enables a comparison to be made between the apparent effects of fruit consumption, cigarette smoking, and sex difference on ascorbic acid levels. The data refer only to subjects aged 75 years and over who were not taking vitamins or oral hematinics. The values for male nonsmokers were intermediate between those for male smokers and females (who were nearly all nonsmokers), but the differences attributable to fruit eating are greater than those attributable to either smoking or sex.

Relationship of PAA and LAA with other variates

Data on the height, weight, skin-fold thickness, and arm circumference of subjects aged 75 years and over were examined for evidence of any association between general nutritional state and ascorbic acid levels. Neither PAA nor LAA correlated significantly with the ratio of weight to the square of height, nor with the ratio of skin-fold thickness to arm circumference.

The relationship between the hematological data and ascorbic acid levels was also examined, and significant correlations were found only in women aged 65 to 69 years. PAA levels were negatively correlated with hematocrit, whereas LAA levels were negatively correlated with both hematocrit and hemoglobin ($P < 0.01$ in each case).

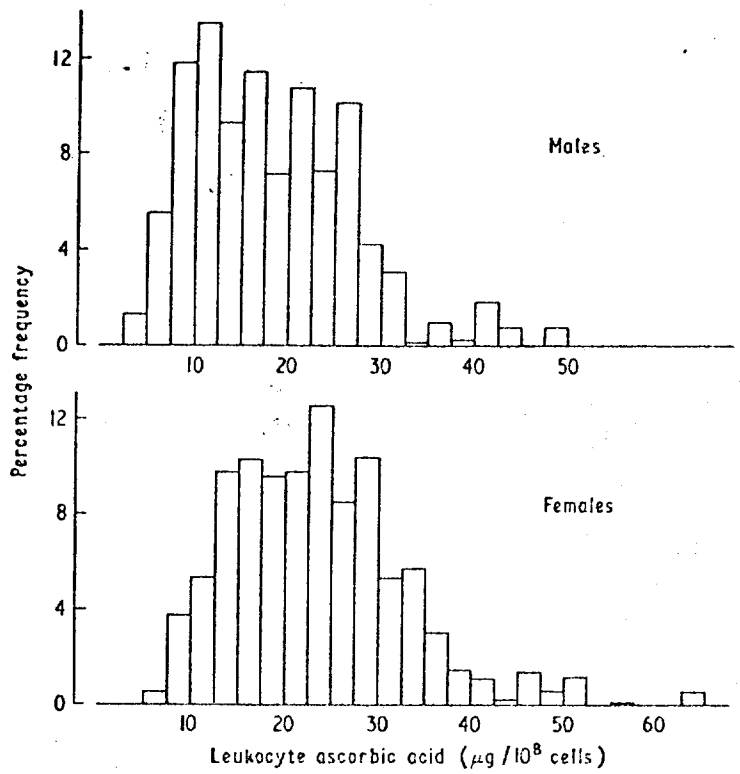


FIG. 2. Distribution of leukocyte ascorbic acid levels.

TABLE 3
Fruit intake and mean ascorbic acid levels of subjects aged 75 years and over and not taking vitamins

	Fruit score					
	0	1–3	4–6	7	Total 0–7	Not known
Males						
Number	71	28	13	21	133	41
Percent of total with known fruit score	53	21	10	16	100	
Mean age, years	79.5	79.4	78.9	79.3	79.4	80.5
Mean PAA, mg/100 ml	0.17	0.20	0.19	0.40	0.21	0.20
Mean LAA, $\mu g/10^8$ cells	13.4	17.0	15.8	22.3	15.9	15.9
Females						
Number	74	80	28	73	255	78
Percent of total with known fruit score	29	31	11	29	100	
Mean age, years	80.0	79.6	79.5	80.3	79.9	81.3
Mean PAA, mg/100 ml	0.21	0.27	0.39	0.47	0.32	0.35
Mean LAA, $\mu g/10^8$ cells	18.6	21.0	22.5	23.4	21.1	20.9

had lower mean LAA levels than nonsmokers. Subjects smoking less than 15 cigarettes per day had ascorbic acid concentrations between those of nonsmokers and heavier smokers.

Table 5 also suggests an inverse association between smoking and diet scores, and the difference between the fruit scores of nonsmokers and heavy cigarette smokers is statis-

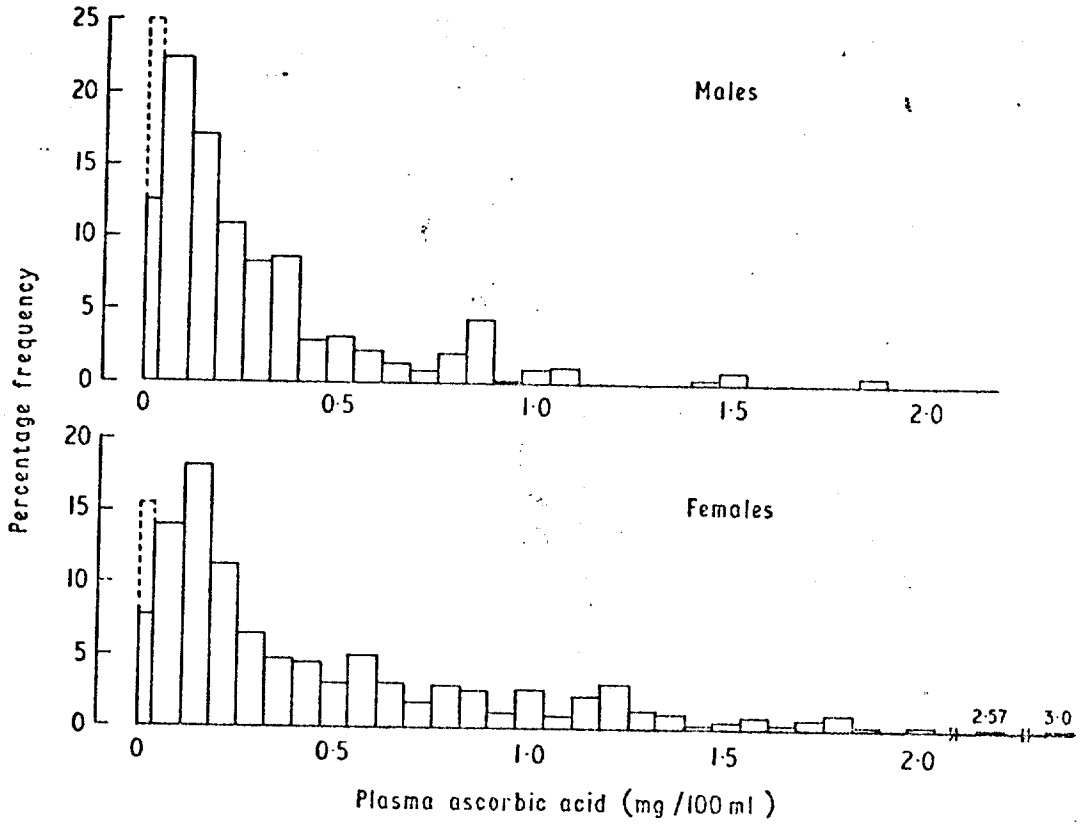


FIG. 1. Distribution of plasma ascorbic acid levels. The percentage frequency of the first group is 12.5 for males and 7.7 for females, but as the class interval is one-half that of the other groups, the correct shape of the distribution is presented by doubling these figures, as shown by the broken lines.

TABLE 2
Mean PAA and LAA levels in different age groups

Age group, years	PAA, mg/100 ml				LAA, $\mu g/10^3$ cells			
	Males		Females		Males		Females	
	No.	Mean ± SE	No.	Mean ± SE	No.	Mean ± SE	No.	Mean ± SE
65–69	48	0.30 ± 0.05	54	0.49 ± 0.06	48	19.9 ± 1.4	54	25.9 ± 1.5
70–74	45	0.27 ± 0.05	46	0.45 ± 0.07	43	17.7 ± 1.2	47	24.0 ± 1.3
75–79	117	0.25 ± 0.03	221	0.41 ± 0.03	117	16.8 ± 0.7	220	22.3 ± 0.5
80–84	54	0.21 ± 0.03	134	0.36 ± 0.03	55	17.0 ± 1.1	134	20.7 ± 0.7
85 and over	31	0.25 ± 0.06	74	0.31 ± 0.04	31	15.1 ± 1.5	74	20.0 ± 1.0

29% of the women said they rarely ate fresh fruit.

The PAA and LAA levels of subjects grouped according to their vegetable scores are shown in Table 4. A positive association exists in each case and is statistically significant ($P < 0.05$). The relationship is rather weak, however, r being 0.21 for PAA in each sex, and 0.17 for LAA in males and 0.13 in females.

Smoking

Smoking was found to be rare among the women in this population. Table 5 shows that, among men aged 75 years and over, smokers

trial, still in progress, to assess the effect of the vitamin on morbidity and mortality.

Results

The numbers seen and response rates obtained are shown in Table 1. PAA and LAA estimations were performed on all these subjects except for five men and four women on whom only one ascorbic acid measurement was made, and one man and one woman from whom insufficient blood was obtained for either estimation. Thirty-four of the subjects (4%) were residents of welfare homes; and 21 (3%) were inpatients in geriatric wards; the remainder were all living at home.

PAA concentrations

Due to the method of measurement, PAA estimations were not continuously distributed but took a series of discrete points. Figure 1 shows the distribution of PAA values, calculated for the whole population aged 65 years and over. This distribution is asymmetrical, the largest group having the lowest levels. No transformation was found that made parametric methods of analysis suitable, so a nonparametric test (Spearman's rank correlation) was used for significance testing. Table 2 shows mean PAA concentrations, which were significantly higher in women than in men at all ages ($P < 0.001$). In both sexes increasing age was accompanied by a significant fall in PAA level ($P < 0.01$ for men; $P < 0.001$ for women). For the women this amounted to 0.09 mg/100 ml over 10 years, whereas in the men the decrease was less steep and not so consistent.

LAA concentrations

The distribution of LAA values, shown in Fig. 2, was also asymmetrical. For subjects aged 75 years and over, the skew was not large enough for logarithmic transformation to produce a more Gaussian distribution, so the data are presented untransformed. Mean LAA levels are given in Table 2, which shows a pronounced sex difference, the women having higher values throughout ($P < 0.001$). The levels fall significantly with increasing age to the extent of 2.2 $\mu g/10^8$ cells in men and 2.5 $\mu g/10^8$ cells in women over a 10-year period ($P < 0.001$ for each).

Dietary information

Table 3 shows the ascorbic acid blood levels of persons aged 75 years and over, grouped according to their frequency of eating fruit. Subjects who had taken vitamin preparations or oral hematinics (which may contain vitamin C) have been excluded from this analysis. The fruit intake of some subjects was not known (because they were not asked or could not remember), but their mean ascorbic acid levels show no evidence suggestive of selective bias.

Persons who ate fruit every day are seen to have substantially higher PAA and LAA levels than those who ate no fruit. The overall correlation coefficients between PAA and fruit score are 0.37 for men and 0.29 for women; between LAA and fruit score they are 0.39 and 0.22, respectively (all $P < 0.001$). The women tended to eat fresh fruit more frequently than the men, the difference being highly significant ($P < 0.001$). Fifty-three percent of the men and

TABLE 1
Numbers seen and response rates by age and sex

Age group, years	Males			Females		
	No. seen	No. refused	Response rate, %	No. seen	No. refused	Response rate, %
65–69	48	6	89	54	9	86
70–74	45	6	88	47	9	84
75–79	119	17	88	222	46	83
80–84	55	9	86	135	21	87
85 and over	31	4	89	74	17	81
Total	298	42	88	532	102	84

Am. J. Clin. Nutr. 27(2): 144-151
1974

1267

Plasma and leukocyte ascorbic acid levels in the elderly[1]

M. L. Burr,[2] M.B., B.S., P. C. Elwood,[3] M.D., D. J. Hole,[4] M.Sc., R. J. Hurley,[5] M.Sc., and R. E. Hughes,[6] Ph.D., F.I.Biol.

ABSTRACT A survey of ascorbic acid blood levels was carried out in the elderly population of a South Wales town. Response rates of 88% for the men and 84% for the women were obtained, and 830 subjects were seen, 93% of whom were living at home. The ascorbic acid concentration was estimated in the plasma and leukocytes (including platelets) and found to decline significantly with advancing age. Mean plasma levels were 0.24 mg/100 ml and 0.37 mg/100 ml in 202 men and 429 women aged 75 years and over; their mean leukocyte levels were 16.6 $\mu g/10^8$ cells and 21.4 $\mu g/10^8$ cells, respectively. These values are considerably lower than those reported from studies of younger persons. Information was obtained about consumption of fresh fruit and green vegetables. Vitamin C blood levels were correlated with both, the effect of the fruit being the stronger. More fruit was eaten by women than by men and more by nonsmokers than by smokers. These differences did not seem to account for the fact that blood ascorbic acid levels were higher in women than in men, and higher in nonsmokers than in smokers. The clinical importance of the low ascorbic acid levels is being investigated by a randomized controlled trial. *Am. J. Clin. Nutr.* 27: 144–151, 1974.

It has been suggested that ascorbic acid deficiency makes an important contribution to the ill health of elderly people in Britain (1, 2). Residents of welfare homes for the aged and geriatric hospital wards are known to have lower ascorbic acid blood levels that those found commonly in younger persons (1, 3, 4). Low levels have also been found in old people living in their own homes, but the general applicability of these findings has been uncertain in view of the small numbers studied (1, 4, 5), the self-selection of those examined (6), or the high proportion of nonrespondents in the sample (7, 8). A survey was therefore carried out to investigate the distribution and clinical importance of plasma ascorbic acid (PAA) and leukocyte (including platelets) ascorbic acid (LAA) concentrations in the elderly inhabitants of a small town.

Methods

The population was defined as comprising all persons aged 65 years and over, resident in a small town in South Wales and on the lists of local general practitioners. Subjects in the age group 65 to 69 and 70 to 74 were sampled in the ratio 1:6 and 1:5, respectively, although all those aged 75 years and over were included. The latter group was supplemented by subjects drawn from two small adjacent villages as well as from the main town. Subjects in residential homes and long-term geriatric wards were included in the survey, whereas those in other hospital wards were excluded. Any who had an acute illness when first contacted were seen on recovery or else counted as "refusals."

Height, weight, triceps skin-fold thickness, and midarm circumference were measured. Blood was taken for estimation of hemoglobin, hematocrit, PAA by the indophenol dye method (9), and LAA by that of Denson and Bowers (10). Information was obtained as to whether or not the subjects had taken any treatment prescribed by a doctor during the 48 hr prior to the interview. Most subjects were also asked to state the usual number of days each week on which they ate fresh fruit, the answer being recorded as a "fruit score," with a possible range from 0 to 7. The frequency of green vegetable consumption was similarly expressed as a "vegetable score." The current smoking habit was also recorded.

Subjects found to have low ascorbic acid levels were subsequently admitted to a randomized controlled

[1] From the Medical Research Council Epidemiology Unit and the University of Wales Institute of Science and Technology, Cardiff, South Wales, United Kingdom.

[2] Scientific Member, Medical Research Council Epidemiology Unit. [3] Assistant Director, Medical Research Council Epidemiology Unit. [4] Medical Statistician, Medical Research Council Epidemiology Unit. [5] Research Assistant, Department of Applied Biology, University of Wales Institute of Science and Technology. [6] Reader in Applied Biology, University of Wales Institute of Science and Technology.

The American Journal of Clinical Nutrition 27: FEBRUARY 1974, pp. 144–151. Printed in U.S.A.

BURNS, J. J.: Biochim. biophys. Acta, in press, 1960.
HOROWITZ, H. H. AND KING, C. G.: J. biol. Chem. **205**: 815, 1953.
ISHIDATA, M. AND HOSOYA, E.: Recent Advances in the Study of Glucuronic Acid in Japan. Glucuronic Acid Symposium of Europe, Zurich, Switzerland, July 13-14, 1959.
LONGENECKER, H. E., FRICKE, H. H. AND KING, C. G.: J. biol. Chem. **135**: 497, 1940.
MAUGHAN, G. B., EVELYN, K. A. AND BROWNE, J. S. L.: J. biol. Chem. **126**: 567, 1938.
ROE, J. H. AND KUETHER, C. A.: J. biol. Chem. **147**: 399, 1943.
SMITH, H. W.: The Kidney, 2nd ed., p. 136, Oxford University Press, New York, 1955.
SMITH, J. N. AND WILLIAMS, R. T.: Biochem. J. **44**: 242, 1949.
STROMINGER, J. L.: Physiol. Rev. **40**: 55, 1960.
TOUSTER, O.: Amer. J. Med. **26**: 724, 1959.

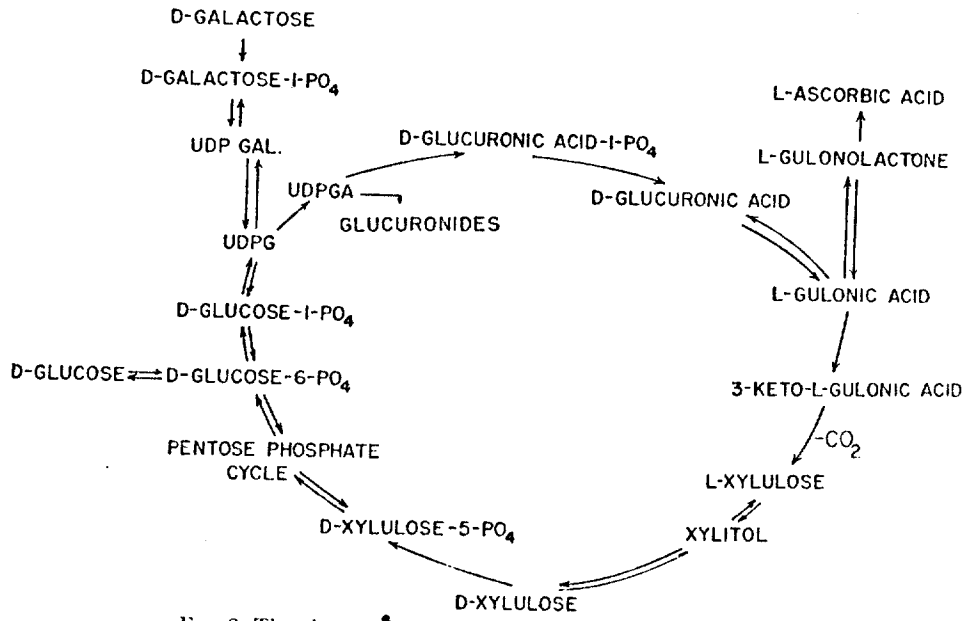


FIG. 2. The glucuronic acid pathway of glucose metabolism.

phenylbutazone; the antihistaminic drugs, diphenhydramine and chlorcyclizine and the carcinogenic hydrocarbons, 3-methylcholanthrene, 3,4-benzpyrene and 1,2,5,6-dibenzanthracene. The enhanced formation of L-ascorbic acid through the glucuronic acid pathway may represent an adaptive response on the part of the body to foreign compounds. Reports have recently appeared indicating that the administration of D-glucuronic acid reduces the toxicity of various foreign compounds in animals by an unknown mechanism (Ishidata and Hosoya, 1959). It is of considerable interest that those drugs which are potent in stimulating the synthesis of L-ascorbic acid such as phenobarbital, barbital, aminopyrine and the previously mentioned carcinogenic hydrocarbons, also increase the activity of liver microsomal enzymes which metabolize various foreign compounds (Conney and Burns, 1959). These results suggest a relationship between the stimulatory effect of foreign compounds on the glucuronic acid pathway and on the activity of drug metabolizing enzymes in liver microsomes.

SUMMARY

Various drugs possessing completely unrelated chemical and pharmacological properties stimulate the biosynthesis of L-ascorbic acid in rats. Among these compounds are included barbital, Chloretone, meprobamate, phenylbutazone, chlorcyclizine, diphenhydramine, orphenadrine, 3-methylcholanthrene and 3,4-benzpyrene. The ability of barbital and Chloretone to enhance the synthesis of free D-glucuronic acid and L-gulonic acid was also observed. The mechanism by which drugs stimulate ascorbic acid biosynthesis is not known but renal factors and drug glucuronide formation are not involved. The possibility was considered that this drug effect on ascorbic acid formation results from increased metabolism of glucose through the glucuronic acid pathway.

