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VOLUME 5

GRAS MONOGRAPH SERIES

ASCORBIC ACID

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prepared for

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

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RESULTS

Tables 1 and 2 show that the administration of high doses of ascorbic acid for 4 days increases the 11-oxysteroid excretion by an average of 110·4 % and decreases the 17-ketosteroid excretion by an average of 35·8 %.

Table 1. *The difference between the 11-oxysteroid content of urine collected during the day preceding the administration of ascorbic acid and that of the fourth day following a daily intake of 4 g.*

Case	Age	Sex	Excretion in urine of 11-oxysteroids (mg./24 hr.) Before administration of ascorbic acid	After administration of ascorbic acid	Increase (%)
A. K.	29	M.	0·468	0·687	46·8
S. K.	30	M.	0·670	1·280	91·0
F. B.	31	M.	0·328	0·644	96·3
I. G.	29	M.	0·441	0·850	92·8
A. A.	30	M.	Undetectable	0·354	—
N. T.	29	M.	Undetectable	0·086	—
A. G.	30	M.	0·176	0·593	238·8
V. O.	29	M.	0·386	0·762	97·4
S. K.	29	M.	0·160	0·311	94·3
A. S.	33	F.	0·270	0·516	91·1
T. K.	38	F.	0·233	0·462	98·2

Table 2. *The difference between the 17-ketosteroid content of urine collected during the day preceding the administration of ascorbic acid and that of the fourth day following a daily intake of 4 g.*

Case	Age	Sex	Excretion in urine of 17-ketosteroids (mg./24 hr.) Before administration of ascorbic acid	After administration of ascorbic acid	Decrease (%)
A. K.	29	M.	18·94	10·12	46·6
S. K.	30	M.	23·63	14·64	37·2
F. B.	31	M.	24·32	14·38	40·9
I. G.	29	M.	19·65	11·60	43·0
A. A.	30	M.	20·34	13·96	31·7
N. T.	29	M.	28·70	20·73	27·8
A. G.	30	M.	27·22	18·53	32·0
V. O.	29	M.	21·77	14·62	37·5
A. S.	33	F.	8·12	5·75	30·0
T. K.	38	F.	7·16	4·14	42·3

DISCUSSION

The results suggest that ascorbic acid increases the production of gluco-corticoids in the adrenal cortex, and decreases the production of androgens. There are two possible ways in which these effects of ascorbic acid may be produced: (1) the effect of ascorbic acid is mediated by the pituitary gland like that of stress and adrenaline, and (2) ascorbic acid acts directly on the adrenal cortex. Adrenaline and stress act with the aid of ACTH secreted by the pituitary gland, and ACTH increases the excretion of 11-oxysteroids and 17-ketosteroids. Ascorbic acid, on the other hand,

increases only the excretion of the 11-oxysteroids and decreases the excretion of the 17-ketosteroids. Since the effect of ascorbic acid differs from that of ACTH, it is probably not mediated by the pituitary gland but is the result of a direct action on the adrenal cortex.

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FIVE-YEAR STORAGE OF FROZEN CONCENTRATED ORANGE JUICE AT -4°, 5°, AND 10° F.

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The purpose of this study was to ascertain the effects of different storage temperatures over an extended period on commercially packed frozen concentrated orange juice. Three years was initially chosen as the length of time for the test. The effects of −4°, 5°, 10°, 20°, and 35° F. storage temperatures on certain quality characteristics of several lots of frozen concentrated orange juice were given at the end of three years in a previous paper (3). The surprisingly good condition of the concentrates stored at −4° F. for three years prompted the continuation of the study.

This paper describes the time-temperature effects resulting from five-year storage at temperatures of −4°, 5°, and 10° F. on the respective qualities of four lots of frozen concentrated orange juice designated as H-51, L-51, O-51, and S-51.

EXPERIMENTAL

Four lots of commercially packed 42° Brix frozen concentrated orange juice were secured in June 1951 from the four largest producers in Florida. Sub-lots of each of the above lots were held in storage for five years at −4°, 5°, and 10° F. Details of the experimental procedures and observations made on these and other lots of concentrate are described in the paper previously mentioned (3). Cloud density measurements were made with the Evelyn photoelectric colorimeter.[2] Tests were also made to ascertain if reducing ions, such as ferrous or stannous, were present in sufficient amount to interfere with the dye titration (1). Samples were tasted by ten members of the laboratory staff. Each sample was presented singly and the tasters were asked to note departure from normal flavor and to indicate acceptability. When cans of concentrate were emptied they were promptly rinsed with water and the interior of the can examined visually for evidence of corrosion or lack of continuity of the enamel.

RESULTS AND DISCUSSION

The optical density measurements of cloud are shown on the graphs Figures 1, 2, and 3 for each of the four lots over the five-year storage period. Optical densities above .45 represent stable cloud; .45 to .15 partial separation; and below .15 complete separation.

At −4° F., as shown in Figure 1, stable cloud was maintained during the whole five years of storage in all samples.

[1]One of the laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

[2]The mention of trade products does not imply that they are preferred by the Department of Agriculture over similar products not mentioned.

At 5° F., as shown in Figure 2, stable cloud was maintained by all lots the first year. During the second year lots H-51 and L-51 showed partial separation and the other two lots retained stable cloud. During the third year lots H-51 and L-51 suffered complete separation. Lot O-51 followed a pattern of steady degradation of cloud and lot S-51 retained stable cloud. During the fourth and fifth year lots H-51 and L-51 showed complete separation, lot O-51 continued to degrade to almost complete separation, and lot S-51, however, retained stable cloud for the entire five-year storage at 5° F.

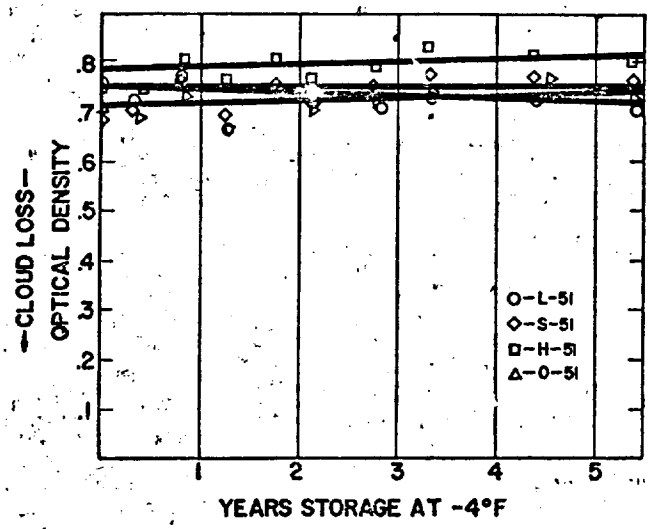

Figure. 1. Stability of cloud in samples stored at -4° F.

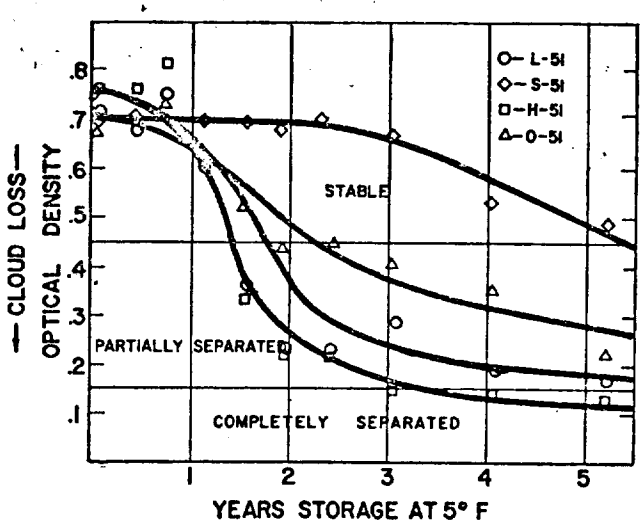

Figure 2. Stability of cloud in samples stored at 5° F.

At 10° F. storage as shown in Figure 3, changes in optical density were much more rapid in all lots. During the first year lots H-51 and L-51 almost reached complete separation, while lot O-51 reached partial separation. Only lot S-51 retained stable cloud. During the second year lot O-51 reached nearly complete separation and lot S-51 showed partial separation. By the end of the third year cloud separation was complete on all lots.

At —4° F. gelation was not observed in any sample stored five years. At 5° F. storage gel lumps were observed at 1½, 2, and 3 years in lots H-51, L-51, and O-51 respectively. Gel lumps never developed in lot S-51. At 10° F. gel lumps were observed in all lots between one and two years.

No measurable difference in ascorbic acid content was found in any lot between portions stored at temperatures of —4°, 5°, and 10° F., and the values found in the reconstituted concentrates after five years were within the normal range for fresh orange juice. It was ascertained by AOAC tests (1) that reducing ions, such as ferrous and stannous, were not present in such quantity as to interfere appreciably with the dye titration for ascorbic acid.