REFERENCES

AXELROD, J., INSCOE, J. K. AND TOMKINS, G. M.: Nature, Lond. **179:** 538, 1957.
BURNS, J. J., MOSBACH, E. H. AND SCHULENBERG, S.: J. biol. Chem. **207:** 679, 1954.
BURNS, J. J. AND EVANS, C.: J. biol. Chem. **223:** 897, 1956.
BURNS, J. J.: J. Amer. chem. Soc. **79:** 1257, 1957.
BURNS, J. J., EVANS, C. AND TROUSOF, N.: J. biol. Chem. **227:** 785, 1957.
BURNS, J. J.: Amer. J. Med. **26:** 740, 1959.
CONNEY, A. AND BURNS, J. J.: Nature, Lond. **184:** 363, 1959.
DOUGLAS, J. F. AND KING, C. G.: J. biol. Chem. **198:** 187, 1952.
ENKLEWITZ, M. AND LASKER, M.: J. biol. Chem. **110:** 443, 1935.
EVANS, C., CONNEY, A. H., TROUSOF, N. AND

bic acid biosynthesis was further shown in experiments with D-galactose-1-C^{14}. Two rats were given 10 mg of the hydrocarbon for 5 days. Four days after the last dose each animal was given 20 mg of D-galactose-1-C^{14} by intraperitoneal injection and urine samples were collected for 24 hours. The conversion of D-galactose-1-C^{14} to L-gulonic acid and L-ascorbic acid averaged 0.61 and 0.50%, respectively. Control experiments in untreated rats showed conversion values of less than 0.06% to each of these urinary products. A marked stimulatory effect of barbital on the conversion of D-galactose-1-C^{14} to urinary D-glucuronic acid, L-gulonic acid and L-ascorbic acid has also been observed (Evans *et al.*, 1960).

DISCUSSION. Recent studies have pointed out the existence in animals of a new route of glucose metabolism, the glucuronic acid pathway[2] (Touster, 1959; Burns, 1959; Strominger, 1960). According to this scheme (fig. 2), D-glucose is oxidized to D-glucuronic acid, which undergoes reduction to L-gulonic acid; the latter acid then serves as the precursor of either L-ascorbic acid or L-xylulose. The enhanced formation of L-ascorbic acid observed in rats following administration of various drugs may now be considered to result from increased metabolism of glucose through the glucuronic acid pathway. This conclusion is supported by the observations that the administration of barbital, Chloretone and 3-methylcholanthrene to rats stimulated the conversion of D-glucose-1-C^{14} to labeled free D-glucuronic acid, L-gulonic acid and L-ascorbic acid. In addition, a marked effect of barbital on the conversion of D-galactose-1-C^{14} to D-glucuronic acid, L-gluonic acid and L-ascorbic acid has been observed.[3] Further evidence for the effect of drugs on the glucuronic acid pathway came from the earlier studies of Enkelwitz and Lasker (1935). Aminopyrine and antipyrine, which stimulate the synthesis of L-ascorbic acid in the rat, also markedly elevate the urinary excretion of L-xylulose in human subjects with essential pentosuria. This observation can be explained in terms of the scheme in figure 2 since administration of these drugs would be expected to increase the formation of L-xylulose from D-glucose. The pentosuric subject is not able to metabolize L-xylulose (Touster, 1959) and thus the pentose would be excreted in the urine.

The mechanism by which drugs stimulate the formation of L-ascorbic acid is not known. The results of this study indicate that a renal mechanism is not involved, since enhanced synthesis of the vitamin was still observed when its excretion was prevented by nephrectomy (table 2). The available evidence indicates that drugs such as Chloretone and barbital increase L-ascorbic acid biosynthesis by stimulating the formation of D-glucuronic acid (Burns and Evans, 1956; Burns *et al.*, 1957). The formation of a glucuronide by the drug is not required since barbital, one of the most potent drugs in exerting this effect, is recovered unchanged in urine (Burns *et al.*, 1957). However, this does not rule out a mechanism involving the formation of a glucuronide with a naturally occurring substrate. It should be noted that borneol and aniline, potent in forming glucuronides, have no effect on the synthesis of L-gulonic acid or L-ascorbic acid. Aniline causes appreciable excretion of free D-glucuronic acid (table 3) which results from the breakdown of a labile aniline N-glucuronide (Smith and Williams, 1949; Axelrod *et al.*, 1957). Whatever mechanism is proposed for the stimulatory effect of drugs on L-ascorbic acid biosynthesis must take into consideration that the amount of glucose metabolized through the glucuronic acid pathway is greater than the amount of drug administered. The amounts of free D-glucuronic acid, L-gulonic acid and L-ascorbic acid excreted in the urine of drug-treated rats are minimum values for the total amount of these acids formed since they are extensively metabolized (Douglas and King, 1952; Burns *et al.*, 1954; Burns and Evans, 1956).

The results of this study and those of Longenecker *et al.* (1940) show that a variety of foreign compounds possessing completely unrelated chemical and pharmacological properties stimulate L-ascorbic acid biosynthesis in the rat. These drugs include the hypnotics, Chloretone and barbital; the analgesics, aminopyrine and antipyrine; the muscle relaxants, orphenadrine and meprobamate; the antirheumatic agent,

[2] This scheme has also been referred to as the glucuronic acid-xylulose pathway, the uronic acid pathway, the C-6 oxidation pathway and the uridine monophosphate shunt.

[3] Increased synthesis of labeled D-glucuronic acid from D-galactose-1-C^{14} has been observed in liver homogenates of rats pretreated with Chloretone, but the specific enzymatic steps involved have not yet been defined (Evans *et al.*, 1960).

TABLE 2

Synthesis of L-ascorbic acid in intact and nephrectomized rats

Expt. No.*	Treatment†	Concentration of L-Ascorbic Acid (mg %)‡				
		Serum	Liver	Testis	Muscle	Brain
A	None	1.21 (0.77–1.44)	28.3 (23.0–31.6)	21.7 (19.9–23.2)	5.4 (4.9–5.6)	47.0 (34.6–68.5)
B	Chloretone 20 mg/day, 3 days	3.0 (2.70–3.20)	39.6 (33.9–44.0)	26.6 (24.8–30.3)	10.6 (9.3–12.5)	43.1 (38.7–48.4)
C	Nephrectomized; sacrificed 24 hr	2.88 (1.80–3.74)	24.0 (19.6–29.6)	23.7 (23.5–24.1)	5.2 (4.7–5.5)	43.6 (42.8–44.2)
D	Chloretone 20 mg/day 3 days; nephrectomized 2nd day; sacrified 24 hr later	19.3 (16.8–21.6)	43.8 (39.4–46.8)	28.1 (25.9–30.0)	9.9 (8.5–11.9)	54.3 (48.9–58.1)
E	Nephrectomized; then 20 mg Chloretone; sacrificed 24 hr	9.95 (8.4–11.1)	27.4 (23.4–34.0)	25.4 (23.9–26.7)	6.95 (6.2–7.7)	39.9 (36.6–42.7)

* Three animals were used in each experiment.

† Chloretone was given by intraperitoneal injection dissolved in 0.2 ml corn oil.

‡ Values in parentheses indicate range obtained in each experiment.

TABLE 3

Effect of compounds on synthesis of D-glucuronic acid, L-gulonic acid and L-ascorbic acid

Compound	Daily* Dosage	Excretion in Urine		% Conversion of D-Glucose-1-C^{14} to†		
		Free D-glucuronic acid	L-Ascorbic acid	D-Glucuronic acid	L-Gulonic acid	L-Ascorbic acid
None	None	<3.0	0.82 ± 0.50**	<0.03‡	<0.03	<0.03‡
Barbital	100 mg orally for 5 days	36 ± 2.3	23 ± 3.2	0.45‡	0.20	0.20‡
Chloretone	50 mg orally for 5 days	—	38 ± 4.0	0.20‡	0.44	0.45‡
Borneol	100 mg orally for 2 days	<3.0§	0.94 ± 0.25	—	<0.06	—
Aniline	50 mg orally for 2 days	31 ± 3.0	0.60 ± 0.15	1.04	<0.03	—
3-Methylcholanthrene	10 mg i.p. for 5 days	—	24.2 ± 4.2	—	0.13	0.18

* Experiments were carried out on last day of drug administration except in the experiment with 3-methylcholanthrene when it was done 5 days after the last dose.

** Average deviation.

† Urine was collected for 24 hours after 10- to 30-mg intraperitoneal dose of D-glucose-1-C^{14} (1.0 μc/mg). The less-than values represent the limit of sensitivity possible. Each radioactive experiment was carried out in duplicate and values represent the averages obtained.

‡ Average of previously published data (Burns *et al.*, 1957).

§ The amount of total D-glucuronic acid excreted in urine in response to borneol was 76 ± 13 mg (Burns *et al.*, 1957).

version of D-glucose-1-C^{14} to urinary free D-glucuronic acid. Furthermore, an appreciable incorporation of C^{14} into L-gulonic acid was observed in the rats receiving barbital, Chloretone or 3-methylcholanthrene, but not in the animals receiving borneol or aniline. The data in table 3 also show that barbital, Chloretone or 3-methylcholanthrene markedly enhanced the synthesis of L-ascorbic acid but no such effect was observed following the administration of either borneol or aniline.

The effect of 3-methylcholanthrene on L-ascor-

TABLE 1

Effect of various compounds on L-ascorbic acid excretion

Expt. No.	Compound	Dosage	Urinary L-Ascorbic Acid* mg/day
Control	None	None	0.46 ± 0.16†
1	Phenylbutazone	50 mg for 3 days by i.p. injection	6.1 ± 0.3
2	Sulfinpyrazone	50 mg for 4 days by i.p. injection	3.5 ± 0.4
3	Oxyphenbutazone	25 mg for 3 days by i.p. injection	4.0 ± 0.7
4	Meprobamate	75 mg orally for 5 days	3.32 ± 0.3
5	Orphenadrine	20 mg orally for 4 days	11.9, 14.4
6	Diphenhydramine	25 mg orally for 4 days	7.6 ± 1.6
7	Chlorcyclizine	12 mg for 6 days by i.p. injection	8.1 ± 1.0
8	3-Methylcholanthrene	Single 10-mg i.p. dose	15.8, 12.3
9	3-Methylcholanthrene	10 mg i.p. for 5 days	20.1 ± 4.2
10	1,2,5,6-Dibenzanthracene	Single 5-mg i.p. dose	6.2, 8.7
11	3,4-Benzpyrene	Single 20-mg i.p. dose	10.0, 6.5
12	3,4-Benzpyrene	10 mg i.p. for 3 days	11.8, 12.6

* L-Ascorbic acid excretion was measured on the last day of drug administration except in expts. 8 to 12 when it was measured 5 days after the last dose. Each value represents the average of from 3 to 5 animals except where values for individual rats are given.

† Average deviation.

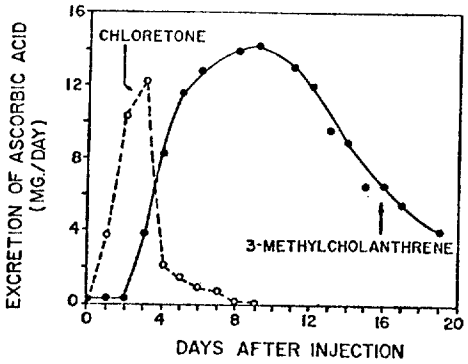


FIG. 1. Stimulation of L-ascorbic acid excretion by 3-methylcholanthrene and Chloretone.

A rat was injected intraperitoneally with a 10-mg dose of 3-methylcholanthrene in 0.5 ml of corn oil, and another rat was injected with a 40-mg dose of Chloretone in 1.0 ml of corn oil.

rats. L-Ascorbic acid is handled by the kidney as a threshold substance (Smith, 1955). Thus a drug such as Chloretone could enhance the urinary excretion of L-ascorbic acid by interfering with the reabsorption of the vitamin through the renal tubular cell. In order to investigate this possibility, the effect of Chloretone on the synthesis of L-ascorbic acid in intact and nephrectomized rats was measured (table 2). The concentration of L-ascorbic acid in the serum of rats pretreated with Chloretone for 3 days increased from an average of 3.0 mg % for the intact rat (Expt. B) to an average of 19.3 mg % 24 hours after nephrectomy (Expt. D). Twenty-four hours following a single 20-mg dose of Chloretone to nephrectomized rats, a significant increase in the serum level of L-ascorbic acid was also observed (Expt. E). The increased amount of L-ascorbic acid synthesized in nephrectomized rats was reflected almost completely by an elevation in serum concentration of the vitamin while little or no increase was observed in its tissue concentration.

Synthesis of free D-glucuronic acid, L-gulonic acid and L-ascorbic acid. The formation of free D-glucuronic acid, L-gulonic acid and L-ascorbic acid was compared following the administration of barbital, Chloretone, aniline, borneol and 3-methylcholanthrene (table 3). Values are given both for the excretion of substances in urine and the extent of conversion of D-glucose-1-C^{14} to the various urinary products. For comparison, previously published data are given in table 3. Barbital and aniline produced a marked increase in the excretion of free D-glucuronic acid. However, administration of borneol did not elevate the excretion of free D-glucuronic acid but did elevate the excretion of total D-glucuronic acid. It will be noted that the administration of barbital, aniline and Chloretone stimulated the con-

borohydride, and the solution was kept at room temperature for 45 minutes. The solution was then acidified and allowed to stand for 30 minutes to decompose any unreacted sodium borohydride. Both this solution and the original formic acid eluate were then analyzed for glucuronic acid by the naphthoresorcinol method (Maughan *et al.*, 1938) and the amount of free D-glucuronic acid in the original urine sample was calculated from the difference in the values. This determination depends on the fact that free D-glucuronic acid is reduced quantitatively by sodium borohydride to L-gulonic acid which does not react with naphthoresorcinol. Since glucuronides are not converted to L-gulonic acid they react with the naphthoresorcinol reagent after treatment with sodium borohydride. This was confirmed in control experiments with glucuronides of borneol, menthol and phenolphthalein.

Free D-glucuronic acid was also demonstrated in the urine of a barbital-treated rat by paper chromatography. A 50-ml aliquot of the aforementioned formic acid eluate from the ion-exchange column was evaporated to a volume of about 5.0 ml and an aliquot of this solution containing about 50 μg of D-glucuronic acid was chromatographed on Whatman 1 mm paper using a 3:1:3 ethyl acetate:water:acetic acid system. The chromatogram was then developed with an alkaline solution of silver nitrate and a spot was obtained with an R_f value similar to that of authentic D-glucuronolactone.[1] No spot for D-glucuronolactone was observed when the chromatographic studies were repeated with an aliquot of the eluate which had been treated with sodium borohydride.

Determination of L-*ascorbic acid.* L-Ascorbic acid was determined in urine by titration with 2,6-dichlorophenolindophenol dye and in serum and tissues by the method of Roe and Kuether (1943).

Isolation procedures. The 24-hour urine samples, preserved either with 0.5 ml of toluene or with 5 ml of 10% oxalic acid, were diluted to 100 ml with water. D-Glucuronic acid, L-gulonic acid and L-ascorbic acid were isolated from aliquots of these diluted urines by specific carrier dilution methods used previously (Burns, 1957; Burns *et al.*, 1957).

RESULTS. *Effect of drugs on* L-*ascorbic acid excretion.* Administration of meprobamate, orphenadrine (Disipal), diphenhydramine (Benadryl) chlorcyclizine and various polycyclic hydrocarbons resulted in a substantial increase in the urinary excretion of L-ascorbic acid (table 1). The effect of phenylbutazone on the excretion of the vitamin was similar to that reported previously for two other pyrazolone derivatives, aminopyrine and antipyrine (Longenecker *et al.*, 1940). Two analogues of phenylbutazone, sulfinpyrazone and oxyphenbutazone, also enhanced the urinary excretion of L-ascorbic acid. Of particular interest is the marked stimulatory effect of the carcinogenic hydrocarbons 3-methylcholanthrene, 1,2,5,6-dibenzanthracene and 3,4-benzpyrene. The striking effect of a 10-mg dose of 3-methylcholanthrene on the urinary excretion of L-ascorbic acid was further shown in figure 1. For comparison, the effect of a 40-mg dose of Chloretone was also given. By 6 days after 3-methylcholanthrene administration, the urinary excretion of L-ascorbic acid was 50 to 75 times greater than the control value and, in fact, during the 19-day period about 140 mg of the vitamin were recovered in the urine. In another experiment, 3 rats were each injected intraperitoneally with 10 mg of 3-methylcholanthrene daily for 3 days and the urinary excretion of ascorbic acid was measured for the following 36 days. The excretion of L-ascorbic acid before the animals received 3-methylcholanthrene was less than 0.1 mg per day which increased to an average of 17 mg per day by 6 days after the last dose of the hydrocarbon. The urinary output of the vitamin declined very slowly and by 36 days after the last injection of 3-methylcholanthrene, the urinary excretion of L-ascorbic acid still averaged 5.8 mg per day.

Various drugs were reported by Longenecker *et al.* (1940) to exert little or no effect on L-ascorbic acid excretion in rats. These included urethane, acetanilide, phenacetin, *p*-aminophenol, phenol, sulfanilamide, codeine, quinine and salicylates. Subsequent studies showed that the administration of borneol, α-naphthol or phenolphthalein also produced no increase in L-ascorbic acid excretion (Burns *et al.*, 1957). In this study Tromexan (125 mg/kg), reserpine (4.0 mg/kg), chlorpromazine (20 mg/kg), chlorzoxazone (150 mg/kg), carbon tetrachloride (1000 mg/kg), sodium bromide (750 mg/kg), nicotinamide (500 mg/kg) and keto-oxyphenbutazone (250 mg/kg) were given daily for 3 to 5 days and they had little or no effect on L-ascorbic acid excretion.

Synthesis of L-*ascorbic acid in nephrectomized*

[1] D-Glucuronic acid present in the formic acid eluate was converted to its lactone upon evaporation of the acidic solution.

Jour Pharmacol and Exptl. Therap 129(2): 132-138. 1960.

8334

OBSERVATIONS ON THE DRUG-INDUCED SYNTHESIS OF D-GLUCURONIC, L-GULONIC AND L-ASCORBIC ACIDS IN RATS

J. J. BURNS, A. H. CONNEY, PETER G. DAYTON, CAROLE EVANS, G. R. MARTIN AND DOLORES TALLER

Laboratory of Chemical Pharmacology, National Heart Institute, National Institutes of Health, Bethesda, Maryland, and New York University Research Service, Goldwater Memorial Hospital, Welfare Island, New York

Received for publication October 22, 1959

When administered to rats, various drugs markedly increase the urinary excretion of L-ascorbic acid (Longenecker *et al.*, 1940). Among the compounds reported to exert this effect are several barbiturates, Chloretone (chlorbutanol), paraldehyde, Trional (sulfonmethylmethane), antipyrine and aminopyrine. Tracer studies (Horowitz and King, 1953; Burns *et al.*, 1954; Burns and Evans, 1956) have shown that this increased excretion results from an acceleration in the rate of synthesis of L-ascorbic acid from glucose through the intermediate formation of D-glucuronic acid and L-gulonic acid. The ability of a drug to form a glucuronide is apparently not required, since barbital, one of the most potent drugs in enhancing the excretion of L-ascorbic acid, is excreted unchanged in urine (Burns *et al.*, 1957). In fact, barbital-treated rats injected with D-glucose-1-C^{14} were shown to excrete significant amounts of labeled free D-glucuronic acid as well as labeled L-ascorbic acid.

In the present paper, results are presented which demonstrate the stimulatory effect of various drugs on L-ascorbic acid biosynthesis other than those reported originally by Longenecker *et al.* (1940). These include phenylbutazone and several of its analogues, various polycyclic hydrocarbons and the antihistaminic drugs, diphenhydramine and chlorcyclizine. The possibility that drugs stimulate L-ascorbic acid formation through a renal mechanism has been excluded by experiments with Chloretone in nephrectomized rats. In addition, the stimulatory effect of barbital, Chloretone and 3-methylcholanthrene, on the synthesis of free D-glucuronic acid, L-gulonic and L-ascorbic acids was measured. It was also shown that borneol and aniline, compounds which are potent in forming glucuronides, did not stimulate the synthesis of L-gulonic acid and L-ascorbic acid. The results presented here furnish evidence for an additional mechanism for D-glucuronic acid formation in response to drugs other than via drug glucuronide formation.