All samples stored at —4° F. for five years were without noticeable flavor deterioration. The flavors at the end of five

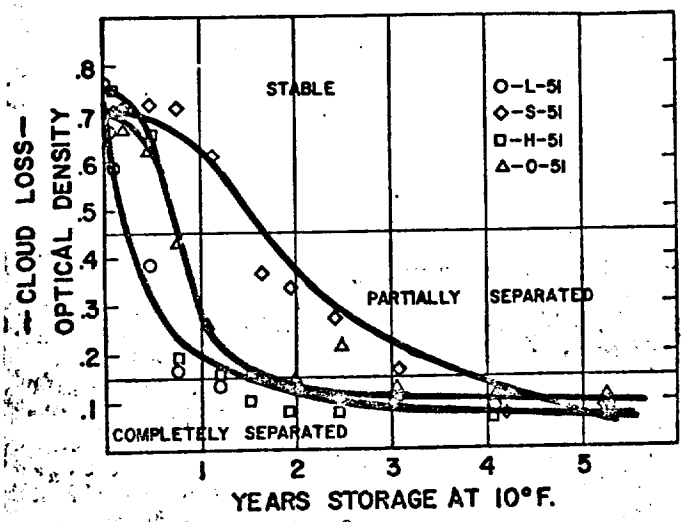

Figure 3. Stability of cloud in samples stored at 10° F.

years at —4° F. were considered to be like those of the same products when first obtained; the flavors were characteristic and no off-flavor was detected. At 5° F. after five-year storage all tasters agreed that samples of lot O-51 were good and that those of lot S-51 were definitely off-flavored (apricot or scorched). Opinion was divided on the flavor of two lots, H-51 and L-51; some tasters felt they were unacceptable, while others reported the flavor to be good. At 10° F. after three-year storage, samples of all four lots of concentrate were definitely off-flavor. These opinions were supported by the observations of changes in cloud density and the development of gelation.

Fully enameled cans were in good condition after five-year storage at —4°, 5°, and 10° F. Cans with plain tin ends which were held at —4° F. showed slight corrosion of the ends; corrosion was more severe at 5° and 10° F. storage temperatures. Cans with no interior enamel showed little damage at —4° and 5° F. and slight corrosion noted in these cans stored at 10° F. for five years.

Those interested in the mechanism of stability may be interested in the composition of the pectin fractions of these concentrates and its relation to gelation which was given in the paper by Huskin and Kew (2).

Below zero storage definitely preserved the quality of these products. It was pointed out in the report at the end of three years (3) that wide differences were found in cloud stability between the commercial lots tested. This variability in stability and the signal effects of sub-zero storage definitely indicate that for the retention of optimum quality, storage temperature should be maintained below zero.

Summary

Cloud remained unchanged during storage for five years at —4° F. in samples of commercial 42° Brix frozen concentrated orange juice. Loss of the cloud was observed in samples held at 5° F. and the rate of cloud loss varied widely. At 10° F. storage partial cloud loss was observed between ½ and 1½ years.

Gelation was not observed in samples stored at —4° F. for five years, but it did occur in three of the four samples stored at 5° F. At 10° F. all samples showed gelation.

Ascorbic acid was found to be very stable in frozen concentrated orange juice.

No off-flavor was detected in any lot of concentrate after five-year storage at —4° F. Flavor was impaired in portions stored at 5° F. and portions stored at 10° F. were unpalatable.

The containers were found to be in good condition after five-year storage, especially the cans with fully enamelled interiors and cans without interior enamel.

For the retention of optimum quality, storage temperatures should be maintained below zero.

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EFFECT OF PROCESSING ON THE VITAMIN C CONTENT OF FOODS[1]

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Although vitamin C is more sensitive to destruction in a general food supply than any other recognized, essential nutrient, it is possible to avoid serious loss of the vitamin during most processing steps if sufficient care is given to a few fundamental considerations. Practically all of the problems relating to destruction of the vitamin center on one type of reaction, oxidation. Furthermore, since the vitamin is not autoxidizable except in alkaline solution, and nearly all foodstuffs are either acid or neutral in reaction, the oxidative destruction is almost completely dependent upon two controllable factors—the presence of a catalyst and exposure to free oxygen (air).

The major aims relative to retention of vitamin C in all processing, then, should be first, to avoid all unnecessary exposure to the air and second, to avoid catalysis of oxidation by destroying the enzymes that are naturally present and by preventing contamination with copper. The following important considerations are directly related to catalytic oxidation: (a) maintaining low temperatures and maximum acidity; (b) keeping agitation and injury to tissues at a minimum; avoiding exposure to (c) sunlight or (d) excessive blanching; (e) controlling harvesting conditions, and (f) avoiding destruction by certain types of bacteria.

There is often much to be gained indirectly from consideration of the processing conditions that will permit a food to retain its maximum content of the antiscorbutic vitamin, because oxidation is destructive of the best flavor and color in most foods, and in addition may cause parallel destruction of other nutrients, such as fats, additional vitamins, and certain amino acids.

Three modern trends in food processing especially reflect attention to the above principles: (1) freezing preservation of fruits and vegetables, with blanching of the latter regulated on the basis of enzyme destruction, (2) utilization of an inert atmosphere, such as steam, during the mechanical treatment as well as during sterilization of canned products, and (3) the introduction of aluminum and stainless-steel equipment to replace copper and copper-contributing alloys.

[1] Contribution No. 404 from the Department of Chemistry, University of Pittsburgh. Approved by the director of the New York State Agricultural Experiment Station for publication as Journal Paper No. 389, July 18, 1940.

STORAGE

Practically speaking, all plant and animal foodstuffs lose their vitamin C content at an appreciable rate when stored as natural products, owing to the respiratory enzymes that are present in all living cells. Such vegetables as potatoes, cabbage, parsnips, rutabagas, carrots, and onions, which possess large bulk and remain essentially "alive" with a low rate of respiration and evaporation, commonly lose about one-half or less of their original antiscorbutic value during a season's storage of several weeks or months, according to Daniel and Munsell (1937)[2]. The best antiscorbutic fruits, such as lemons, grapefruit, oranges, and apples, as shown by Batchelder and Overholser (1936), also lose gradually about one-half of their original ascorbic acid content while in a marketable condition. In sharp contrast to the above, such foods as fresh spinach, as shown by Tressler, Mack, and King (1936); lettuce, according to Wheeler, Tressler, and King (1939); green corn, as indicated by Richardson, Mayfield, and Davis (1937); green peas, according to Fenton and Tressler (1938), Mack, Tressler, and King (1936), and Fellers and Stepat (1936); and green beans, lose their antiscorbutic value rapidly at room temperature and fairly rapidly even at cold-storage temperatures.

Fresh meats lose vitamin C steadily during ordinary cold-storage practice. A short tenderizing treatment at an elevated temperature does not, however, appreciably accelerate the rate of destruction. It is evident from the observations of Stefansson (1935-36) and others that frozen fresh meat and American market meat may retain enough vitamin C to be fully protective.

Fresh high-quality raw milk that is held in refrigeration without exposure to copper-containing equipment at any stage commonly loses about 10 per cent of its vitamin C content per day, but the rate of loss is much more rapid in market milk because of contamination with copper.

DEHYDRATION

Fruits and vegetables dried according to common home and commercial practice are practically devoid of vitamin C because of enzymic oxidation. Special precautions may be taken, however, to prevent much of the loss. Sulfite treatment, according to Morgan (1935), and drying *in vacuo* both afford a measurable degree of protection. Evaporated (sulfited) apples, peppers, and citrus juice powders may show a fairly good retention of vitamin C. Renner (1935) found that powdered milk also retains its antiscorbutic value fairly well if produced and stored under favorable conditions. Most green leafy foods lose their vitamin C content during dehydration. Dry-cured meats are practically devoid of vitamin C.

[2] The publication of Daniel and Munsell gives a summary with references and quantitative data covering the literature to 1936. Appended to the present discussion will be found complete bibliography subsequent to 1936.

BLANCHING

The practice of blanching is more important in relation to vitamin C retention than has been commonly recognized. As discussed in more detail in the section on freezing, blanching should be adequate to destroy the oxidative enzymes while causing a minimum loss of the vitamin by diffusion from the product being blanched.

CRUSHING AND SLICING

Enzyme systems that can catalyze the oxidation of ascorbic acid are apparently present in the cells of all the higher plants and animals. While the tissues are essentially "living," the vitamin is protected from oxidation by the organization of functions within the cell, but when the tissues are disorganized, as by crushing, slicing, or freezing and thawing, the vitamin is oxidized and destroyed with extreme rapidity in practically all foods

TABLE 1

Loss of Ascorbic Acid From Vined Peas Held for Various Periods of Time[1]

Holding time	Temperature	Ascorbic acid content	
		Fresh basis	Dry basis
hr.	*°C.*	*mg. per gm.*	*mg. per gm.*
0	4	.28	1.20
3	4	.26	1.14
9½	4	.26	1.14
3	27	.24	1.02

[1] From Jenkins, R. R., Tressler, D. K., and Fitzgerald, G. A., 1938. Vitamin C content of vegetables. VIII. Frozen peas. Food Research 3, 136.

except those characterized by high acidity. In plant juices, especially, the rate of oxidation is nearly always very rapid, but in acid juices, such as the citrus juices and tomato juice, according to McElroy, Munsell, and Stienbarger (1939) and Kohman, Eddy, and Gurin (1933), the rate of loss is relatively slow.