METHODS AND MATERIALS. *Radioactive material and procedures.* D-Glucose-1-C^{14} and D-galactose-1-C^{14} with a specific activity of 1.0 μc/mg and 1.5 μc/mg, respectively, were obtained from the National Bureau of Standards, Washington, D.C. The method for collection and preparation of samples and their assay for C^{14} were the same as those used previously (Burns *et al.*, 1957).

Experimental animals. Male albino rats of the Wistar strain weighing 250 to 325 g were used. The animals were maintained on a basal diet of evaporated milk and water for at least 7 days before each experiment (Burns *et al.*, 1957).

Determination of free D-glucuronic acid in urine. Free D-glucuronic acid was estimated in urine by the naphthoresorcinol procedure of Maughan *et al.* (1938), modified as follows. The 24-hour urine sample was diluted to 100 ml and the solution was passed through an Amberlite IR-4B anion-exchange column in the acetate form (Burns *et al.*, 1954). D-Glucuronic acid was eluted with 2 N formic acid, and 120 ml of eluate were then collected. This step removed large amounts of neutral substances in urine that reacted with the naphthoresorcinol reagent. Control experiments gave quantitative recoveries when 25 mg of D-glucuronic acid were carried through this ion-exchange step. Since the naphthoresorcinol reagent reacts with both free and conjugated D-glucuronic acid, the amount of free D-glucuronic acid was determined as follows. Five ml of the formic acid eluate was adjusted to pH 7.0 with 1 N NaOH. To this was added 1 ml of 0.4 M sodium

21 C for extended periods. The amount of sodium ascorbate to be added to evaporated milk to assure the retention of the minimum legal amount of vitamin C after sterilization and storage for one year at 21 C may be calculated for each plant from the formula given below:

$$W = \frac{100}{100\text{-}x} \times \frac{100}{100\text{-}y} \times \frac{14\text{-}V}{0.88}$$

where W = mg of sodium ascorbate to be added per 100 ml of evaporated milk,

x = per cent loss during sterilization, using upper confidence limit,

y = per cent loss during storage, using upper confidence limit,

V = mg of natural vitamin per 100 ml of control evaporated milk present after sterilization and storage,

0.88 = correction factor for converting the weight of ascorbic acid into the weight of sodium ascorbate, and

14 mg per 100 ml is equivalent to the minimum legal requirement for vitamin C level in enriched evaporated milk in Canada.

Suggested amounts of sodium ascorbate to be added to evaporated milk. Based upon the formula, and using the upper confidence limits at the 5% probability level for vitamin C losses during sterilization and the subsequent 12 months of storage at 21 C, values of W for Plants A, B, and C were 25.9, 28.8, and 27.6 mg sodium ascorbate per 100 ml of evaporated milk, respectively. Assuming the specific gravity of evaporated milk to be 1.06, these values for W are equivalent to 243, 272, and 261 g per 1,000 kg, respectively. The differences in these amounts of sodium ascorbate are partly explained by the natural vitamin C content of the control samples. The average values of V for Plants A, B, and C after 12 months of storage were 1.3, 0.4, and 1.0 mg ascorbic acid per 100 ml, respectively. If these three values for the natural vitamin were averaged and used in the formula, then the amounts of sodium ascorbate required to be added would become 252, 262, and 263 g per 1,000 kg evaporated milk, respectively.

If the natural vitamin C content of the milk is to be considered when calculating the amount of sodium ascorbate to be added, each plant must make periodical checks for the vitamin content of the nonenriched freshly sterilized evaporated milk and from this estimate the amount expected to be present after 12 months of storage. Because the average amount of natural vitamin C present after this storage period was only 0.9 mg per 100 ml, it would seem more practical to disregard it for purposes of calculating the amount of sodium ascorbate to be added, thereby eliminating the need for processing and testing nonenriched control samples. The natural vitamin could then be considered as a safety factor. Therefore, if V is disregarded in the formula, the amounts of sodium ascorbate to be added at Plants A, B, and C are 268, 292, and 280 g per 1,000 kg evaporated milk, respectively. These amounts should insure that the vitamin C levels remaining in the evaporated milk after 12 months of storage at 21 C or less will meet the minimum legal standard.

Acknowledgments

The cooperation of Cow and Gate (Canada) Limited, The Borden Company Limited, and the Carnation Company Limited, who supplied the evaporated milk samples for this investigation, is appreciated. The help and constructive criticism of Dr. E. J. Reedman, Consultant Chemist to Hoffmann-La Roche Limited, in various phases of this investigation, also is acknowledged.

References

(1) Canada Department of National Health and Welfare. 1966. Office consolidation of the Food and Drug Act and the Food and Drug Regulations, no. B. 08.027. Queen's Printer, Ottawa.

(2) Conochie, J. 1958. The stability of sodium ascorbate in evaporated milk. Australian J. Dairy Technol., 13: (2), 89.

(3) Demers, P., D. Fraser, R. Goldbloom, R. MacLean, H. Medovy, A. Morrison, J. Webb, and W. Cochrane. 1965. Epidemiological study of scurvy in Canada, 1961-63. Canad. Med. Ass. J., 93: 573.

(4) Doan, F. J., and D. V. Josephson. 1943. Observations on the ascorbic acid content of evaporated milk. J. Dairy Sc., 26: 1031.

(5) Josephson, D. V., and F. J. Doan. 1944. Further observations dealing with the behavior of ascorbic acid in evaporated milk. J. Dairy Sci., 27: 686.

(6) Medovy, H. 1959. A plea for vitamin C fortified milk. Canad. Med. Ass. J., 80: 213.

(7) Pelletier, Omer. 1967. Determination of ascorbic acid in enriched evaporated milk. J. Ass. Offic. Anal. Chemists, 50: 817.

(8) Whelen, W. S., M. D. Fraser, and M. D. Robertson. 1958. The rising incidence of scurvy in infants. Canad. Med. Ass. J., 78: 177.

(9) Woessner, W. W., K. G. Weckel, and H. Schuette. 1940. The effect of commercial practices on ascorbic acid and dehydroascorbic acid in milk. J. Dairy Sci., 23: 1131.

min C content of both the enriched and control evaporated milk samples was determined. The difference in these two values was assumed to be the residual of the added vitamin C. The loss of added vitamin C during sterilization was calculated by subtracting the residual amount from that added to the milk. Similarly, storage losses of the added vitamin were estimated by subtracting the difference between the vitamin C content of the enriched and nonenriched samples at the end of a given storage period from the residual present after sterilization.

Results and Discussion

Loss of vitamin C during sterilization and storage. Data regarding the loss of added vitamin C during commercial sterilization of cans of evaporated milk are presented in Table 1. The losses in the enriched milk of Plant B were consistently higher than either of the other plants. Observations at the time of sampling cannot account for this difference, but it is possible that Plant B operations were such as to cause more of the added ascorbic acid to be oxidized to dehydroascorbic acid which was destroyed later during sterilization.

From the data presented in Table 2, it may be observed that over 50% of the losses of vitamin C occurred during the first four months of storage at 21 C. Most of the oxygen present in the cans was probably exhausted during this time. Losses tended to level off between the eighth and twelfth months of storage. The destruction of vitamin C was considerably greater in the evaporated milk stored at 36 than at 21 C. This finding is in agreement with observations made by Conochie (2) and Josephson and Doan (5). It is unlikely, however, that evaporated milk would be stored for prolonged periods at temperatures as high as 36 C. Therefore, data regarding the vitamin C losses in evaporated milk stored at 21 C would be of greater practical interest to evaporated milk manufacturers.

The average amounts of natural vitamin C present in the freshly sterilized nonenriched samples were 1.8, 0.7, and 1.4 mg per 100 ml for Plants A, B, and C, respectively. After 12 months of storage, these values dropped to 1.3, 0.4, and 1.0.

At the 5% probability level, the upper confidence limits for loss of added vitamin C during sterilization were 24.4, 31.2, and 27.0% for Plants A, B, and C, respectively, as indicated in Table 3. Similarly, it was calculated that the upper confidence limits for the loss of vitamin C after storage for 12 months at 21 C for Plants A, B, and C, respectively, were 26.1, 21.9, and 26.6% of the vitamin C present at the commencement of storage.

The required amount of vitamin C as calculated from the legal minimum standards is 14 mg per 100 ml of evaporated milk. It is presumed that evaporated milk will ordinarily be sold within one year from date of manufacture, and that it will not likely be stored much above

TABLE 1. Loss of added vitamin C during sterilization of commercial evaporated milk.

Sample number[a]	Ascorbic acid (mg/100 ml) Added	Present after sterilization[b]	Lost during sterilization	Loss (%)	Avg plant loss (%)
A-1	24.8	19.8	5.0	20.2	
A-2	24.8	19.3	5.5	22.2	
A-3	24.8	20.3	4.5	18.1	
A-4	24.8	21.3	3.5	14.1	18.7
B-1	24.8	18.7	6.1	24.6	
B-2	24.8	17.8	7.0	28.2	
B-3	24.8	17.4	7.3	29.4	
B-4	24.8	18.0	6.8	27.4	27.4
C-1	24.8	19.1	5.7	23.0	
C-2	24.8	20.3	4.5	18.1	
C-3	26.2	21.7	4.5	17.2	
C-4	26.2	19.7	6.5	24.8	20.8

[a] Letters indicate plants, digits indicate sets of samples, 1 and 2 for winter and 3 and 4 for summer.

[b] Each value represents the mean of analyses of four cans of evaporated milk.

TABLE 2. Per cent loss of added vitamin C in evaporated milk during storage at 21 C and 36 C.

Storage period (months)	Plant	Storage temperature 21 C Mean	21 C Standard deviation	36 C Mean	36 C Standard deviation
4	A	11.30	1.45	32.52	2.79
	B	10.45	2.09	27.52	0.56
	C	12.42	1.71	27.18	5.52
8	A	19.47	3.21	47.80	4.50
	B	15.30	1.61	42.56	1.88
	C	17.78	3.27	43.95	2.21
12	A	19.60	3.31	64.55	4.73
	B	18.03	2.00	56.35	2.40
	C	21.13	2.86	55.42	2.76

TABLE 3. Per cent loss of added vitamin C in evaporated milk during commercial sterilization.

Plant	Per cent loss of added vitamin C Mean	SD	95% Confidence limits
A	18.65	2.99	12.7 and 24.4%
B	27.43	1.90	23.7 and 31.2%
C	20.75	3.72	14.5 and 27.0%

J. Dairy Sci. 51(6): 921-923

OUR INDUSTRY TODAY

Stability of Vitamin C in Enriched Commercial Evaporated Milk [1]

D. H. BULLOCK, S. SINGH, and A. M. PEARSON
Department of Dairy Science, University of Guelph
Guelph, Ontario, Canada

Abstract

During commercial sterilization of winter and summer milk, losses in per cent of added vitamin ranged from 14.1 to 22.2, 24.6 to 29.4, and 17.2 to 24.8, respectively, in milk from three plants. The storage losses of vitamin C (expressed as a per cent of that present at the beginning of storage) for all plants after 4, 8, and 12 months at 21 C averaged 11.4, 17.5, and 19.7%, respectively. The vitamin C content of evaporated milk enriched by addition of sodium ascorbate at the rate of 266 g per 1,000 kg and stored at 21 C for 12 months remained above the minimum legal standard (Canada Food and Drugs Act), which is equivalent to 14 mg vitamin C per 100 ml of evaporated milk.

A decade ago physicians and others interested in public health expressed concern about the increasing incidence of infantile scurvy in Canada (3, 8). They publicized that infantile scurvy was the outcome of a vitamin C deficiency in the diet. Medovy (6) stated that there was a real need for the inclusion of vitamin C in milk as it is used universally in infant feeding. Thus, as a health measure aimed primarily at preventing infantile scurvy, and also to improve general vitamin C nutrition, the Food and Drug Directorate, Department of National Health and Welfare, Ottawa, permitted addition of vitamin C to evaporated milk beginning in March, 1964. Although the manufacturer is not obliged to add vitamin C to evaporated milk, if it is added the milk must contain at least 70 mg of vitamin C per liter when reconstituted to the original moisture content (1).

Insufficient information was available in the industry concerning the stability of added vitamin C in commercial evaporated milk and its relative loss of potency in processing and storage. Therefore, the objective of this study was to determine the percentage retention of vitamin C in commercial evaporated milk after processing in different plants and after subsequent storage at different temperatures.

Experimental Procedures

Collection of canned evaporated milk samples. Cans of evaporated milk containing about 454 g of product were obtained from three manufacturing plants in Ontario. They are identified here as Plants A, B, and C. Both enriched and nonenriched evaporated milk were sampled, the latter to provide the control samples. Two sampling periods were used, January 1966, to supply winter milk, and June 1966, to supply summer milk. At each sampling period, two sets of samples were obtained from each plant by sampling twice within a five-day period. Enriched and control samples received the same commercial processing treatment. Because vitamin C determinations were made after four storage periods (0, 4, 8, and 12 months) at two temperatures (21 and 36 C), using four cans to estimate the vitamin C level for each set of the experimental variables, the total number of cans of evaporated milk analyzed was 3 (plants) × 2 (seasons) × 2 (enriched and nonenriched) × 2 (storage temperatures) × 4 (storage periods) × 2 (sets of samples) × 4 (cans analyzed per estimate) = 768.

Level of addition of vitamin C. Except for the milk obtained from Plant C in June, 1966, sodium ascorbate additions were made just prior to canning at the rate of 266 g per 1,000 kg of evaporated milk. In the exception mentioned above, rate of addition was 281 g per 1,000 kg. Because sodium ascorbate is equivalent to 88% of its weight of ascorbic acid, the amounts added per 100 ml were 24.8 and 26.2 mg, respectively.

Estimation of loss of added vitamin C. Doan and Josephson (4) and Woessner et al. (9) observed that dehydroascorbic acid was rapidly destroyed in evaporated milk during processing and storage. In addition, because the method of analysis used by the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa (7), determines only the reduced form of ascorbic acid (thereby defining the requirements for enrichment of evaporated milk in terms of reduced ascorbic acid), the estimates of vitamin C throughout this study were based on the determination of the reduced form. In this method, the ascorbic acid content of the sample is determined by titrating with 2,6-dichlorophenolindophenol after removal of other reducing substances by precipitation. Correction is made for traces of interfering reducing substances which still may be present by using formaldehyde blanks. Formaldehyde condenses readily with ascorbic acid at pH 3.5, while reductones do not condense appreciably.

Within 24 hours after sterilization, the vita-

[1] Supported in part by a grant from Hoffmann-La Roche Limited, Montreal, Quebec, Canada

The slowing of the oxidation rate by any one antioxidant was closely related to the method by which it was added to the meal. Use of a solvent increased the effectiveness considerably (table 5).

The antioxidant effectiveness also was increased by increasing the thoroughness of mixing. Thus in one case where Santoquin was stirred in by hand (employing a solvent), an oxidation rate of 12.0 microliters per hour per gram was obtained, whereas when the antioxidant was added (in a solvent) by grinding twice a value of 3.0 microliters per hour per gram was obtained.

Table 5 - Effect of Using Solvent to Incorporate Antioxidant in Tuna Meal 1/

Antioxidant	Used for Incorporation of Antioxidant	
	No Solvent	Solvent
	Microliters Per Hour Per Gram of Oxygen Uptake	
DPPD	7.0	3.8
BHA	7.4	5.8
BHT	8.0	6.7
Control.	27.5	27.5

1/ Yellowfin-albacore mixture.

SUMMARY

1. Hematin compounds have been shown to play a major role in catalysis of oxidation of fish oils. The content of such compounds, measured in nine species of fish, varied from 5.4×10^{-5} M to 0.1×10^{-5} M.

2. Various combinations of antioxidants were developed for application in retarding oxidation of oil in fish tissue. Some of these were far more effective than were the usual antioxidants used alone or with a single synergist. A mixture of tocopherol, ascorbic acid and citric acid, for example, was about 80 times as effective as was ascorbic acid alone or mixtures of ascorbic acid and of citric acid.

3. The effectiveness of the most widely-used commercial food and feed antioxidants was compared.

4. The rate of oxygen adsorption in fish meals was measured, and certain commercial antioxidants were found to be effective in retarding oxidation. The method of mixing the antioxidant with the meal was found to be of considerable importance in determining the effectiveness of the antioxidant.

USE OF CHEMICALLY-TREATED ICE FOR FISH

"Storage of Fish in Ice Containing Antibiotics or Chemical Storage Products, Annual Report, 1954." Institute of Chemical and Technical Investigations of the Fisheries Department, Bergen, 1955, pp. 11-12.

The experiments were done on cod, one part of which was stored in ice containing aureomycin, and the other, used as a control sample, in pure ice. The temperature range was 0° to -2° C. (32° and 28.4° F.). After 2 days, 97 percent of bacteria were destroyed. Between -0.5 and +1° C. (31.1° and 30.2° F.), aureomycin is still more efficient: 99.2 percent of bacteria are destroyed.

The use of foromycene, a chemical storage product, gave similar results.

ple, was about 80 times more effective in retarding oxidation than was citric acid and ascorbic acid alone.

COMMERCIAL ANTIOXIDANTS: For certain applications to fishery products, it is possible to use some of the more potent antioxidants that are commercially available. A series of tests were carried out employing some of these commercial antioxidants in a model system.

This system consisted of 10 milliliters of linoleic acid (60 percent), 20 milliliters of phosphate buffer (pH 7.0), 0.25 milliliters of Tween 40, and sufficient hemoglobin solution to effect a concentration of 5×10^{-5} M in the final mixture. To this was added the particular antioxidant system under test, and the oxygen adsorption was measured for at least 1 hour in a Warburg apparatus at a temperature of 37° C.

Table 3 - Oxidation Rate of Fish Meals and Freeze-Dried Fish at 30° C.

Sample	Oxidation Rate
	Microliters Per Hour Per Gram
Menhaden meal 1/	10
Tuna meal 1/	3
Freeze-dried:	
Pilchard	300
Mackerel	300
Tuna	28
Pink salmon (light meat)	44
Pink salmon (dark meat)	370
Chum salmon (light meat)	2
Chum salmon (dark meat)	34
Sole	3
Halibut	2
Cod	0.8

1/ These commercial meals had been stored for several months and hence already had gone through the rapid initial oxidation.

The results obtained with the various substances tested are shown in table 2. It can be seen that the antioxidant Santoquin gave the greatest protection against oxidation. This particular antioxidant is not now approved by the Food and Drug Administration for general commercial use.

OXIDATION OF OIL IN FISH MEALS

RATE OF OXIDATION OF MEALS: In this work, both commercial fish meals and freeze-dried fish were used. The latter may be considered as fish meals dried under ideal conditions. The commercial fish meals used in this work had been stored for a period of months before the studies on rate of oxidation were started. They therefore already had gone through the initial high rate of oxidation, which was not the case with the freeze-dried samples. Table 3 shows the oxidation rate for these samples, the moisture content of which had been adjusted to 5 percent. The rate of oxidation was measured in a Warburg apparatus at 30° C.

Table 4 - Oxidation Rate of Tuna Meal 1/ Protected by Various Antioxidants

Antioxidant	Oxidation Rate of Tuna Meal
	Microliters Per Hour Per Gram
Santoquin	3.0
DPPD	3.8
BHA	5.8
BHT	6.7
Propyl gallate	17.0
NDGA	22.0
None (control)	27.5

1/ Yellowfin-albacore mixture.