From a theoretical point of view at least, it is interesting to note that the enzymes most active in causing vitamin C destruction are characterized by their content of active copper. The ascorbic acid oxidase, which acts directly on the vitamin, as found by Stotz (1940) and Stotz, Harrer, and King (1938), and polyphenol oxidase, which according to Kubowitz (1938) and Dalton and Nelson (1938) oxidizes ascorbic acid by means of a quinone carrier, are both copper-proteins. Two additional enzymes that can act on the vitamin are apparently iron-containing catalysts, cytochrome oxidase, and peroxidase.

FREEZING

Work at the Massachusetts and New York State Agricultural Experiment Stations and in certain other laboratories has indicated the necessity of prompt handling of fruits and vegetables at each step in the process of preparing these products for freezing, if the maximum retention of

ascorbic acid is to be attained. Thus, vegetables must be washed, blanched, and cooled or else refrigerated promptly after harvest, since nearly all of the vegetables now frozen commercially lose vitamin C rapidly if allowed to stand at ordinary room temperatures. During a warm night freshly harvested snap beans were found to lose one-half of their total ascorbic acid content. On standing three hours at 26.7°C. (80°F.) vined (threshed) peas were observed to lose approximately 15 per cent of their ascorbic acid (Table 1).

Blanching (scalding) prior to freezing is necessary in order to inactivate the action of oxidizing enzymes. Since blanching causes some loss of ascorbic acid, however, by solution in the hot water commonly used for the purpose, the blanching treatment should be just sufficient to inactivate the oxidizing enzymes. Forty-five to 60 seconds of blanching time is required for the inactivation of the oxidizing enzymes at 93°C. (199.4°F.). This causes a loss of approximately 16 per cent of the ascorbic acid. On the other hand, 153 seconds of blanching causes a loss of about 35 per cent of the vitamin (Table 2).

TABLE 2

Effect of Length of Blanch[1] in Water at 93°C.(199.4°F.) on Ascorbic Acid Content of Peas[2]

Time	Total solids	Ascorbic acid content	
		Fresh basis	Dry basis
sec.	*pct.*	*mg. per gm.*	*mg. per gm.*
0	22.5	.25	1.11
60	22.2	.21	.95
85	23.1	.20	.87
128	21.8	.17	.78
153	21.8	.16	.73

[1] A Scott blancher was used. [2] From Jenkins, R. R., Tressler, D. K., and Fitzgerald, G. A., 1938. Vitamin C content of vegetables. VIII. Frozen peas. Food research 3, 136.

After the vegetables are blanched and cooled, relatively little loss of ascorbic acid occurs provided the vegetables are quickly inspected, packaged, and chilled. In the case of vegetables such as peas, which are graded for quality in brine flotation equipment (a quality separator), there is a small additional loss of ascorbic acid by diffusion into the brine (Table 3).

Freezing itself causes no loss of ascorbic acid from properly blanched vegetables. The frozen vegetables must be stored at low temperatures, e.g., below —17.8°C. (0°F.), or else there will be a slow loss during long-continued storage (Figs. 1 and 2).

Even though the vitamin C content of the frozen vegetable is lower than when freshly picked, the final vitamin C content of the two is substantially the same because cooking causes a smaller loss of this vitamin from the frozen than from the fresh vegetable (Table 4). The reason for the smaller loss of ascorbic acid during the cooking of frozen products is because both blanching and freezing tenderize vegetables, thus shortening the cooking period.

PASTEURIZATION

When carried out in aluminum, stainless-steel, or glass equipment pasteurization of acid fruit juices can be accomplished, even to the point of sterilization, in continuous-flow equipment with only slight destruction of ascorbic acid. When similarly protected from copper contamination and excessive aeration in continuous-flow equipment, milk can also be pasteurized with only approximately 10 per cent loss in vitamin C content. In the commercial handling of milk, tests for loss in ascorbic acid before, during, and after pasteurization provide one of the most effective ways of anticipating undesirable changes in flavor because the oxidative catalysis of copper that may be dissolved from equipment affects the vitamin content of

TABLE 3

Vitamin C Content of Peas During Preparation for Freezing[1]

Treatment	Total solids	Ascorbic acid content	
		Fresh basis	Dry basis
	pct.	*mg. per gm.*	*mg. per gm.*
Freshly vined peas	24.1	.23	.96
After washing	23.1	.23	1.00
After blanching	24.0	.21	.88
Before shaker spray	24.8	.22	.89
Before quality separator	25.1	.21	.84
After quality separator	23.9	.18	.75
Before picking belt	23.3	.16	.69
After picking belt	22.4	.16	.71
Before filling	22.9	.16	.70
After packaging	21.3	.16	.75

[1] From Jenkins, R. R., Tressler, D. K., and Fitzgerald, G. A., 1938. Vitamin C content of vegetables. VIII. Frozen peas. Food Research 3, 134.

the milk before other changes are evident (personal observations; cf. also the paper on "Riboflavin, Vitamin C, and Flavor of Dairy Products" by Sharp and Hand on page 139).

COOKING

The effect of cooking upon the vitamin C content of foods has been studied in great detail in a number of laboratories. It seems fair to state that the losses involved in cooking are not so much due to the heat treatment as to other factors. The losses are primarily of three types: first, a brief initial loss owing to residual air in the tissues and a transitory period of enzymic activity before the proteins are coagulated; second, a mechanical loss caused by diffusion of ascorbic acid into the cooking water; and third, a continued oxidation that is dependent upon the degree of exposure to air and catalysis by copper and/or hemochromogens. The latter are apparently formed largely by decomposition of heme-containing substances, such as hemoglobin and cytochromes, in the food material. The first factor can be controlled in part by deaeration and quick initial heating. The second type of loss can be controlled best by using the least possible amount

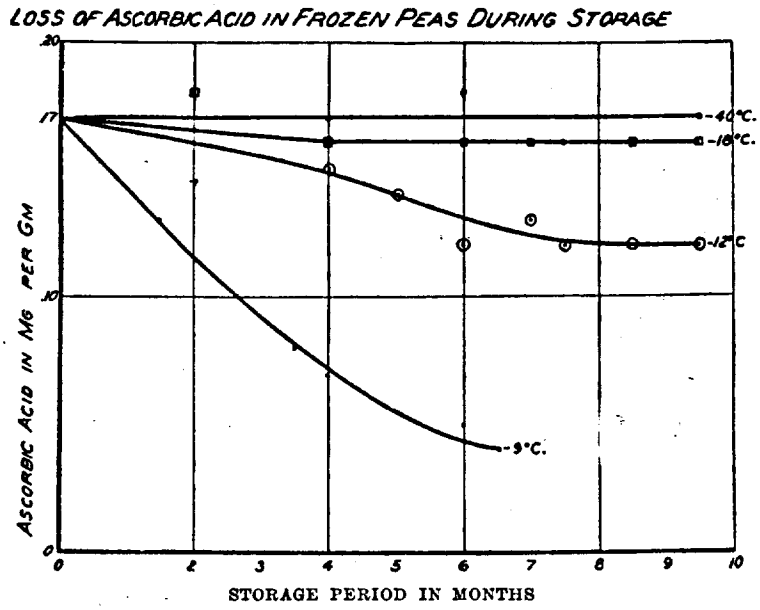

FIG. 1. [1]

[1] From Jenkins, R. R., Tressler, D. K., and Fitzgerald, G. A., 1938. Vitamin C in vegetables. Storage temperatures for frosted vegetables. The British Association of Refrigeration. Proceedings of General Conference on Refrigeration. Plate XIV.

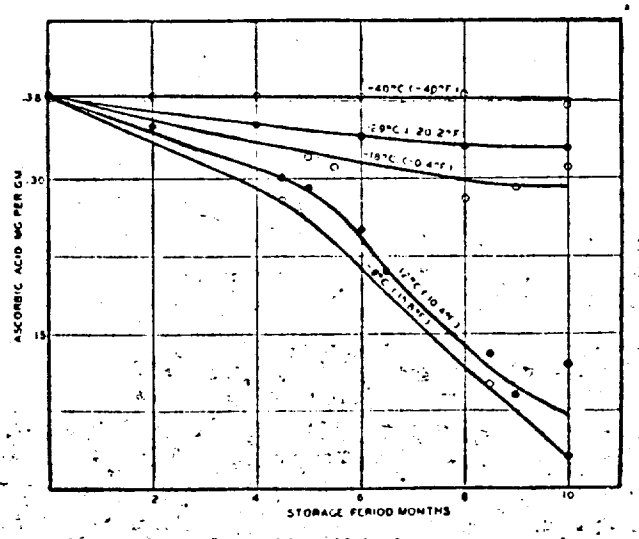

FIG. 2. Loss of ascorbic acid in frozen asparagus.[1]

[1] Tressler, D. K., Moyer, J., and McIntosh, J., 1940. Storage of frozen

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of cooking water, a short cooking time, and as little chopping or cutting of the material as will prove satisfactory in the product. The third type of destruction can best be prevented by avoiding exposure to the air and by utilizing a short cooking time. It is evident to any careful observer that home cooking methods tend to be much more negligent in relation to all three types of loss than one usually finds in a commercial establishment. An exception is the restaurant practice of holding vegetables on a steam table.