EFFECT OF COMMERCIAL ANTIOXIDANTS: The oxidation rates of fish meals to which had been added various commercial antioxidants were measured. Antioxidants were added to meals that had been prepared commercially as described in the preceding paragraph. In all cases, the antioxidants reduced the rate of oxygen uptake (table 4).

meat and from 67.2 x 10^{-5} M to 49.6 x 10^{-5} M in the dark meat. Spectral absorption curves of aqueous extracts of the samples before and after oxidation showed a shift

Table 2 - Protective Factors for Antioxidants, With or Without Synergists, in a Linolenate Model System

Antioxidant 1/	Protective Factor 2/ in Presence or Absence of Indicated Synergist				
	None	Citrate	Citrate + Ascorbate	Ascorbate	10X Ascorbate
BHA	4.0	4.8	2.0	2.0	9.0
2X BHA	8.6				
BHT	4.9	8.7	1.4	1.4	7.1
2X BHT	15.9				
BHA + BHT	13.5				
NDGA	2.6				
2X NDGA	9.2				
DPPD	2.4				
2X DPPD	54				
Santoquin	8.9				
2X Santoquin	80				

1/ The concentration of antioxidants and synergists was 10^{-4} M in final solutions except that it was 2 or 10 times this amount when 2x or 10x respectively, are indicated.
BHA = butylated hydroxyanisole; BHT = butylated hydroxytoluene;
NDGA = nordihydroguaiaretic acid; DPPD = N, N-diphenyl-p-phenylenediamine.

2/ Protective factor = $\frac{\text{Time for system to absorb 300 } \mu \text{ } 1.O_2}{\text{Time for control to absorb 300 } \mu \text{ } 1.O_2}$

in maxima from 542 and 578 millimicrons for the unaltered samples to 500 and 630 millimicrons for the oxidized ones. This shift indicates a transformation of oxyhemoglobin (or oxymyoglobin) to methemoglobin (or metmyoglobin).

RATE OF OXIDATION IN FISH MEAT: The rate of oxygen adsorption by cubes of different species of fish were measured in a Warburg apparatus at 0° C. The samples were treated with aureomycin to retard bacterial decomposition. Measurements were made on both the light and the dark meat. The results are shown in table 1.

ROLE OF ANTIOXIDANTS

NATURALLY-OCCURRING ANTIOXIDANTS: Certain antioxidants occur in foodstuffs and are harmless for use as added antioxidants to foods. These include such substances as ascorbic acid, citric acid, and alpha tocopherol. The latter substance occurs in the vitamin E group present in fish oils. Initial work was concentrated on the effect of these types of antioxidants. Oxygen-absorption tests were carried out in model systems in which in most cases an emulsion of (1) one of the fatty acids found in fish-oil glycerides with (2) hemoglobin and a buffer solution was employed. The use of this model system permitted reproducible results to be obtained over a period of time, whereas the use of a fish oil rather than of a pure fatty acid as a test material would have made it impossible to obtain repeat results because of the changes that occur in fish oils with storage and the impossibility of getting new lots of fish oils that are chemically identical to those used in earlier work.

In one test, results using the model system were compared with those obtained employing menhaden oil, and it was found that parallel types of results were obtained. The rate of oxidation was greater and the effect of a given antioxidant was less with menhaden oil, but the comparative effectiveness of different antioxidants was the same in the model system as in the menhaden-oil system. In general, it was found that citric acid and ascorbic acid synergize with tocopherol in inhibiting oxidation. A mixture of alpha tocopherol and citric acid and ascorbic acid, for exam-

CATALYTIC EFFECT OF HEMATIN COMPOUNDS: The effect of the hematin compounds of fish meat on the catalysis of oil oxidation next was determined. This work was carried out by measuring the oxygen uptake of a salt of an unsaturated fatty acid, ammonium linoleate, when shaken in a Warburg respirometer. With the reaction being carried out at 20° C. and at a pH of 9.0, aqueous extracts of fish were added to the ammonium linoleate substrate and their effect on the rate of oxidation was determined. The fact that the catalysis was due primarily to hematin compounds and not to some other biocatalysts in the fish was confirmed by repeating the experiment in the presence of cyanide, in which case no catalytic effect was observed. Cyanide inhibits hematin catalysis of unsaturated fatty acids oxidation.

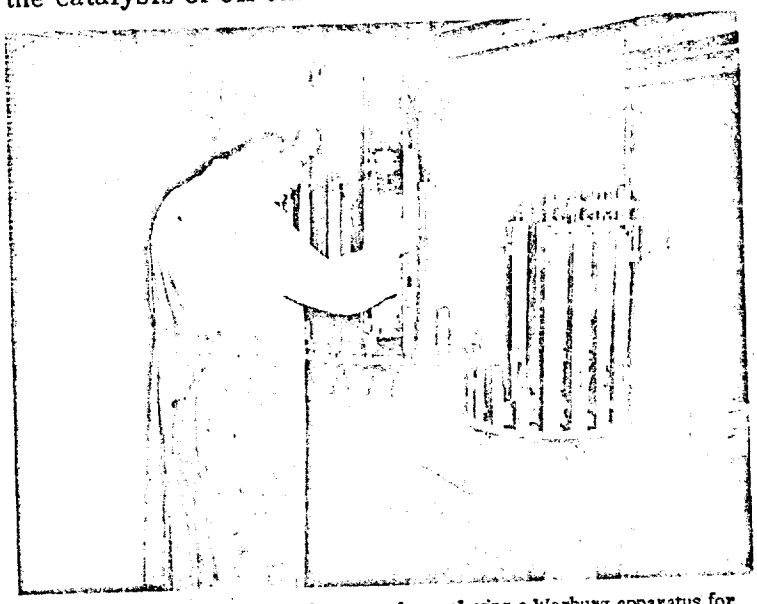


Fig. 2 - Measuring oxidation of tuna meals employing a Warburg apparatus for measurement of oxygen adsorption.

In another series of experiments (1) cubes of fish meat and (2) extracted fish oil from different species were measured for rate of oxygen uptake in a Warburg apparatus.

The results of all these experiments showed a direct correlation between the hematin-compound content of the various species of fish and the catalytic effect on the linoleate oxidation. The rate of oxidation of the fish meat also was correlated with the content of hematin compounds.

Table 1 - Oxidation Rate of Cubes of Light and Dark Meat of Fish in a Warburg Apparatus at 0° C.

Species	Rate of Oxidation	
	Light Meat	Dark Meat
	Microliters of Oxygen Uptake Per Hour Per Gram	
Chum salmon	0	17
Pink salmon	0	13
Tuna	0	13
Sheepshead	0.4	1.5
Lake chub	0.6	1/
Pilchard	7	1/
Rockfish	1	1/
Cod	0.5	1/
Mackerel	8	1/

1/ For these species, no separation of light and dark meat was attempted. The cubes of meat used, however, were mostly light meat.

CATALYTIC EFFECT OF PROTEINS: Some experiments were carried out to determine the catalytic effect of various proteins on the oxidation of fish oils. It was shown that all of the proteins studied had a measurable effect, but that none of these proteins approached hemoglobin in catalyzing the oxidation reaction.

HEMATIN-COMPOUND CHANGES DURING OXIDATION: During oxidation of oil in fish tissue, the hematin compounds that catalyze the oxidation are chemically altered, and their concentration diminishes. Cubes of tuna meat, cut from both the light and the dark tissue, were allowed to oxidize in a Warburg apparatus at 0° C. The content of hematin compound diminished from 2.1×10^{-5} M to 1.0×10^{-5} M in the light

1172

Commercial Fisheries Review (Rev. 19) (May 57 Supplement) 5A: a 27-31, 1957.

Rev 19 No 5a, a 27-31 1957

OXIDATIVE DETERIORATION IN FISH AND FISHERY PRODUCTS

Part II - Progress on Studies Concerning Mechanism of Oxidation of Oil in Fish Tissue

By W. D. Brown,* A. W. Venolia,* A. L. Tappel,**
H. S. Olcott,** and M. E. Stansby***

ABSTRACT

Studies showed that the mechanism of oxidation of fish oils in the meat of fish or in fish meal differs from that of extracted oils owing to the activity of biocatalysts in the tissues. The fish meat content and activity of hematin, found to be the primary catalytic agent in such oxidation, was studied. Activities of the proteins of the meat were found to be of lesser importance. Studies were made on naturally-occurring and commercial antioxidants. A mixture of the natural antioxidants citric acid, ascorbic acid, and tocopherol was found to be 80 times as effective, owing to synergism, than was citric acid or ascorbic acid alone. Method of addition of the antioxidant to the test system was found to be of importance in retarding oxidation.

The portion of the program on which progress is reported in this paper deals with the mechanism of oil oxidation while the oil still is associated with the fish tissue or while it is present in fish meal. The mechanism of the oxidation is different under these conditions from that in extracted oils because of the presence in the tissue of certain biocatalysts that affect the course of the oxidation.

Fig. 1 - Colorimetric determination of tocopherol in king salmon oil.

This phase is basic to applications whether involving changes in oil in situ in the meat of stored fish, in fish meals, or in the extracted oil itself because, in all these cases, the oil starts out in the meat and some changes always take place initially in the meat before any manufacturing steps have been started. Accordingly, chief emphasis in the initial stages of this program has been toward elucidating the mechanism of oxidation of oil in fish meat.

HEMATIN CATALYSIS

HEMATIN-COMPOUND CONTENT OF FISH: In the first phase of the work a number of species of fish were analyzed for their content of hematin compounds, substances that have been shown to be powerful biocatalysts for oil oxidation in other foods. Nine species of fish were examined, and the hematin compounds were found to vary in content from 5.4×10^{-5} M in pilchard down to 0.1×10^{-5} M in cod.

*Chemist, U. S. Fish and Wildlife Service, Davis, Calif.
** University of California, Department of Food Technology, Davis and Berkeley, Calif.
*** Chief, Pacific Coast and Alaska Technological Research, Fishery Technological Laboratory, Branch of Commercial Fisheries, U. S. Fish and Wildlife Service, Seattle, Wash.

Nature (London) 1972 238 (5362) 277 (ENG)

[7] Kennedy, G. Y., *Chemical Zoology* (edit. by Florkin, M., and Scheer, B. T.), 4, 311 (Academic Press, New York and London, 1969).
[8] Falk, J. E., Dresel, E. I. B., Benson, A., and Knight, B. C., *J. Biol. Chem.*, 63, 87 (1956).
[9] Brockmann, A., and Schodder, H., *Chem. Ber.*, 74, 73 (1941).
[10] Nicholas, R. E. H., *Biochem. J.*, 48, 309 (1951).
[11] Muir, H., and Neuberger, A., *Biochem. J.*, 45, 163 (1949).
[12] Chu, T. C., and Chu, E. J., *J. Biol. Chem.*, 208, 537 (1954).
[13] Fox, D. L., and Millott, N., *Proc. Roy. Soc.*, B, 142, 392 (1954).
[14] Czeczuga, B., *Marine Biology*, 5, 141 (1970).
[15] MacMunn, C. A., *J. Physiol.*, 7, 240 (1886).
[16] Clarke, G. L., and Denton, E. J., *The Sea* (edit. by Hill, M. N.), 1, 456 (Interscience, New York and London, 1962).

Vitamin C Requirements and Oral Contraceptives

ANIMAL experiments[1-4] indicate that oestrogens increase the rate of ascorbic acid breakdown and lower tissue levels. Vitamin C is poorly stored by humans and widespread subclinical deficiencies occur. We have investigated levels of ascorbic acid in blood cells from healthy women, some of whom were taking oral contraceptives. We have also studied pregnant women and others receiving an injected contraceptive. None was receiving supplementary vitamins.

Several brands of "combined-type" oral contraceptives were in use, while the injected product was 'Depo-Provera' (150 mg medroxyprogesterone acetate every 3 months).

Blood was collected by venepuncture and 3.0 ml. added to a special diluting agent[5], any remainder being added to plastic tubes containing EDTA. A combined leucocyte-platelet button was prepared from the former, and a relatively pure platelet button from the latter. Ascorbic acid was determined by 2,4-dinitrophenylhydrazine[5] and expressed as $\mu g/10^8$ cells for the leucocytes and as mg/g for the platelets. Mean values for each group are set out in Tables 1 and 2. We also measured ascorbic acid in blood cells from small groups of young women before and after beginning treatment with steroid contraceptives, and in others receiving no treatment. These results are shown in Table 3.

85 women took part in the main study; 31 were untreated non-pregnant controls (mean age 27 yr), 18 were pregnant (mean 24 yr), while 39 were receiving steroid contraceptives (mean 27 yr). Each group contained European, African and Asian women. For those in the contraceptive group, 21 had been under treatment for under 6 months, 4 for between 6 and 12 months, and 14 for over 1 year.

Our results show that ascorbic acid in both leucocytes and platelets is significantly lower in women taking steroid contraceptives than in untreated controls or in pregnant women. Only the injected contraceptive appeared not to influence the vitamin C concentration. Cell ascorbic acid was also significantly lower in the 5 women studied before and after beginning oral contraceptives, but not in the woman starting 'Depo-Provera', nor in the untreated group.

It seems likely that oral contraceptive steroids increase the breakdown of ascorbic acid, perhaps by their stimulant action on liver release of ceruloplasmin[6], a copper-containing protein with ascorbate oxidase activity[7]. It seems clear that many women receiving oral contraceptives may be in an induced hypovitaminotic C condition and require supplementary vitamin. It is possible that some of the reported side-effects of "the pill" may be a consequence of this vitamin lack.

Table 1 Leucocyte Ascorbic Acid Content

Group	No.	Ascorbic acid: ($\mu g/10^8$: mean ± s.d.)	*P* value* (*t*-test)
All controls	31	39 ± 28	—
African controls	12	40 ± 24	NS
All contraceptives	39	26 ± 22	< 0.01
Norgestrel products	15	27 ± 21	< 0.02
Ethynodiol diacetate products	6	23 ± 19	< 0.02
Lynestranol products	4	25 ± 10	< 0.05
Norethisterone acetate products	7	26 ± 17	< 0.02
Other products	3	25 ± 19	< 0.02
Depot contraceptive	4	38 ± 17	NS
All pregnant	18	34 ± 21	NS
First trimester	1	19	—
Second trimester	9	33 ± 23	NS
Third trimester	8	37 ± 21	NS

* Each group is compared with the complete control group: *P* values greater than 0.05 are listed as NS (not significant).

Table 2 Platelet Ascorbic Acid Content

Group	No.	Ascorbic acid: (mg/g wet weight) mean ± s.d.	*P* value
All controls	10	0.26 ± 0.11	—
Oral contraceptives *	10	0.19 ± 0.13	< 0.01
Depot contraceptive	5	0.28 ± 0.14	NS
Pregnant	5	0.24 ± 0.10	NS

* As the group is small, it has not been subdivided by product: it contained 3 patients taking 'Gynovlar', 5 taking 'Ovral' or 'Eugynon' (equivalent) and 2 taking 'Ovulen' 50.

Table 3 Leucocyte and Platelet Ascorbic Acid before and after Treatment with Oral Contraceptives

Group	Age of subject	Product	Interval (months)	Ascorbic acid Leucocyte 1	Leucocyte 2	Platelet 1	Platelet 2
Contraceptive	31	'Lyndiol' 2.5	2	38	19	0.30	0.25
	22	'Minilyn'	1	65	38	0.27	0.22
	23	'Depo-Provera' *	1	28	29	0.24	0.26
	35	'Neogynon'	1	29	16	0.19	0.15
	23	'Eugynon'	2	32	28	0.23	0.16
	20	'Ovulen' 50	2	45	32	0.28	0.20
Control	28	None	1	31	34	0.24	0.27
	20	None	1	23	28	0.18	0.20
	19	None	1	28	31	0.26	0.28
	21	None	1	38	32	0.28	0.25
	18	None	1	42	38	0.24	0.19

Ascorbic acid concentrations for leucocytes are $\mu g/10^8$ cells and for platelets mg/g wet weight. The values shown under column 1 are pre-treatment and under column 2 post-treatment results.

* Injectable contraceptive.

MICHAEL BRIGGS
MAXINE BRIGGS

Department of Biochemistry,
University of Zambia,
PO Box 2379,
Lusaka, Zambia

Received March 20; revised April 24, 1972.

[1] di Martini, Marrubini, G., and Silo, N., *Arch. Fisiol.*, 50, 94 (1950).
[2] Mosonyi, J., *Hoppe-Seyler's Z. Physiol.*, 242, 158 (1936).
[3] Clemetson, C. A. B., *Lancet*, ii, 1037 (1968).
[4] Saroja, N., Mallikarjuneswara, V. R., and Clemetson, C. A. B., *Contraception*, 3, 269 (1971).
[5] Denson, K. W., and Bowers, E. F., *Clin. Soc.*, 21, 157 (1961).
[6] Russ, E. M., and Raymunt, J., *Proc. Soc. Exp. Biol. Med.*, 92, 465 (1956).
[7] Osaki, S., McDermott, J. A., and Frieden, E., *J. Biol. Chem.*, 239, 3570 (1964).

aciduria, since some urine may be contaminated by feces in the metabolism cages.

The volume of urine in animals on the ascorbic acid deficient diet was greater than in animals on fox chow. This, in turn, may have accounted for the occasional reduction in concentration of various urinary constituents such as glucose, protein, occult blood, and nucleoprotein. It is not obvious to what constituent of the deficient diet were related the slightly lower colonic temperature, slight leucocytosis, and slightly greater hair loss and skin soiling seen in animals on this diet.

Summary

The death rate and most of the clinical and pathological signs of chronic toxicity to an oral 0.1 L-LD_{50} of benzylpenicillin in albino rats on a diet deficient in ascorbic acid were similar to those in corresponding animals on a diet of fox chow. Such differences as did exist could be largely explained on the basis of factors in the deficient diet other than the deficiency of ascorbic acid.

Supplementation of the deficient diet with added ascorbic acid had little significant effect upon the toxicity syndrome. Synthesis of ascorbic acid in albino rats, therefore, was not affected to the point of becoming a factor determining signs of toxicity to this daily dose of benzylpenicillin.

logical differences noted between the two groups.

When animals on the ascorbic acid deficient diet were given benzylpenicillin, their food and water intake, colonic temperature, and urinary concentration of occult blood and nucleoprotein were lower and their urinary volume, hair loss, and fur soiling were greater than in animals on fox chow and given benzylpenicillin (Table 1). Death rates and other clinical signs (Table 1) and most values for organ weights (Table 2) and water levels (Table 3) were identical in the two groups. Histopathologically, rats on vitamin C deficient diet exhibited the same signs as those on fox chow (which have been previously reported[2]) except that vascular congestion was more marked in the stomach, cecum, kidneys, lungs, and thymus gland.

The addition of 1-ascorbic acid to the deficient diet did little to modify the effects of the diet on chronic benzylpenicillin toxicity as may be seen by data summarized in Table 1 to 3. There were also no consistent histopathological differences.

Discussion

The results of this project demonstrated that the hematuria seen during chronic benzylpenicillin toxicity in the albino rat is not augmented by placing the animals upon a diet deficient in ascorbic acid. The concentration of urinary occult blood was actually decreased in animals on the deficient diet and to the same extent as in animals given the deficient diet plus supplementary ascorbic acid. No evidence was obtained to prove, therefore, that the hematuria of chronic benzylpenicillin poisoning in the albino rat is due to impaired synthesis of ascorbic acid.

Other signs of toxicity from benzylpenicillin in animals on the ascorbic acid deficient diet were similar, for the most part, to those in animals on the deficient diet plus supplementary ascorbic acid. The effects of the diet were due, therefore, to factors other than the absence of ascorbic acid.