Rigid attention to the above three avenues of loss can be made the basis for improvements in the quality of cooked products in most homes and commercial institutions both from the point of view of nutritional value and in relation to attractiveness of the product.

TABLE 4

Ascorbic Acid in Fresh, Frozen, and Thawed Peas (Thomas Laxton Variety)[1]

Material	Cooking time	Ascorbic acid				
		Retained in drained peas		Found in cooking water		Destroyed
	min.	*mg. per gm.*	*pct.*	*mg. per c.c.*	*pct.*	*pct.*
Fresh peas	0	0.23	100	0	0	....
Fresh peas	16	0.11	42	0.13	48	10
Frozen peas	0	0.17	100	0	0	....
Frozen peas	8	0.12	59	0.07	36	5
Thawed peas	0	0.17	100	0	0	....
Thawed peas	8	0.12	56	0.08	39	5

[1] From Fenton, Faith, and Tressler, D. K., 1938. Losses of vitamin C during the cooking of certain vegetables. J. Home Econ. 30, 719.

Among the more extensive studies of vitamin C losses during cooking, one may cite the investigations carried out in the U. S. Bureau of Home Economics, the New York State Agricultural Experiment Station, the National Canners Laboratory, the Hermetikkindustriens Laboratorium at Stavanger, and the laboratory of Messrs. Chivers and Sons, Ltd., Cambridge.

In summation of the reports in the literature it is evident that the rate of destruction of vitamins in foodstuffs during cooking varies greatly with different products, but that in the case of steaming and boiling, the losses are usually between 10 and 25 per cent; baking, frying, and roasting cause somewhat larger losses, generally in the range of 30 to 60 per cent. On the other hand, a lack of careful attention to cooking losses may readily result in losses that range from 50 to 90 per cent.

CANNING

The principles outlined in the discussion of cooking losses apply to most phases of home and commercial canning. It is evident from many published papers, cf., Daniel and Munsell (1937), that commercial canning of acid fruits and fruit juices can be accomplished with losses of less than 20 per cent of the fresh-juice values. Retention of 90 per cent of the orig-

inal antiscorbutic value is not uncommon. To all practical purposes such products, if prepared with reasonable care, are as valuable and reliable as the corresponding fresh produce. The neutral-type vegetables, such as spinach, asparagus, peas, green beans, and kale, undergo greater losses in the order of 40 to 60 per cent. It is interesting to note, however, that some of the firms producing such highly disintegrated and sensitive products as neutral sieved baby foods can place stable products on the market with an antiscorbutic value that is 35 to 65 per cent of the value of the fresh produce. When one considers the losses in vitamin C that are commonly met with during regular marketing practices and home cooking, the

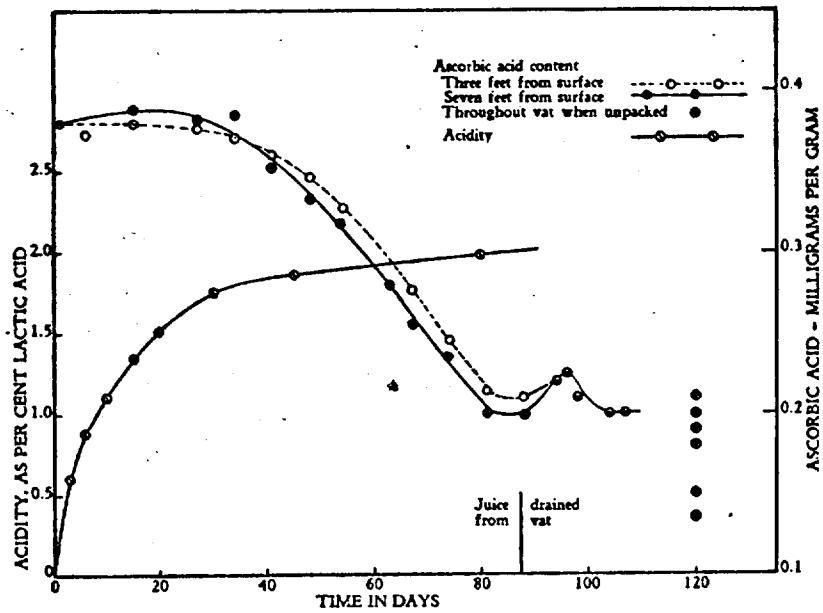

FIG. 3. Ascorbic acid content of kraut in vat No. 62.[1]

[1] From Pederson, C. S., Mack G. L., and Athawes, W. L., 1939. Vitamin C content of sauerkraut. Food Research 4, 40.

production of such canned products with roughly one-half of their original antiscorbutic value represents an outstanding achievement in relation to public health.