Several effects of the deficient diet were due to the animals eating less of it than of fox chow. This apparently lead to some impairment in growth of certain organs. Impaired growth was not as marked as might have been expected from the decreased food intake presumably because the caloric intake was not appreciably lowered. Reduced food intake was probably responsible for a reduction in water intake. The deficient diet contained 20% of wheat bran which may have accounted for the soft stools and possibly the

Table 3

WATER LEVELS IN ORGANS OF ALBINO RATS AFTER 8 WEEKS ON A DIET DEFICIENT IN ASCORBIC ACID OR ON FOX CHOW, WITH AND WITHOUT DAILY ADMINISTRATION OF BENZYLPENICILLIN AND ASCORBIC ACID.*

Organ	No Benzylpenicillin		Benzylpenicillin-Treated		
	Ascorbic Acid Deficient Diet	Fox Chow	Ascorbic Acid Deficient Diet	Ascorbic Acid Deficient Diet Plus Ascorbic Acid	Fox Chow
Adrenal glands	219 ± 38	203 ± 37	197 ± 31	196 ± 18	214 ± 36
Brain	353 ± 5*	344 ± 10	353 ± 33	348 ± 22	340 ± 17
Gastrointestinal tract:					
Cardiac stomach	307 ± 52	294 ± 41	283 ± 23*	300 ± 39	318 ± 34
Pyloric stomach	323 ± 32	305 ± 32	323 ± 11	330 ± 26	324 ± 26
Small bowel	337 ± 24	323 ± 50	334 ± 33	351 ± 32	348 ± 46
Cecum	349 ± 44	364 ± 57	401 ± 49	393 ± 52	392 ± 21
Colon	[illegible]5 ± 24	400 ± 39	377 ± 42*	382 ± 18*	410 ± 28
Heart	332 ± 9*	349 ± 13	330 ± 11	317 ± 43	332 ± 25
Kidneys	318 ± 11*	337 ± 19	326 ± 15	317 ± 31	338 ± 17
Liver	238 ± 28	251 ± 25	242 ± 16	240 ± 10	240 ± 18
Lungs	355 ± 10	350 ± 34	337 ± 20	349 ± 20	348 ± 31
Mesentery-omentum	122 ± 75	77 ± 27	89 ± 33	80 ± 31	105 ± 55
Muscle (Abd. wall)	276 ± 10*	256 ± 19	278 ± 14	257 ± 25*	278 ± 16
Salivary glands (Submax.)	299 ± 13	298 ± 17	320 ± 90	276 ± 24*	294 ± 9
Skin	148 ± 25	157 ± 24	136 ± 30*	143 ± 36	165 ± 30
Spleen	334 ± 11*	322 ± 16	325 ± 13	325 ± 12	323 ± 9
Testicles	644 ± 9	639 ± 34	649 ± 11	646 ± 11	660 ± 35
Thymus gland	354 ± 59*	271 ± 51	304 ± 28	298 ± 22	320 ± 25
Residual Carcass	207 ± 15	202 ± 27	198 ± 17	211 ± 18	213 ± 45

Water levels were measured as grams water per 100 grams dry weight of tissue and are expressed as mean ± standard deviation. Means in animals on a diet deficient in ascorbic acid which differed at P = 0.05 from corresponding means in animals on a diet of fox chow are indicated by an asterisk.

1047

Table 1

A SUMMARY OF MEASUREMENTS MADE OVER A PERIOD OF 8 WEEKS UPON ALBINO RATS MAINTAINED ON A DIET DEFICIENT IN ASCORBIC ACID OR ON FOX CHOW, WITH AND WITHOUT DAILY ADMINISTRATION OF BENZYLPENICILLIN AND ASCORBIC ACID.

Measurement	No Benzylpenicillin		Benzylpenicillin-Treated		
	Ascorbic Acid Deficient Diet	Fox Chow	Ascorbic Acid Deficient Diet	Ascorbic Acid Deficient Diet Plus Ascorbic Acid	Fox Chow
Deaths: % of animals at 56 da.	0	0	20	20	30
Weekly gain in body wt.: Gm.	13.5 ± 5.7	16.0 ± 4.8	11.5 ± 8.0	5.8 ± 5.2*	14.0 ± 6.6
Food intake: Gm. /Kg. / 24 hrs.	54.4 ± 11.3*	85.9 ± 13.7	53.6 ± 15.2*	63.1 ± 17.5*	88.5 ± 17.5
Water intake: ml. /Kg. / 24 hrs.	102.5 ± 29.6	111.0 ± 20.9	173.7 ± 43.5*	196.4 ± 39.0	200.5 ± 35.6
Colonic temp.: °F.	100.2 ± 0.7	100.8 ± 0.7	99.5 ± 0.9*	99.4 ± 0.8*	100.1 ± 0.8
Urine vol.: ml. /Kg. /24 hrs.	17.3 ± 9.9	12.1 ± 10.3	42.3 ± 21.3*	63.0 ± 19.9*	29.6 ± 14.0
Urine pH	5.9 ± 0.4*	6.5 ± 0.7	6.4 ± 0.7	6.4 ± 0.2	6.4 ± 0.4
Urine glucose: Gm. /100 ml.	0.2 ± 0.3	0.2 ± 0.3	0.3 ± 0.3	0.0 ± 0.0*	0.2 ± 0.3
Urine protein: mg. /100 ml.	48 ± 24 *	136 ± 33	31 ± 19	22 ± 13 *	40 ± 21
Urine occult blood: 1+ to 4+	0.2 ± 0.3	0.0 ± 0.0	0.1 ± 0.3*	0.1 ± 0.2*	0.5 ± 0.3
Urine nucleoprotein: 1+ to 4+	0.4 ± 0.3	±	0.3 ± 0.3*	0.7 ± 0.3	0.7 ± 0.3
Blood hemoglobin: Gm. /100 ml.	14.9 ± 0.9	15.6 ± 1.3	14.4 ± 0.9	14.1 ± 1.0	14.2 ± 0.8
Blood Leucocytes: 10^3/cu. mm.	22.6 ± 2.8*	14.7 ± 2.0	16.3 ± 3.3	16.7 ± 2.1*	14.7 ± 2.1
Hair loss: % of total hair	0.0 ± 0.0	0.0 ± 0.0	24 ± 6 *	23 ± 5 *	18 ± 5
Skin soiling: % of total area	0.0 ± 0.0	0.0 ± 0.0	28 ± 9 *	29 ± 7 *	23 ± 4
Pallor: 1+ to 4+	0.5 ± 0.2*	0.0 ± 0.0	0.6 ± 0.2	0.5 ± 0.2	0.6 ± 0.2
Abdominal bloating: 1+ to 4+	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 0.2	0.9 ± 0.2	1.1 ± 0.2
Diarrhea: 1+ to 4+	2.3 ± 0.6*	0.0 ± 0.0	4.0 ± 0.0	4.0 ± 0.0	4.0 ± 0.0

The results are expressed as mean ± standard deviation of weekly measurements except as noted. Means in animals on a diet deficient in ascorbic acid which differ at P = 0.05 from corresponding means in animals on fox chow are indicated by an asterisk.

Table 2

FRESH WEIGHTS OR ORGANS OF ALBINO RATS AFTER 8 WEEKS ON A DIET DEFICIENT IN ASCORBIC ACID OR ON FOX CHOW, WITH AND WITHOUT DAILY ADMINISTRATION OF BENZYLPENICILLIN AND ASCORBIC ACID. *

Measurement	No Benzylpenicillin		Benzylpenicillin-Treated		
	Ascorbic Acid Deficient Diet	Fox Chow	Ascorbic Acid Deficient Diet	Ascorbic Acid Deficient Diet Plus Ascorbic Acid	Fox Chow
Adrenal glands	0.044 ± 0.006	0.046 ± 0.013	0.044 ± 0.008	0.041 ± 0.007	0.045 ± 0.010
Brain	1.76 ± 0.10	1.70 ± 0.10	1.75 ± 0.14	1.78 ± 0.17	1.82 ± 0.07
Gastrointestinal tract:					
Cardiac stomach	0.281 ± 0.046*	0.369 ± 0.090	0.294 ± 0.036*	0.302 ± 0.051*	0.340 ± 0.022
Pyloric stomach	0.926 ± 0.121	1.000 ± 0.25	1.021 ± 0.133*	1.099 ± 0.122	1.161 ± 0.131
Small bowel	5.58 ± 0.95	5.84 ± 0.91	6.00 ± 1.00	5.75 ± 0.92	5.56 ± 0.52
Cecum	1.29 ± 0.18	1.27 ± 0.29	2.92 ± 0.61*	2.43 ± 0.64	2.15 ± 0.19
Colon	1.57 ± 0.23*	2.12 ± 0.57	1.51 ± 0.24	1.70 ± 0.78	1.49 ± 0.18
Heart	0.926 ± 0.070*	0.963 ± 0.123	0.796 ± 0.111	0.731 ± 0.115	0.796 ± 0.073
Kidneys	2.37 ± 0.18*	2.99 ± 0.18	2.32 ± 0.24	2.03 ± 0.22*	2.38 ± 0.26
Liver	13.2 ± 1.3*	15.5 ± 2.03	11.5 ± 1.5	10.5 ± 1.5	12.0 ± 1.6
Lungs	1.45 ± 0.29*	2.11 ± 0.61	1.11 ± 0.40	1.09 ± 0.18	1.16 ± 0.12
Mesentry-omentum	3.71 ± 1.06	4.10 ± 1.61	3.70 ± 0.88	3.37 ± 1.09	3.50 ± 0.95
Muscle (Abd. wall)	10.85 ± 0.75*	12.40 ± 2.61	11.31 ± 1.80	10.33 ± 2.05	11.70 ± 0.96
Salivary glands (Submax.)	0.533 ± 0.071	0.535 ± 0.081	0.580 ± 0.112*	0.507 ± 0.108	0.454 ± 0.049
Skin	72.3 ± 9.5*	80.3 ± 9.2	60.5 ± 9.3	54.3 ± 8.8	63.5 ± 9.8
Spleen	0.619 ± 0.098*	0.813 ± 0.114	0.452 ± 0.079	0.393 ± 0.056*	0.482 ± 0.089
Testicles	3.26 ± 0.22	3.01 ± 0.49	3.20 ± 0.26	2.96 ± 0.39	3.06 ± 0.19
Thymus gland	0.246 ± 0.030	0.229 ± 0.102	0.178 ± 0.044	0.163 ± 0.040	0.186 ± 0.045
Residual Carcass	178.4 ± 8.1	187.2 ± 17.8	156.2 ± 16.5	143.0 ± 24.7	168.2 ± 17.7

Weight was measured as grams wet weight at autopsy immediately after death and the results are expressed as mean ± standard deviation. Means in animals on a diet deficient in ascorbic acid which differed at P = 0.05 from corresponding means in animals on a diet of fox chow are indicated by an asterisk.

clinical report

Clin Med 70: 2223-9 Dec 63

Ascorbic Acid and Benzylpenicillin Toxicity

ELDON M. BOYD, M.D.,* *Kingston, Ontario, Canada*

▶*No evidence was found that deficiency of ascorbic acid was a factor appreciably influencing the chronic toxicity syndrome of albino rats to an oral 0.1 L-LD$_{50}$ of benzylpenicillin. Differences which did exist were explained on the basis of factors in the deficient diet other than ascorbic acid.*◀

Hematuria and renal tubular congestion have been recorded amongst the signs of acute[1] and chronic[2] oral toxicity of benzylpenicillin in the albino rat. It appeared possible that the hematuria might have been due to increased capillary fragility associated with impaired synthesis of ascorbic acid. The normal rat is capable of synthesizing its requirement of ascorbic acid,[3] but synthesis from glucose[4] has been reported impaired under certain circumstances apparently due to inanition and inhibited growth.[5] To determine if similar impairment of synthesis could have accounted for the hematuria and possibly other toxic signs, the chronic oral toxicity of benzylpenicillin was measured in albino rats placed on diets deficient in, or containing an excess of, ascorbic acid.

Method

The project was performed upon groups of 10 to 20 male albino rats weighing 203 ± 12 (mean ± standard deviation) grams. One group was placed upon a vitamin C deficient test diet, prepared from the formula of Woodruff et al[6] by General

*Department of Pharmacology, Queen's University. This study was supported by a grant from The National Vitamin Foundation.

1. Boyd, E. M., et al., *Arch. internat. pharmacodyn.*, 123:295-304,1960.
2. Boyd, E. M., & Selby, M. J., *Antib. & Chemo.*, 12:249-262,1962.
3. Schour, I., & Massler, M., Chapter 6 in Farris, E. J., & Griffith, J. Q., (Editors), *The Rat in Laboratory Investigation*, (Second Edition), J. B. Lippincott Company, Philadelphia, 1949.
4. Brown, G. M., *Physiol. Rev.*, 40:531-868, 1960.
5. Mapson, L. W., *The Biosynthesis of Ascorbic Acid*, in Harris, R. S., Marrian, G. F., and Thimann, K. V. (editors), *Vitamins and Hormones*, Academic Press Inc., New York, 13:71-100,1955.
6. Woodruff, C. W., et al., *J. Biol. Chem.*, 178:861-868,1949.

Biochemicals,[7] and given daily, six days a week, 5.34 Gm. of benzylpenicillin potassium per Kg. of body weight. The benzylpenicillin was freshly dissolved in distilled water each day and administered by intragastric tube in a volume of 10.0 ml. per Kg. The dose of benzylpenicillin was equivalent to the 0.1 L-LD$_{50}$ as estimated by Boyd and Selby.[2] A second group was treated in the same manner except that L-ascorbic acid was added to the diet at a concentration of 300 mg. per Kg. of diet, equivalent to an intake averaging about 15 mg. per Kg. of body weight per day. The third group was given benzylpenicillin and kept upon a standard laboratory diet of Purina fox chow. Two remaining groups were given no benzylpenicillin and maintained upon the vitamin C deficient diet and fox chow respectively. Measurements, noted in Table 1, were made by techniques described by Boyd and Selby[2] at weekly intervals for eight weeks after which survivors were autopsied. At autopsy, gross and microscopic pathology and weight and water content were recorded upon the organs listed in Tables 2 and 3. Statistical methods outlined by Waugh[8] and Croxton[9] were used.

Results

Clinical data are summarized in Table 1 and autopsy data in Tables 2 and 3.

Animals on the ascorbic acid deficient diet without benzylpenicillin were similar in appearance to those on fox chow alone. Daily intake, of the ascorbic acid deficient diet expressed as Gm. per Kg. of body weight was lower than that of fox chow, but since the vitamin C deficient diet contained approximately 50 per cent more calories per gram, the caloric intake was practically identical in the two groups. Stools of the group on ascorbic acid deficient diet were somewhat soft and not pelleted and there were certain other differences as noted in Table 1. The weights of some organs were lower in the group on vitamin C deficient diet (Table 2) and there were some significant differences in water content (Table 3). Histologically, the reduced weight of the spleen was seen to be at the expense of the red pulp, the smaller kidneys had some degree of capillary congestion in the loop region, and the increased water content of the brain was associated with minor vascular congestion. Otherwise there were no histo-

7. GBI Bulletin D15, *Biological Test Diets for Experimental Animals*, General Biochemicals, Chagrin Falls, Ohio, 1961.
8. Waugh, A. E., *Elements of Statistical Method*, (Third Edition), McGraw-Hill Book Company, Inc., New York, 1952.
9. Croxton, F. E., *Elementary Statistics with Applications in Medicine*, Prentice-Hall, Inc., New York, 1953.

1042

0994

plasma levels, suggesting that cortisone causes an increase in the kidney threshold to ascorbic acid.

4. The pattern of serum cholesterol response to ascorbic acid was reversed during cortisone therapy in the patients studied. Prior to cortisone therapy the level of serum cholesterol was unchanged in the first hour following intravenous administration of ascorbic acid, whereas in the same patients during cortisone therapy, the level of serum cholesterol was elevated in the first hour. The possible relationship of this elevation to ascorbic acid storage in the adrenal gland is discussed.

REFERENCES

1. Booker, W. M.; Hayes, R. L.; Sewell, M. B., and Dent, F. M.: Experimental studies in ascorbic acid metabolism, *Am. J. Physiol.* **166**: 374, 1951.
2. Booker, W. M.; Hayes, R. L., and Dent, F. M.: Influence of adreno-cortical hormones on the day-to-day blood levels and excretion of ascorbic acid in dogs, *Fed. Proc.* **9**: 14, 1950.
3. Hayes, R. L.; Booker, W. M., and Sewell, M. B.: Ascorbic acid metabolism in patients with certain gingival disturbances, *Fed. Proc.* **8**: 70, 1949.
4. Lewin, E., and Wassen, E.: Effect of combined injections of desoxycortone acetate and ascorbic acid on rheumatoid arthritis, *Lancet* **2**: 993, 1949.
5. Kellgren, J. H.: Desoxycortone acetate and ascorbic acid in rheumatoid arthritis, *Lancet* **2**: 1128, 1949.
6. Le Vay, D., and Loxton, G. E.: Desoxycortone acetate and ascorbic acid in the treatment of arthritis, *Lancet* **2**: 1134, 1949.
7. Schaffenburg, C.; Masson, G. M., and Corcoran, A. C.: Interrelationships of desoxycorticosterone, cortisone and vitamin C in the genesis of mesenchymal lesions, *Proc. Soc. Exper. Biol. & Med.* **74**: 358, 1950.
8. Selye, H., and Pentz, E.: Pathogenetical correlations between periarthritis nodosa renal hypertension and rheumatic lesions, *Canad. M. A. J.* **49**: 264, 1943.
9. Sayers, G.; Sayers, M., and Long, C. N. H.: The effects of pituitary adrenotrophic hormone on the cholesterol and ascorbic acid content of the adrenal of the rat and the guinea pig, *Endocrinology* **38**: 1, 1948.
10. Dent, F. M.; Hayes, R. L., and Booker, W. M.: Further studies on possible antagonism between cholesterol and ascorbic acid, *Fed. Proc.* **10**: 291, 1951.
11. Mindlin, R. L., and Butler, A. M.: The determination of ascorbic acid in plasma; a macromethod and micromethod, *J. Biol. Chem.* **122**: 673, 1938.
12. Bloor, W. R.; Pelkan, K. P., and Allen, D. M.: Determination of fatty acids and cholesterol in small amounts of blood plasma, *J. Biol. Chem.* **52**: 191, 1922.
13. Weinbach, A. P.: A micro method for the determination of sodium, *J. Biol. Chem.* **110**: 95, 1935.
14. Shohl, A. T., and Bennett, A. B.: A micro method for the determination of potassium as iodoplatinate, *J. Biol. Chem.* **78**: 643, 1928.
15. Booker, W. M.; Hayes, R. L., and Dent, F. M.: Hypercholesterolemia as a possible adrenal response to ascorbic acid administration, *Fed. Proc.* **10**: 17, 1951.

In this connection, however, it is significant that the Addisonian patient and adrenalectomized animals show no significant increase in cholesterol following ascorbic acid administration. Nor does the administration of adrenal steroids, in the absence of the adrenal glands, result in an increased level of serum cholesterol (15). If our thesis is correct, that the increase in serum cholesterol concentration is concomitant with increased storage of ascorbic acid in the adrenal gland, it may be tenable that patients during treatment with cortisone are storing administered ascorbic acid in the adrenals in contradistinction to their response during the pre-treated periods. We have shown (1), as have Schaffenburg, Masson and Corcoran (7), that cortisone increases adrenal ascorbic acid storage in experimental animals. The role of the endogenous adrenocortical hormone in ascorbic acid and cholesterol metabolism, however, is not clear cut. It is well known that, following ACTH administration and during stress, the cholesterol and ascorbic acid content of the adrenal decreases. During such conditions, it is thought that adrenocortical activity is increased. We have called attention (3) to experiments in which the fall in adrenal ascorbic acid and cholesterol could be blocked by the administration of adrenocortical hormones. Also we have pointed out the increased deposition of ascorbic acid in the adrenal glands of animals receiving ascorbic acid and adrenocortical extract, compared with the deposition in animals receiving only ascorbic acid. It is understandable, therefore, that patients under cortisone therapy should excrete less ascorbic acid than before. We are not clear, however, as to the role ascorbic acid may be playing in the formation of adrenocortical hormones or its influence on the functional state of the adrenal gland. Schaffenburg, Masson and Corcoran (6) have mentioned that the adrenal gland in scurvy, in experimental arthritis and in Addison's disease is depleted of its ascorbic acid, and is therefore deficient in glucosteroid. If this view is correct, ascorbic acid may play an outstanding part in the formation of the adrenocortical hormone and in the state of function of the adrenal gland.

SUMMARY AND CONCLUSION

1. Experiments are described showing the influence of cortisone on ascorbic acid metabolism in patients.

2. When ascorbic acid is given intravenously during cortisone therapy, there is a greater increase in the level of blood cell and plasma ascorbic acid and the levels remain increased longer than in the same patients prior to cortisone therapy. The increase in the blood cell value is particularly noteworthy.

3. When ascorbic acid is given intravenously during cortisone therapy, there is decreased urinary excretion of ascorbic acid in spite of increased

ascorbic acid in the first, second and third hours. It is significant that the percentage increase was greater than that of normal subjects at all time intervals. The increase in tolerance to ascorbic acid during cortisone treatment is again shown in Figure 3. During cortisone treatment the urinary output of ascorbic acid was far less than in the same patients before cortisone was administered. In the first hour the pre-treated cortisone patients excreted approximately 150 per cent more ascorbic acid than did the same patients during cortisone treatment and approximately 250 per cent more than did the normal subjects. Note that in the second hour, individuals in all categories showed a decreased output, but in the third hour the patients undergoing cortisone treatment showed an excretion which was decreased far below that of normal subjects. The changes in the level of serum cholesterol following ascorbic acid administration in the pre-treated and treated periods can be seen in Figure 4. It is interesting that prior to cortisone treatment the cholesterol did not change in the first hour following ascorbic acid administration, but decreased as much as 42 and 20 per cent in the second and third hours, respectively; whereas the same patients during cortisone therapy showed an increase of approximately 33 per cent in serum cholesterol in the first hour, followed by decreases, of 21 and 5 per cent in the second and third hours, respectively. The statistical significance of the data presented in Figures 1, 2, 3 and 4 is expressed in the legends for the figures.

DISCUSSION

The results of these studies are in agreement with experimental observations made in these laboratories that adrenal steroids reduce the urinary excretion of ascorbic acid and cause higher blood levels to be built up, particularly in the blood cells. The increase in cell content following the administration of ascorbic acid during cortisone therapy is outstanding, and favors the view that cortisone increases cell permeability. Increase in the kidney threshold to ascorbic acid may explain the increase of ascorbic acid in the plasma concomitant with reduced excretion in the urine.

The meaning of the changes in the level of blood cholesterol before and during cortisone treatment is not clearly defined at this time. We postulate that the normal functioning adrenal gland stores ascorbic acid upon administration to the organism; this storage may be increased by exogenous hormones. There is evidence to show that as the adrenal ascorbic acid increases, the adrenal cholesterol decreases (1). We believe, therefore, that the elevated serum cholesterol may represent, in part, cholesterol which has been released by the adrenal gland. Conclusion on this point will have to await attempts to calculate the actual amount of cholesterol that may be leaving the gland following ascorbic acid administration.

plasma ascorbic acid in the first hour, although in the second hour the value decreased 18 per cent and in the third hour, 32 per cent. The change in the tolerance to ascorbic acid and the influence of cortisone on the permeability of the blood cells to ascorbic acid can be seen in Figure 2. Note the lack of increase in cell ascorbic acid in the first hour after ascorbic acid was administered to patients prior to cortisone treatment, and the very slight increase in the second and third hours; whereas the same patients during cortisone treatment showed tremendous increases in cell

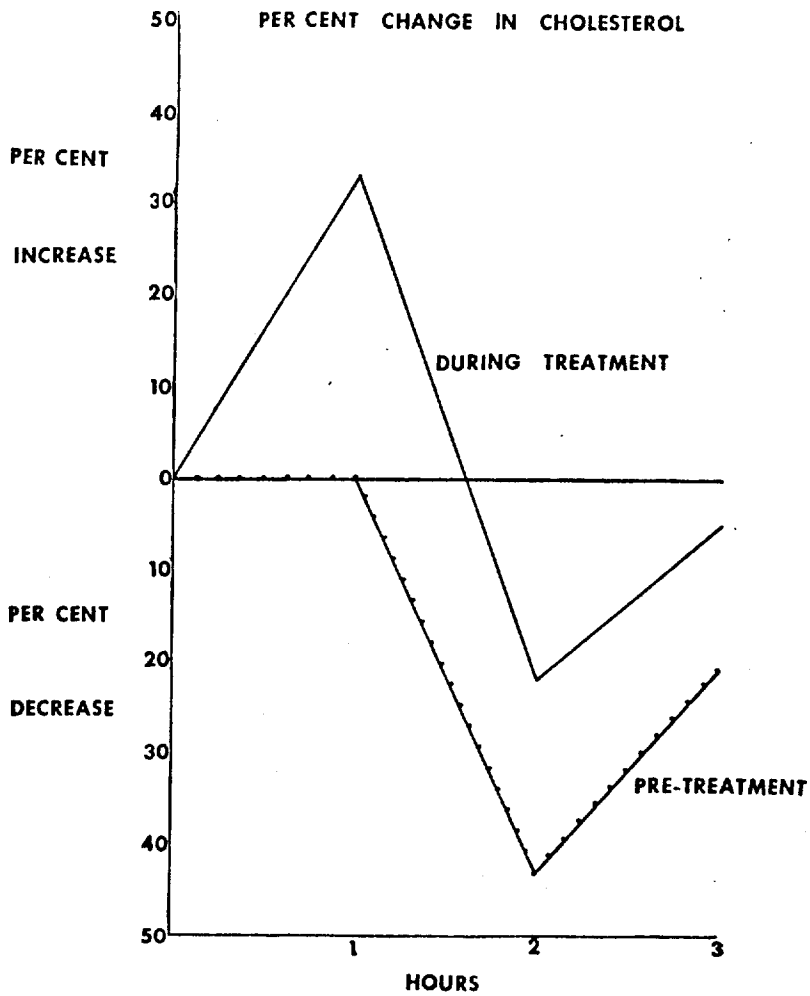


FIG. 4. Showing the mean percentage change in the total serum cholesterol in 20 patients before and during cortisone therapy, following administration of ascorbic acid intravenously.

P.= <.01 (pre-treated and treated periods compared at first hour.)

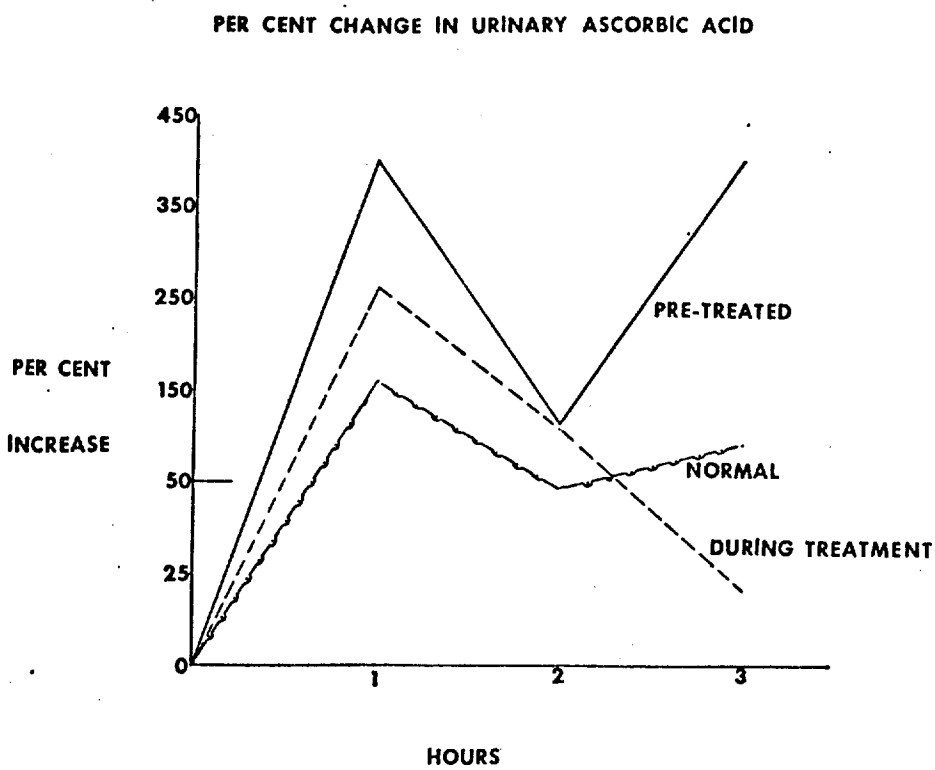


FIG. 3. Showing the mean percentage change in the urinary excretion of ascorbic acid in 20 patients before and during cortisone therapy, following administration of 300 mg. of ascorbic acid intravenously; and the results in 10 normal subjects following the same ascorbic acid dosage.

P = <.01 (pre-treated and treated periods compared at first hour.)

stances, a second test was made during the period in which cortisone was administered.

RESULTS

Figure 1 shows the percentage changes in plasma ascorbic acid following its administration to normal subjects, to patients before cortisone treatment and to the same patients during cortisone treatment. Note the great increase in the level of plasma ascorbic acid in the normal subjects in the first and second hours and the return almost to the initial level by the third hour. On the other hand, in the patients before cortisone treatment, the plasma ascorbic acid showed a decrease in the first hour, returned to the initial level in the second hour and decreased again in the third hour. During cortisone treatment, the ascorbic acid tolerance curve changed toward that of normal subjects as shown by the 20 per cent increase in

the method of Mindlin and Butler (11). Urinary ascorbic acid was determined by titration with standard 2, 6 dichloroindophenol. Serum cholesterol concentration was determined by the method of Bloor *et al.* (12), blood chlorides by the well known procedure of silver nitrate precipitation and titration against standard potassium thiocyanide, blood sodium by the Weinback modification of the Butler and Tuthill method (13), and potassium by the method of Shohl and Bennett (14).

Following one to ten days of continuous cortisone therapy, beginning the dose at 300 mg. daily and reducing it to 50–75 mg. daily, the ascorbic acid tolerance and blood chemistry changes were again studied under the identical conditions described for the pre-cortisone period. In some in-

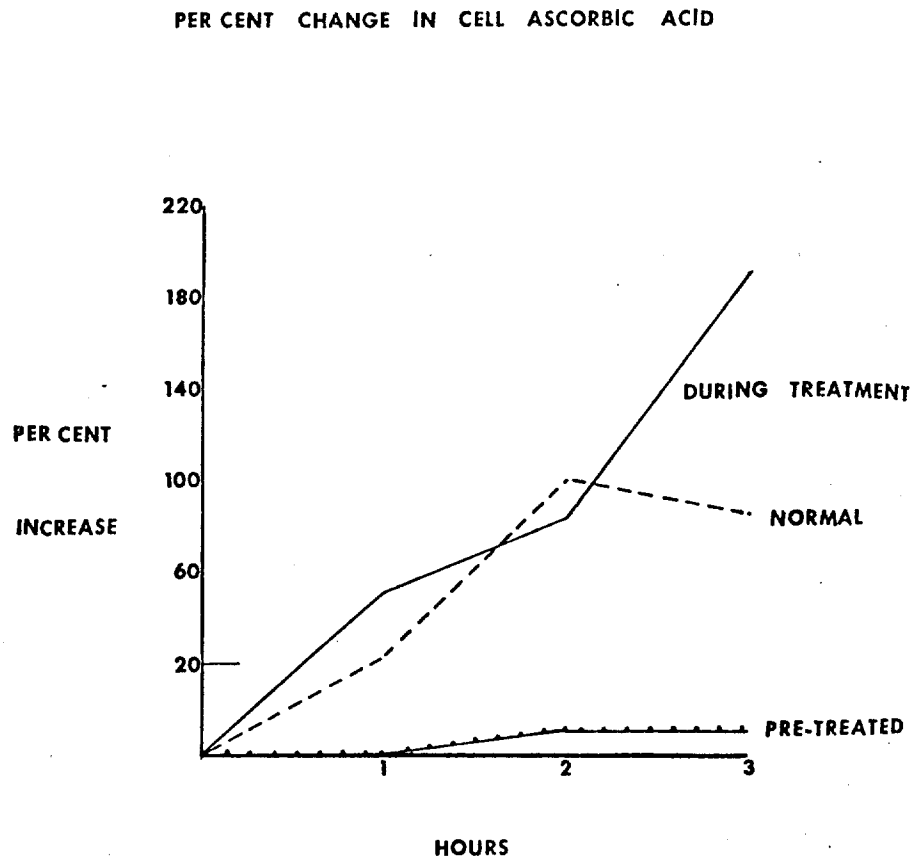


FIG. 2. Showing the mean percentage change in the blood cell ascorbic acid in 20 patients before and during cortisone therapy, following administration of 300 mg. of ascorbic acid intravenously; and the results in 10 normal subjects, following the same ascorbic acid dosage.

P = <.01 (pre-treated and treated periods compared at first hour.)

and during treatment with cortisone may well serve as a means to shed more light on the question of the role of ascorbic acid in adrenocortical hormone formation and function; hence the importance of this work.

METHODS

Observations were made on a group of 30 patients. This group included patients with rheumatoid arthritis, chronic ulcerative colitis, psoriasis, scleroderma, disseminated lupus erythematosus, leukemia and drug sensitivity.

Before cortisone treatment all patients were arranged for a study of tolerance and of blood chemistry changes following ascorbic acid administration. A blood sample was drawn and urine was collected with the patient in a fasting state, following which 300 mg. of ascorbic acid was administrated intravenously. Blood and urine were collected hourly thereafter for three hours. Ascorbic acid was determined on plasma and cells, using

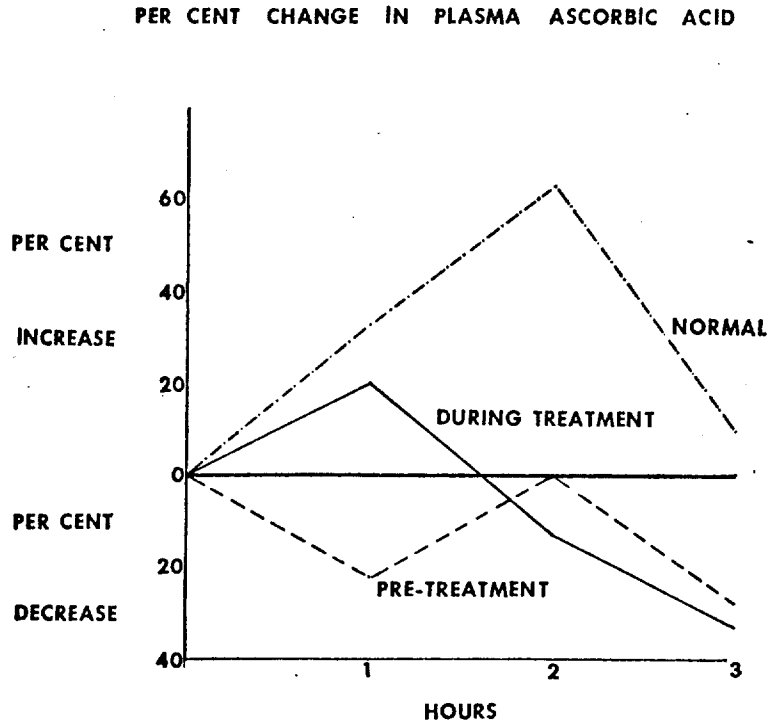


FIG. 1. Showing the mean percentage change of the plasma ascorbic acid of 20 patients before and during cortisone therapy, following the intravenous administration of 300 mg. of ascorbic acid Also shown is the mean percentage change of plasma ascorbic acid in 10 normal subjects, following administration of 300 mg. of ascorbic acid intravenously. P = <.01 (pre-treated and treated periods compared at first hour.)

J. Clin. Endocrinol. Metab. 12(3): 346-353 1952

0994

EFFECT OF CORTISONE ON TOLERANCE TO ASCORBIC ACID AND LEVEL OF BLOOD CHOLESTEROL IN MAN*†

WALTER M. BOOKER, PH.D., JOHN B. JOHNSON, M.D., RAYMOND L. HAYES, D.D.S., RILEY F. THOMAS, M.D., WALTER HENRY, M.D., JAMES R. TUREMAN, M.D. AND FRANCES DENT, B.S.

From the Departments of Pharmacology and Medicine, College of Medicine, and the Department of Oral Medicine, College of Dentistry, Howard University, Washington, D. C.

INTRODUCTION

EXPERIMENTAL studies with animals (1, 2, 3) as well as clinical studies have been reported, which suggest a relationship between ascorbic acid metabolism and adrenocortical hormones. The therapeutic use of ascorbic acid in large doses and of ascorbic acid in conjunction with adrenocortical hormone administration has been proposed in the treatment of collagenous diseases (4, 5, 6, 7). Our own experimental observations and those of others on the diminution of ascorbic acid excretion following adrenocortical hormone administration provoked our curiosity as to what might occur in the various patients during cortisone therapy. Selye (8) postulates that a major factor in the pathogenesis of certain diseases, now treated with cortisone, is failure of the particular organism to adapt to stress. It has been shown repeatedly that during stress adrenal ascorbic acid and cholesterol decrease (Sayers, 9). In a previous report (10), we have suggested that the increase in serum cholesterol following ascorbic acid administration may represent adrenal gland release of cholesterol concomitant with storage of ascorbic acid in the gland, although it is questionable whether the hypercholesterolemia is due entirely to adrenal gland response.

A comparison of the changes in the ability to handle ascorbic acid and in the patterns of cholesterol response to ascorbic acid in patients before

Received for publication June 18, 1951.

* An abstract of this work was presented before the Society for Pharmacology and Experimental Therapeutics, Fall Meeting, November 14–16, 1950, Boston, Massachusetts.

† This work was supported in part by a grant-in-aid from the U. S. Public Health Service to the College of Dentistry, and by the Food and Drug Administration under a project agreement with the Department of Medicine, Howard University, and Freedmen's Hospital.

0967

GIBSON, Q. H., and HARRISON, D. C. (1947). Familial idiopathic methaemoglobinaemia, five cases in one family. *Lancet* **2**, 941–943.

GOLDSTEIN, G. M., and DOULL, J. (1970). Effect of hyperbaric oxygen on *p*-aminopropiophenone and sodium nitrite-induced methemoglobinemia. *Pharmacologist* **12**, 242.

GRAYBIEL, A., LILIENTHAL, J. L., JR., and RILEY, R. L. (1945). The report of a case of idiopathic congenital (and probably familial) methemoglobinemia. *Bull. Johns Hopkins Hosp.* **76**, 155–162.

HORNIG, D., WEBER, F., and WISS, O. (1971). Uptake and release of [1-^{14}C]ascorbic acid and [1-^{14}C]dehydroascorbic acid by erythrocytes of guinea pigs. *Clin. Chem. Acta* **31**, 25–35.

JACOB, H. S., and JANDL, J. H. (1966). A simple visual screening test for glucose-6-phosphate dehydrogenase deficiency employing ascorbate and cyanide. *N. Engl. J. Med.* **274**, 1162–1167.

JAFFÉ, E. R. (1959). The reduction of methemoglobin in human erythrocytes incubated with purine nucleosides. *J. Clin. Invest.* **38**, 1555–1563.

JAFFÉ, E. R. (1966). Hereditary methemoglobinemias associated with abnormalities in the metabolism of erythrocytes. *Amer. J. Med.* **41**, 786–798.

JAFFÉ, E. R., and NEUMANN, G. (1964). A comparison of the effect of menadione, methylene blue and ascorbic acid on the reduction of methaemoglobin in vivo. *Nature (London)* **202**, 607–608.

KILGORE, L., STASCH, A. R., and BARRENTINE, B. F. (1964). Relation of ascorbic acid to nitrate content of turnip greens and to methemoglobin formation. *Amer. J. Clin. Nutr.* **14**, 52–55.

KING, E. J., WHITE, J. C., and GILCHRIST, M. (1947). A case of idiopathic methaemoglobinaemia treated by ascorbic acid and methylene blue. *J. Pathol. Bacteriol.* **59**, 181–188.

KOCIBA, R. J., and SLEIGHT, S. D. (1970). Nitrite toxicosis in the ascorbic acid-deficient guinea pig. *Toxicol. Appl. Pharmacol.* **16**, 424–429.

LAYNE, W. R., and SMITH, R. P. (1969). Methylene blue uptake and the reversal of chemically induced methemoglobinemias in human erythrocytes. *J. Pharmacol. Exp. Ther.* **165**, 36–44.

NASON, A., and EVANS, H. J. (1955). Nitrate reductase from *Neurospora*. *Methods Enzymol.* **2**, 411–415.

SCHAFFERT, R. R., and KINGSLEY, G. R. (1955). A rapid simple method for the determination of reduced, dehydro- and total ascorbic acid in biological materials. *J. Biol. Chem.* **212**, 69–68.

SCHOW, S. R. (1966). A serial study of ascorbic acid depletion in the guinea pig. *J. Dent. Res.* **45**, 1232.

SIEVERS, R. F., and RYON, J. B. (1945). Congenital idiopathic methemoglobinemia, favorable response to ascorbic acid therapy. *Arch. Intern. Med.* **76**, 299–307.

SMITH, R. P. (1967a). The oxygen and sulfide binding characteristics of hemoglobins generated from methemoglobin by two erythrocytic systems. *Mol. Pharmacol.* **3**, 378–385.

SMITH, R. P. (1967b). The nitrite methemoglobin complex—its significance in methemoglobin analyses and its possible role in methemoglobinemia. *Biochem. Pharmacol.* **16**, 1655–1664.

SMITH, R. P., and RUSSELL, J. T. (1969). A hemoglobin variant induced by methylene blue and low oxygen tensions. *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **28**, 479.

STOEWSAND, G. S. (1970). Influence of sex and dietary ascorbic acid on nitrite-induced methemoglobinemia in Japanese quail. *Proc. Soc. Exp. Biol. Med.* **133**, 1166–1168.

TERUUCHI, J., and MOCHIZUKI, H. (1959). Relationship between blood level and urinary excretion of vitamin C. *J. Vitaminol.* **5**, 223–228.

WAISMAN, H. A., BAIN, J. A., RICHMOND, J. B., and MUNSEY, F. A. (1952). Laboratory and clinical studies in congenital methemoglobinemia. *Pediatrics* **10**, 293–305.

of congenital methemoglobinemia the net effects of ascorbate would weigh heavily on the severity of the enzymatic deficiency. Thus, lower ascorbate concentrations might well have dramatic effects, and our results are not in conflict with clinical experience. In otherwise normal individuals with an acute chemically induced methemoglobinemia ascorbate offers little advantage over spontaneous methemoglobin reductase activity except perhaps in the case of hydroxylamine.

Although it is almost inconceivable that a blood ascorbate concentration of 5 mM could ever be achieved in a human subject, Tables 1 and 6 contain results which suggest adverse effects on the red cell. In guinea pig red cells 5.0 mM ascorbate seems to promote methemoglobin formation (Table 1). When one compares methemoglobin levels and oxygen capacities for 0.5 and 5.0 mM ascorbate in human cells at 24 and 36 hr (Table 6) the oxygen capacity at the higher ascorbate level is no better than for the lower ascorbate level even though there is a large difference in the amounts of methemoglobin present. This observation suggests the presence of a pool of inert pigment (sulfhemoglobin, choleglobin, etc. (Jacob and Jandl, 1966) with the higher ascorbate concentration. Indeed, continued incubation through 48 hr resulted in such damage to hemoglobin that turbidity in solutions precluded the spectrophotometric determination of methemoglobin.

A different phenomenon, however, is implicit in the data of Table 6 where recovery of oxygen capacity after 12 hr of incubation with 5.0 mM ascorbate appears to lag behind methemoglobin reduction. Because the oxygen capacity continues to improve over the next 12 hr (without additional methemoglobin reduction) this deficit cannot be ascribed to "sulfhemoglobin" which would represent an irreversible pool of inert pigment. Nor, can it be ascribed to deoxyhemoglobin since these preparations were thoroughly equilibrated with air prior to the oxygen capacity measurements. A similar phenomenon has been observed with methylene blue reduction of methemoglobin (Smith, 1967a). It has been tentatively ascribed to the presence of an unknown intermediate between methemoglobin and oxyhemoglobin which reacts abnormally with ferricyanide to consume oxygen (Smith and Russell, 1969). It is noteworthy that oxygen-consuming processes are associated with the nitrite- and the hydroxylamine-oxyhemoglobin reactions (Cranston and Smith, 1971).

REFERENCES

BARCROFT, H., GIBSON, Q. H., HARRISON, D. C., and MCMURRAY, J. (1945). Familial idiopathic methaemoglobinemia and its treatment with ascorbic acid. *Clin. Sci.* **5**, 145–157.

BODANSKY, O., and GUTMANN, H. (1947). Treatment of methemoglobinemia. *J. Pharmacol. Exp. Ther.* **90**, 46–56.

BRAY, G. D. (1960). A simple efficient scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* **1**, 279–285.

CARNRICK, M., POLIS, B. D., and KLEIN, T. (1946). Methemoglobinemia, treatment with ascorbic acid. *Arch. Intern. Med.* **78**, 296–302.

CRANSTON, R. D., and SMITH, R. P. (1971). Some aspects of the reactions between hydroxylamine and hemoglobin derivatives. *J. Pharmacol. Exp. Ther.* **177**, 440–446.

DEENY, J., MURDOCK, E. T., and ROGAN, J. J. (1943). Familial iodiopathic methaemoglobinemia with a note on the treatment of two cases with ascorbic acid. *Brit. Med. J.* **1**, 721–723.

EDER, H. A., FINCH, C., and MCKEE, R. W. (1949). Congenital methemoglobinemia. A clinical and biochemical study of a case. *J. Clin. Invest.* **28**, 265–272.

DISCUSSION

From the results of Tables 1 and 2 it is concluded that ascorbic acid per se plays no physiological role either in nitrite inactivation or in methemoglobin reduction in the guinea pig. Several possibilities remain to be explored in attempts to reconcile results presented here with those of Kociba and Sleight (1970). A sex difference may be responsible; only male guinea pigs were used in our studies. Kociba and Sleight (1970) observed a difference between scorbutic and control pregnant females, but not in fetuses apparently of either sex from these dams. In an additional key series of their experiments sex was not specified. Our control animals were supplemented with ascorbic acid whereas those of Kociba and Sleight (1970) were supplemented with cabbage. It is possible that cabbage may contain unknown factors which inactivate nitrite, reduce methemoglobin, or indirectly retard the absorption of injected chemicals from sc depots.

In contrast to results obtained with guinea pig red cells (Table 1), a high concentration of ascorbate under identical conditions did significantly attenuate the methemoglobinemic response of human red cells to sodium nitrite (Table 5). Although ascorbate inactivates nitrite, this effect within the time scale of the observation is more likely due to direct methemoglobin reduction (Jaffé, 1959) than to nitrite destruction (Table 4). The data, therefore, imply at least a modest species difference between human and guinea pig red cells. The guinea pig erythrocyte is permeable in both directions to ascorbate and to dehydroascorbate, and no differences in permeability exist between red cells from control and from scorbutic animals (Hornig *et al.*, 1971). Differences in routes or rates of intraerythrocytic biotransformation of ascorbate, or in the mechanisms or kinetics of the ascorbate-methemoglobin reactions may, therefore, play roles.

Differences were also encountered in the effects of ascorbate when tested in combination with various methemoglobin-generating chemicals. (Table 5.) When evaluated on the basis of the percent of the total methemoglobin reduced with and without ascorbate, 5.0 mM ascorbic acid was more effective in the case of hydroxylamine than in the case of either nitrite or phenylhydroxylamine. It must be noted, however, that a direct reaction between hydroxylamine and ascorbate has not been ruled out, and that control methemoglobin levels in the case of hydroxylamine were lower than with the other agents. The observation is of some interest because hydroxylamine methemoglobinemia is resistant to reversal by methylene blue (Layne and Smith, 1969). Moreover, if phenylhydroxylamine is an accurate model for methemoglobin formation by *p*-aminopropiophenone, it is significant that the former is refractory to ascorbate whereas the latter is exacerbated by hyperbaric oxygen (Goldstein and Doull, 1970). Thus, a spectrum of efficacy may eventually emerge among various combinations of methylene blue, hyperbaric oxygen, and ascorbate in the management of different chemical methemoglobinemias.

In examining a number of human cases of congenital methemoglobinemia (NADH-methemoglobin reductase deficiency) successfully treated with ascorbate (Barcroft *et al.*, 1945; Carnrick *et al.*, 1946; Deeny *et al.*, 1943; King *et al.*, 1947; Sievers and Ryon, 1945; Waisman *et al.*, 1952), whole blood ascorbic acid levels seldom exceeded 2 mg/100 ml) during active methemoglobin reversal. As superimposed on spontaneous methemoglobin reductase activity, we observed a significant effect of 0.5 mM ascorbate after 12 hr (Table 6), but not with 0.05 mM ascorbate even after 36 hr. The latter concentration more nearly approximates blood ascorbate levels in the general population. In any given case

62% increase in oxygen capacity. It had previously been determined that ascorbate per se did not interfere in oxygen capacity determinations.

TABLE 6

CHANGES IN METHEMOGLOBIN LEVEL AND OXYGEN CAPACITY WITH TIME OF NITRITED HUMAN RED CELL SUSPENSIONS INCUBATED WITH AND WITHOUT ASCORBIC ACID[a]

Ascorbate (mM)	Percent methemoglobin		Oxygen capacity (μl)	
	Control	$NaNO_2$	Control	$NaNO_2$
		$t = 0$		
0.0	3.7 ± 2.2	98.2 ± 3.1	(100)	(0)
		$t = 12$ hr		
0.0	9.3 ± 1.9	80.2 ± 17.4	91.9 ± 11.6	8.9 ± 2.8
0.05	8.9 ± 0.9	96.3 ± 5.5	91.9 ± 2.9	8.6 ± 1.3
0.5	4.3 ± 0.7	64.4 ± 23.1[b]	94.7 ± 6.9	35.6 ± 4.3[d]
5.0	4.4 ± 1.9	1.6 ± 1.5[c]	102.9 ± 5.9	61.7 ± 2.9[d]
		$t = 24$ hr		
0.0	7.3 ± 0.8	55.5 ± 2.7	80.2 ± 11.1	22.1 ± 8.9
0.05	6.7 ± 0.6	47.0 ± 6.3	74.0 ± 31.5	22.8 ± 11.8
0.5	5.5 ± 1.3	27.7 ± 6.0[d]	86.0 ± 8.2	70.3 ± 2.1[c]
5.0	0.9 ± 1.5[b]	2.0 ± 0.1[e]	79.0 ± 1.0	79.5 ± 8.9[b]
		$t = 36$ hr		
0.0	10.6 ± 3.8	30.0 ± 5.3	82.0 ± 8.9	53.3 ± 8.4
0.05	10.0 ± 4.7	31.4 ± 4.1	84.9 ± 6.1	53.8 ± 2.2
0.5	11.4 ± 3.6	17.1 ± 0.8	82.5 ± 2.6	75.9 ± 0.4[b]
5.0	5.4 ± 0.6	2.1 ± 2.3[b]	71.6 ± 0.2	67.9 ± 15.3

[a] Values are means ± SD for no less than 3 observations.
[b,c,d,e] Significantly different from values obtained without ascorbate in the same time interval, $p < 0.05$, 0.02, 0.01, or 0.001, respectively.

At 24 hr both 0.5 and 5.0 mM ascorbate continued to accelerate methemoglobin reduction significantly. The 70% decrease in methemoglobin with 0.5 mM ascorbate was paralleled by an approximately 70% increase in oxygen capacity. Although no further methemoglobin reduction could occur between 12 and 24 hr with 5.0 mM ascorbate, the oxygen capacity increased during this period. Thus, increases in oxygen capacity at high ascorbate levels appear to lag behind methemoglobin reduction. By 36 hr spontaneous methemoglobin reduction had caught up with reduction induced by 0.5 mM ascorbate but 5.0 mM ascorbate showed a continued effect. The oxygen capacity at 0.5 mM ascorbate continued to parallel methemoglobin reduction. Although the experimental errors are large, the oxygen capacity at 5.0 mM ascorbate is not significantly different from control preparations despite a significantly lower methemoglobin level.

Because differences are recognized among methemoglobinemias generated by various chemicals in terms of the response to methylene blue (Layne and Smith, 1969) and to hyperbaric oxygen (Goldstein and Doull, 1970), it appeared desirable to test ascorbate against other agents. For this purpose human red cell suspensions were employed as shown in Table 5. After 1 hr of incubation none of the tested concentrations of ascorbate alone led to significant methemoglobin accumulation. Indeed, in contrast to the results with guinea pig erythrocytes (Table 1) the 5.0 mM ascorbate concentration produced a significant attenuation of the methemoglobinemic response to sodium nitrite. From the 1 hr results with 5.0 mM ascorbate in Table 4 it seems unlikely that this effect can be ascribed to ascorbate destruction of nitrite. The effects of 5.0 mM ascorbate in a phenylhydroxylamine-methemoglobinemia were similar in magnitude to the case with nitrite. Although direct chemical inactivation of phenylhydroxylamine by ascorbate has not been ruled out, it also appears unlikely because phenylhydroxylamine was added at a 10-fold lower concentration than nitrite. A somewhat larger effect of ascorbate was observed in the case of hydroxylamine. With the latter, direct inactivation by ascorbate remains problematical.

TABLE 5

METHEMOGLOBIN LEVELS GENERATED 1 HR AFTER THE ADDITION OF $NaNO_2$ (2.5 mM), H_2NOH (2.5 mM) OR PHENYLHYDROXYLAMINE (0.25 mM) TO WASHED HUMAN RED CELL SUSPENSIONS INCUBATED WITH AND WITHOUT ASCORBIC ACID[a]

Ascorbate (mM)	Methemoglobin-Generating agent			
	None	$NaNO_2$	H_2NOH	$\phi NHOH$
	Percent methemoglobin			
0.0	2.5 ± 1.3	68.0 ± 5.4	42.3 ± 3.6	70.7 ± 3.2
0.05	2.8 ± 1.4	67.5 ± 4.6	46.3 ± 2.6	70.4 ± 5.3
0.5	1.2 ± 1.4	61.6 ± 7.7	43.4 ± 4.0	69.8 ± 2.2
5.0	1.7 ± 1.1	58.1 ± 3.0[b]	20.7 ± 3.3[c]	55.6 ± 2.1[b]

[a] Values are means ± SD for no less than 3 observations.
[b] Significantly different from levels obtained without ascorbate, $p < 0.01$.
[c] Significantly different from levels obtained without ascorbate, $p < 0.001$.

It seemed possible that the distinctly unphysiological levels of ascorbate used in the ·xperiments above might have adverse effects on the erythrocyte. We observed no evilence of gross hemolysis, but the presence of "sulfhemoglobin" (Jacob and Jandl, 1966) ›r other abnormal blood pigments was not ruled out. In the experiments of Table 6 ›uman red cells were first exposed to nitrite to generate high levels of methemoglobin. 'he cells were then washed with fresh media and incubated without or with ascorbate. 'hanges in oxygen capacity as well as methemoglobin were followed with time. After 2 hr of incubation both 0.5 and 5.0 mM ascorbate had produced a significant acceleraion of methemoglobin reduction as compared with control preparations without ıscorbate. This significant reductive activity was accompanied by significant increases ›n the oxygen capacity (Table 6). At 0.5 mM ascorbate a 34% decrease in methemoglobin was accompanied by an approximately 36% rise in oxygen capacity. Surprisingly, however, at 5.0 mM ascorbate a 96% decrease in methemoglobin was accompanied by only a

TABLE 3

RATE OF ABSORPTION OF RADIOACTIVITY AFTER SUBCUTANEOUS INJECTION OF TRACER AMOUNTS OF $KS^{14}CN$ IN GUINEA PIGS[a,b]

Observation	Control	Scorbutic	*P* value
Body weights (g)	458 ± 80	342 ± 75	<0.05
Blood ascorbate (mg 100/ml)	1.68 ± 0.60	0.60 ± 0.06	<0.01
	Counts/min per 100 μl plasma		
Minutes after $KS^{14}CN$			
5	187 ± 58	192 ± 92	NS
10	338 ± 104	289 ± 120	NS
15	432 ± 83	354 ± 102	NS
30	503 ± 51	478 ± 74	NS
60	478 ± 57	478 ± 52	NS
90	391, 383	386, 496	
120	399, 492	384, 436	

[a] Dose of $KS^{14}CN$ was 2 μCi and 0.5 μmole/kg.
[b] All reported values are means ± SD for no fewer than 3 and no more than 6 animals, except for paired observations on radioactivity at 90 and 120 min.

These results could be reconciled with the findings of Kociba and Sleight (1970) if one postulated that ascorbate was responsible for the maintenance of a factor in sc tissue (but not blood) which could inactivate nitrite. Using 10% homogenates of sc tissue and skeletal muscle from our injection sites in normal animals, however, we were unable to show a significant inactivation of added sodium nitrite. Nitrite was added in amounts equivalent to those injected into intact animals, and its presence was followed colorimetrically for 2 hr.

Ascorbate-nitrite is widely recognized as an example of chemical incompatibility. Therefore, the influence of ascorbic acid on nitrite stability was tested under our conditions (KRPG, 37 C, pH 7.4) as shown in Table 4. Only at 5.0 mM ascorbate and after 2 hr of incubation was a small, but statistically significant disappearance of nitrite observed.

TABLE 4

STABILITY OF $NaNO_2$ IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF ASCORBIC ACID[a]

Ascorbate (mM)	Minutes of incubation			
	30	60	90	120
	Assayed nitrite (mmole/l)			
0.0	2.23 ± 0.15	2.28 ± 0.14	2.65 ± 0.50	2.47 ± 0.11
0.05	2.29 ± 0.12	2.36 ± 0.11	2.42 ± 0.08	2.47 ± 0.18
0.5	2.29 ± 0.12	2.36 ± 0.11	2.36 ± 0.11	2.58 ± 0.00
5.0	1.91 ± 0.00	2.07 ± 0.03	2.00 ± 0.06	2.10 ± 0.02[b]

[a] Values are means ± SD for no fewer than 3 observations.
[b] Significantly different from levels obtained without ascorbate, $p < 0.02$.

TABLE 1

METHEMOGLOBIN LEVELS GENERATED BY THE ADDITION OF $NaNO_2$ (2.5 mM) TO WASHED GUINEA PIG RED CELL SUSPENSIONS INCUBATED WITH AND WITHOUT ASCORBIC ACID[a]

Ascorbate (mM)	Minutes of incubation			
	30	60	90	120
	Percent methemoglobin			
0.0	27.6 ± 1.8	22.2 ± 4.7	17.2 ± 3.5	15.0 ± 3.0
0.05	31.7 ± 3.5	22.2 ± 2.7	18.4 ± 2.5	12.5 ± 4.5
0.5	29.0 ± 1.9	22.2 ± 2.3	19.6 ± 3.7	15.0 ± 3.5
5.0	32.7 ± 4.1	24.8 ± 2.3	22.2 ± 2.5[b]	19.1 ± 3.6[c]

[a] Values are means ± SD for no fewer than 3 observations.
[b] Significantly different from levels obtained without ascorbate, $p < 0.05$.
[c] Significantly different from levels obtained without ascorbate, $p < 0.01$.

In contrast to the findings of Kociba and Sleight (1970) after sc nitrite, the results of Table 2 show that intra-arterial nitrite did not generate significantly higher levels of methemoglobin in scorbutic guinea pigs than in control animals. Although the mean body weight of the scorbutic guinea pigs was not significantly different from the control animals, a highly significant difference in blood ascorbate levels was observed. These blood ascorbate levels are comparable to those measured by Kociba and Sleight (1970) in the scorbutic (0.34 mg/100 ml) and control (0.88 mg/100 ml) groups.

TABLE 2

METHEMOGLOBIN LEVELS GENERATED BY INTRA-ARTERIAL $NaNO_2$, 40 MG/KG, IN CONTROL AND SCORBUTIC GUINEA PIGS[a]

Observation	Control	Scorbutic	*P* value
Body weights (g)	492 ± 35	470 ± 90	NS
Blood ascorbate (mg/100 ml)	1.78 ± 0.51	0.38 ± 0.09	<0.001
Minutes after $NaNO_2$	Percent methemoglobin		
0	0.78 ± 0.25	0.58 ± 0.26	NS
30	29.6 ± 8.5	23.4 ± 6.3	NS
60	21.2 ± 8.1	14.7 ± 4.7	NS
90	11.8 ± 5.8	7.3 ± 4.1	NS
120	5.1 ± 2.3	2.8 ± 1.0	NS

[a] All reported values are means ± SD for no fewer than 3 and no more than 6 animals.

To test for differences in absorption from sc depots between control and scorbutic animals, the experiments of Table 3 were performed. Instead, of nitrite, $KS^{14}CN$ was injected sc on the medial surface of a hind limb. The changes in radioactivity in arterial plasma were then followed for 2 hr. At none of the indicated sampling times was a significant difference encountered between control and scorbutic animals. With these groups significant differences in both mean body weight and blood ascorbate were found.

supplemented with ether. After tracheostomy, a carotid artery was cannulated with polyethylene tubing. Heparin 20 mg/100 ml in physiological saline was used periodically as a flush to keep the cannula patent. Control blood samples of 2.5 ml were collected in heparinized tubes for methemoglobin (Smith, 1967b) and ascorbic acid (Schaffert and Kingsley, 1955) analyses. A dose of 40 mg/kg sodium nitrite was then introduced into the cannula which was then rinsed with heparin-saline. At periodic intervals 0.5-ml blood samples for methemoglobin analyses were taken.

In a similar series of experiments nitrite was omitted, but tracer amounts (2 μCi and 0.5 μmole/kg) of $KS^{14}CN$ were injected sc on the medial surface of a hind limb. At various times blood samples were taken as above and centrifuged. Plasma (0.1 ml) was added to 15 ml of Bray (1960) solution for scintillation counting.

For in vitro experiments blood was obtained by cardiac puncture from guinea pigs maintained on a normal diet. The plasma and buffy coat were removed and the cells washed 3 times in Krebs-Ringer-phosphate-glucose (KRPG) media, pH 7.4. Incubation volumes were 3 ml made up from various combinations of 1 ml packed red cells, 1 ml stock ascorbate solution (0.15, 1.5, or 5.0 mM), 1 ml stock sodium nitrite (7.5 mM) or appropriate volumes of KRPG. All reagents were prepared in the latter media. Ascorbate solutions were added and mixed before nitrite addition. The final heme concentration was approximately 5.0 mM. These mixtures were incubated under air at 37°C in an oscillating shaker. Aliquots were periodically removed for methemoglobin analyses. In some experiments red cells were omitted and nitrite was determined colorimetrically by the method of Nason and Evans (1955).

Similar experiments were performed with human red cells originally drawn into K_3HEDTA. In addition to nitrite the effects of ascorbate were followed with hydroxylamine and phenylhydroxylamine-generated methemoglobinemias. The effects of ascorbate on methemoglobin reduction and restoration of oxygen capacity (Smith, 1967a) over extended periods of incubation were also tested in red cell suspensions previously made methemoglobinemic by exposure to nitrite. These cells were washed 3 times in fresh media before addition of ascorbate. Incubation media also contained 0.5 mg/ml sodium penicillin G and 0.5 mg/ml streptomycin. During these longer incubations the pH fell a full unit in all preparations. All statistical comparisons were by paired or independent samples *t* tests.

RESULTS

Table 1 summarizes the methemoglobin levels generated by the addition of a constant amount of sodium nitrite to suspensions of guinea pig red cells incubated without and with various concentrations of ascorbic acid. In the absence of ascorbate, a 28% level of nitrite-methemoglobin was reduced to 15% over 90 min, presumably as a result of spontaneous (NADH) methemoglobin reductase activity. Concentrations of 0.05 and 0.5 mM ascorbate were without effect on methemoglobin reduction. At 5.0 mM ascorbate significantly higher levels of methemoglobin were found at 90 and 120 min in comparison with the appropriate controls. This result would not have been predicted from the work of Kociba and Sleight (1970), but it is in accord with the findings of Kilgore *et al.* (1964). Such an effect could be due to an inhibition of spontaneous methemoglobin reductase activity or to actual methemoglobin formation by this high concentration of ascorbate (Jacob and Jandl, 1966).

the present investigation. The depletion of adrenal ascorbic acid is closely associated with the enhanced secretory function of the gland. However, the exact role of ascorbic acid during increased adrenocortical activity still remains to be defined.

The authors are grateful to the Ministry of Defence (R. & D. Organization), Government of India, New Delhi, and the Council of Scientific & Industrial Research, New Delhi, for providing financial assistance.

References

1. Roe, J. H. & Kuether, C. A., *J. biol. Chem.*, **147** (1943), 399.
2. Hanel, H. K. & Dam, H., *Acta chem. scand.*, **9** (1955), 677.
3. Birge (Jr), S. J. & Peck, W. A., *Biochem. biophys. Res. Commun.*, **22** (1966), 532.
4. Gero, E., *Biochim. biophys. Acta*, **92** (1964), 160.
5. Rajalakshmi, R., Mehta, T. & Subbulakshmi, G., *Indian J. exp. Biol.*, **2** (1964), 238.
6. Page, E. & Babinean, L. M., cited in *Biochemistry and physiology of nutrition*, Vol. I, edited by G. H. Bourne & G. W. Kidder (Academic Press Inc., New York), 1953.
7. Monier, M. M. & Weiss, R. J., *Proc. Soc. exp. Biol. Med.*, **80** (1952), 446.
8. Murphy, S. D., *Ann. N.Y. Acad. Sci.*, **160** (1969), 366.
9. Sayers, G., *Physiol. Rev.*, **30** (1950), 241.
10. Lahiri, S. & Llyod, B. B., *Biochem. J.*, **84** (1962), 478.
11. Yates, F. E., Leeman, S. E., Glenister, D. W. & Dallman, M. F., *Endocrinology*, **69** (1961), 67.
12. Yates, F. E. & Urquhart, J., *Physiol. Rev.*, **42** (1962), 359.
13. Pincus, G., cited in *The human adrenal cortex*, edited by A. H. Curie, T. Symington & T. K. Grant (E. & S. Livingstone Ltd, Edinburgh), 1962, 85.

concentrations by appropriate dose regimens of ascorbic acid (Jaffé, 1966). The effects of ascorbate in such cases, however, are much less dramatic and far more delayed than those of methylene blue (Jaffé and Neumann, 1964). In a number of reported cases (Barcroft *et al.*, 1945; Deeny *et al.*, 1943; Gibson and Harrison, 1947; Graybiel *et al.*, 1945; King *et al.*, 1947; Waisman *et al.*, 1952) distinct effects of ascorbate were delayed for as long as 3–5 days after the institution of therapy. This delay coincides with the time required to achieve maximal steady-state levels of ascorbate in blood (Teruuchi and Mochizuki, 1959) when it is given po.

Limited clinical and experimental experience indicates ascorbate is much less satisfactory for the management of acute chemically induced methemoglobinemias. Carnrick *et al.* (1946) treated a single case of nitrite poisoning with ascorbate. As measured "in the morning" a methemoglobin level of 3.9 g/100 ml blood was reduced to 1.9 g/100 ml by 6:30 PM after 2 iv injections of ascorbate totaling 700 mg. In contrast, Eder *et al.* (1949) generated 2.5 g/100 ml methemoglobin in a healthy individual with iv nitrite and found its half-life to be only 3 hr in the absence of any treatment. The half-life of a 60% *p*-aminopropiophenone-methemoglobinemia in dogs was 4 hr without treatment. Large iv doses of ascorbate (200 mg/kg) reduced the half-life by a factor of only 1 to 2 whereas 1 mg/kg methylene blue had a 6 to 11-fold effect (Bodansky and Gutmann, 1947).

Recently, Kociba and Sleight (1970) reported that sc sodium nitrite produced significantly higher levels of methemoglobin in ascorbic acid-deficient guinea pigs than in control animals. Several aspects of that report are troublesome. Control animals were supplemented with cabbage instead of ascorbic acid. Since the sodium nitrite was given sc, possible effects on its absorption should have been considered. When nitrite was given to pregnant guinea pigs, methemoglobin levels in scorbutic dams were also significantly higher than those in the control dams. In contrast, methemoglobin levels in the scorbutic fetuses were identical with those in the control fetuses. Finally, these results are not in accord with brief experiments reported by Kilgore *et al.* (1964). The latter investigators gave sodium nitrite by mouth to guinea pigs and found methemoglobin levels in the control animals (also supplemented with green leafy vegetables) to be the same as, or even slightly higher, than those in deficient animals. Supplementation with 0.5% ascorbate in the diet of Japanese quail had no effect on the methemoglobin levels generated by feeding 0.5% sodium nitrite (Stoewsand, 1970). Additional experimentation to resolve these apparent contradictions appeared desirable.

METHODS

Male English short hair guinea pigs (355–400 g) were obtained from a commercial source.[3] After several days on a normal diet and water, they were divided into 2 groups and both were given only pelleted ascorbic acid-deficient diet.[4] The control group was also given ascorbic acid solutions, 1 g/l, instead of water. Control and experimental animals were used alternately after periods of 3–5 wk (Schow, 1966). Animals were anesthetized with 30–60 mg/kg ip sodium pentobarbital. Anesthesia was sometimes

[3] Cam Research Institute, Inc., Wayne, New Jersey 07470.
[4] Nutritional Biochemicals Corporation, Cleveland, Ohio 44128.

0967

TOXICOL. APPL. PHARMACOL., 21: 176-85, 1972.

TOXICOLOGY AND APPLIED PHARMACOLOGY 21, 176-185 (1972)

Ascorbic Acid and Chemically Induced Methemoglobinemias[1]

JOHN Z. BOLYAI, ROGER P. SMITH,[2] AND CLARKE T. GRAY

Department of Pharmacology and Toxicology and Department of Microbiology, Dartmouth Medical School, Hanover, New Hampshire 03755

Received March 18, 1971

Ascorbic Acid and Chemically Induced Methemoglobinemias. BOLYAI, JOHN Z., SMITH, ROGER P., and GRAY, CLARKE T. (1972). *Toxicol. Appl. Pharmacol.* **21**, 176-185. Sodium nitrite was added to suspensions of washed guinea pig red cells in concentrations which converted about a third of the total blood pigment to methemoglobin. Various aliquots also contained added ascorbic acid in concentrations of 0.05, 0.5, or 5.0 mM ("physiological" levels are between 0.05 and 0.1 mM). Methemoglobin levels were followed for 2 hr at 37°C. None of the tested concentrations of ascorbate significantly attenuated methemoglobin formation. On the contrary, significantly higher methemoglobin levels were sometimes found with 5.0 mM ascorbate. Guinea pigs were maintained several weeks on a scorbutogenic diet with or without ascorbate supplementation in their water. When blood ascorbate levels were significantly different, both groups were given intra-arterial sodium nitrite, 40 mg/kg, and methemoglobin levels were followed with time as above. At none of the sampling times was there a significant difference in the methemoglobin levels between the 2 groups. As judged by the appearance of radioactivity in blood at various times after sc injection, the two groups also showed no difference in the rates at which they absorbed $KS^{14}CN$. In human red cell suspensions 5.0 mM ascorbate significantly attenuated the methemoglobinemic response to tested concentrations of sodium nitrite, hydroxylamine and phenylhydroxylamine at 1 hr, but lower concentrations of ascorbate were without effect. When human red cells were first exposed to nitrite, then washed and ascorbate added, significant acceleration of methemoglobin reduction was observed with 5.0 mM ascorbate at 12, 24, and 36 hr, and with 0.5 mM ascorbate at 12 and 24 hr. In contrast, the oxygen capacities of suspensions with 5.0 mM ascorbate were significantly higher than control only at 12 and 24 hr, whereas with 0.5 mM ascorbate they were significantly higher at 12, 24, and 36 hr. In plain buffer, 5.0 mM ascorbate produced a small but significant loss of the tested concentration of sodium nitrite within 2 hr, but not at shorter intervals or at lower ascorbate concentrations.

Abnormally high circulating levels of methemoglobin in human cases of NADH-methemoglobin reductase deficiency can be reduced and maintained near normal

[1] Supported by Grants AP-00260, HE-14127, and AI-05806. A preliminary report of this work was presented at the meeting of the Federation of American Societies for Experimental Biology, Chicago, Illinois, April, 1971: Abstract, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* 30, 221 (1971).

[2] Recipient of USPHS Research Career Program Award 1-K3-GM-31, 784 from the National Institutes of Health.

© 1972 by Academic Press, Inc.

0914

Bodansky, M. 1938
Introduction to Physiological Chemistry, 4th Edition
John Wiley and Sons, Inc., New York, pp. 594-604

0908

ive action of sulfhydryl compounds such as cysteine and glutathione on the autoxidation of ascorbic acid is well known[3]. A simple lowering by ascorbic acid of the oxidation–reduction potential of the medium enough to initiate growth appears unlikely due to the inability of cysteine to replace ascorbic acid. The greater than catalytic amounts of ascorbic acid required and the activity of the *d*-isomer rules out a growth-factor effect.

The significance of the present findings with respect to the induction of hepatomas in the rat by the feeding of azo-dye carcinogens is uncertain. However, DAB and other carcinogens are known to increase the ascorbic acid level in the liver of rats[4]. Furthermore, rats deficient in riboflavin have a reduced content of ascorbic acid in the tissues[5], and the induction of hepatomas by DAB is favored in riboflavin-deficient rats[6]. Also, it should be noted that certain carcinogenic hydrocarbons are potent in stimulating ascorbic acid biosynthesis as well as inducing the synthesis of several liver-microsomal enzymes (*e.g.*, azo-dye demethylase) which metabolize foreign compounds[7] implying that vitamin-C deficiency may decrease the activity of such enzymes.

This investigation was supported in part by Public Health Service Research Grant C-2869 from the National Cancer Institute to Dr. D. A. SALZBERG and in part by the Max Fleishmann Fund of the Palo Alto Medical Research Foundation. The technical assistance of Mrs. D. LIEBES is gratefully acknowledged.

Palo Alto Medical Research Foundation,
Palo Alto, Calif. (U.S.A.)

DOLORES BOBB

[1] D. BOBB, D. LIEBES AND D. A. SALZBERG, *J. Bacteriol.*, 84 (1962) 242.
[2] H. J. VOGEL, *Microbial Genet. Bull.*, 13 (1956) 42.
[3] W. E. KNOX, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 2, Academic Press, New York, 1960, p. 253.
[4] J. P. GREENSTEIN, *Biochemistry of Cancer*, 2nd edition, Academic Press, New York, 1954, p. 335.
[5] B. SURE, R. M. THEIS AND R. T. HARRELSON, *J. Biol. Chem.*, 129 (1939) 245.
[6] C. J. KENSLEP, K. SUGIURA, N. F. YOUNG, C. R. HALTER AND C. P. RHOADS, *Science*, 93 (1941) 308.
[7] A. H. CONNEY, G. A. BRAY, C. EVANS AND J. J. BURNS, *Ann. N.Y. Acad. Sci.*, 92 (1961) 115.

Received July 29th, 1963

against inhibition by the dye occurred with both strains if ascorbic acid was also present in the medium. The ascorbic acid effect was most easily seen with the higher concentrations of the dye. At a given dye concentration, increasing amounts of the vitamin produced progressively greater protective effects. There was no superimposed effect of ascorbic acid on the pH of the growing cultures. Beyond a molar ratio of vitamin/dye of about 100:1 no further protective effects were manifested. Growth curves showed that ascorbic acid reduced the lag period incurred with MeMAB. The medium and the mycelial mats retained the bright yellow color of the dye whether or not ascorbic acid was present.

In the above experiments the vitamin was filter sterilized and added aseptically to the medium after autoclaving. The same results were obtained when the vitamin was added to the medium prior to autoclaving for 10 min at 15 lb pressure.

TABLE II

EFFECT OF GSH AND CYSTEINE ON MeMAB INHIBITION

Figures represent 3-day growth in mg of strain 51602 on complete medium (5 μg B_2 per 20 ml).

MeMAB supplement (μg per 20 ml)	*Concentrations (m moles)*							
	GSH				*Cysteine*			
	0	*1.5*	*3*	*5*	*0*	*1*	*3*	*5*
None	48	50	49	46	59	59	57	33
50	49	52	44	38	48	50	41	22
100	10	10	17	22	3	1	2	2

The *l*-ascorbic acid could be replaced by the isomer, *d*-isoascorbic acid, as well as by dehydroascorbic acid. Cysteine was ineffective while GSH was slightly effective at very high concentrations (Table II). These compounds were all filter sterilized.

Furthermore, the ascorbic acid-protective effect was nullified in the presence of either cysteine or GSH (Table III). The growth inhibition of strain 51602 on complete medium by cysteine plus ascorbic acid, but not by either compound alone at these concentrations, cannot be explained. On medium containing 75 μg MeMAB per 20 ml, 1.5 mM cysteine gave added growth inhibition.

The protection offered by ascorbic acid in preventing the toxicity of MeMAB for *N. crassa* would appear to depend on the autoxidation of the vitamin. The protec-

TABLE III

PREVENTION OF ASCORBIC ACID EFFECT BY CYSTEINE AND GSH

Figures represent 3-days growth in mg of strain 51602 on complete medium (5 μg riboflavin per 20 ml).

MeMAB supplement (μg per 20 ml)	*Control*	*Concentrations (m moles)*				
		3.0 l-ascorbic acid	*1.5 cysteine*	*0.75 GSH*	*1.5 cysteine + 3.0 l-ascorbic acid*	*0.75 GSH + 3.0 l-ascorbic acid*
None	52	50	57	52	28	58
50	47	46	—	48	—	48
75	26	45	13	—	6	—
100	4	38	11	7	trace	3

0908

Biochim Biophys Acta 78: 795-7, 13 Dec '63

P. 1325

Ascorbic acid protection against toxicity of an azo dye carcinogen for *Neurospora crassa*

While riboflavin has been alleged to overcome azo-dye inhibition of growth of microorganisms (see BOBB *et al.*[1], for references), to our knowledge a direct effect by ascorbic acid has not previously been reported. The synergistic effect of methylene blue on azo-dye growth inhibition (unpublished observations) led us to test the capacity of an easily oxidizable substance to reverse the inhibition by the carcinogen. This report deals with the ability of ascorbic acid to overcome the growth inhibitory effect of MeMAB on *N. crassa*.

Growth experiments were performed in 125-ml erlenmeyer flasks containing 20 ml of medium N (ref. 2) which contained per l of solution: 2.5 g sodium citrate, 5.0 g KH_2PO_4, 2.0 g NH_4NO_3, 0.2 g $MgSO_4 \cdot 7\ H_2O$, 0.1 g $CaCl_2 \cdot 2\ H_2O$, 5 mg biotin, 20 g sucrose, and trace elements; pH 5.8. For growth of the riboflavin-deficient strain 51602, 5 μg of riboflavin per 20 ml was included. After inoculation with a standardized conidial suspension, duplicate cultures were incubated at 31° without shaking. Growth was determined by drying the mycelial mats to constant weight and averaging the duplicate values.

As reported previously, all Neurospora strains tested were inhibited in their growth by the presence of the azo-dye carcinogen MeMAB, inhibition being manifested primarily by a prolonged lag phase rather than by decreased growth rate or growth capacity[1]. The wild-type strain STA was relatively resistant and the riboflavin-deficient strain 51602 was the most sensitive to azo-dye inhibition.

TABLE I

THE ABILITY OF *l*-ASCORBIC ACID TO REVERSE THE GROWTH INHIBITORY EFFECTS OF MeMAB ON *N. crassa*

Concentrations of medium constituents are given in μg per 20 ml. The figures represent weight of mycelial mats in mg.

Strain	*Days of growth*	*Additions to basal medium*	*Ascorbic acid (m moles)*			
			0	*1*	*2*	*3*
51602	3	5 B_2	53	56	53	52
		5 B_2 + 50 MeMAB	48	47	50	51
		5 B_2 + 75 MeMAB	28	32	39	36
		5 B_2 + 100 MeMAB	3	10	39	43
			0	1.5	3	
STA	2	None	61	58	59	
		100 MeMAB	47	63	64	
		125 MeMAB	22	50	58	

As shown in a representative experiment (Table I), *l*-ascorbic acid *per se* did not affect the growth of either strain on a complete medium. Growth of both strains was progressively inhibited by increasing amounts of MeMAB. A definite protection

Abbreviations: MeMAB, 3′-methyl-4-monomethylaminoazobenzene; DAB, 4-dimethylaminoazobenzene.