FERMENTATION AND PICKLING

From the limited data available it is apparent that vitamin C is not utilized or destroyed directly by the action of most bacteria and yeasts, but some organisms clearly do destroy the vitamin. Hence one should always consider the possibility of such destruction during fermentation processes. Aside from the immediate effect of the microörganisms it is evident that in all such processes the vitamin is subjected to direct loss through oxidation. Probably the only fermented foodstuff that contributes significant amounts of vitamin C in the American dietary is sauerkraut.

Cabbage loses little ascorbic acid during the fermentation process (Fig. 3). After fermentation ceases, a slow steady loss of ascorbic acid occurs during storage. If the kraut is canned, there is a destruction of approximately 20 per cent of its vitamin C content. After canning there is little loss of ascorbic acid.

In conclusion, it is evident that the food manufacturer needs the aid of the food technologist and biochemist in controlling his processes, if he is to produce foods of high vitamin C content.

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FURTHER NOTES ON THE DETERMINATION OF ASCORBIC ACID AND SULPHUR DIOXIDE IN FRUIT JUICES

By H. F. W. KIRKPATRICK

In a previous paper[1] a method was described for the separation and determination of ascorbic acid and sulphur dioxide in fruit juices. Use of this method in conjunction with direct titration of ascorbic acid in commercial concentrated citrus juices with dichlorophenol-indophenol showed that the sulphur dioxide caused no appreciable interference. It was also shown that, when using the iodometric method, combination occurs between the sulphur dioxide and the hexoses present in the juice, as a result of which a large part of the sulphur dioxide is not titratable with iodine, although it may be removed completely by steam-distillation. Further work has shown that the fact that ascorbic acid can be titrated with indophenol in concentrated citrus juices without interference by sulphur dioxide is due to the same combination with hexoses. Since sulphur dioxide in the absence of these sugars decolorises indophenol, a study has been made of the conditions necessary for the inhibition of this reaction, viz., the concentration of sugar, sulphur dioxide, and ascorbic acid and the time required for the formation of the sugar–sulphite compound. It has been found that sulphur dioxide in amounts up to 1300 p.p.m. does not affect the titration until the sugar concentration of the juice falls below 20%, the critical amount of sulphur dioxide being about 1000 p.p.m. at 15% and 500 p.p.m. at 10%.

The methods used must be followed in detail if reproducible results are to be obtained. The separation method, as described previously for the iodine titration, has been modified to enable an accurate determination of the sulphur dioxide to be made when using indophenol. Metaphosphoric acid has been substituted for trichloroacetic acid, a suggestion for which the author is indebted to Dr. L. W. Mapson. The dye solution, as before, was a 0·05% solution in a buffer solution of p_H 7·0, standardised iodometrically. The procedures used are as follows.

Direct titration.—A weight of sample sufficient to give a titration of 3–5 ml. with the dye solution is diluted with 10 ml. of 2 w/v metaphosphoric acid and titrated until the pink tinge persists for 10 seconds. When titrating the juices a control is essential to determine the correct end-point. The titration should occupy about 2 minutes.

Separation method.—10–25 g. of the sample (according to concentration) are weighed into a 100-ml. Kjeldahl flask, diluted with a little water if necessary, and the sulphur dioxide is removed by distillation and titrated as previously described. The liquid in the flask, after cooling, is washed with water, previously boiled and cooled in a current of nitrogen, into a 100-ml. measuring flask, 10 ml. of 20 w/v metaphosphoric acid are added, and the liquid is made up to volume. If a greater volume is more suitable, the amount of acid added should be proportionally increased. Aliquot parts of this solution are titrated exactly as in the direct titration except that no dilution or acid is necessary.

(a) *Sugar concentration.*—The effect of the sulphur dioxide on the direct titration with indophenol in the presence of various concentrations of sugar was determined by using solutions of pure glucose and invert sugar, the latter being prepared from commercial invert sugar syrup by dilution with water. To these solutions, ascorbic acid, sulphur dioxide, and citric acid were added and, after keeping for several days, the determinations were carried out by the methods described.

The losses of ascorbic acid and sulphur dioxide which occurred on keeping these solutions rendered useless a comparison of the amounts found with those added, except where the concentration of sugar was at least 40%. In consequence a comparison is made of the results given by direct titration and by the separation method. The results for the glucose solutions are given in Table I, and for the invert sugar solutions in Table II. The percentages shown are all calculated to weight in weight.

TABLE I

Concn. of glucose %	SO_2 found* p.p.m.	Ascorbic acid found % Direct titration	Separation method
1	700	0·199	0·186
5	850	0·192	0·179
10	760	0·166	0·164
15	750	0·153	0·149
25	800	0·143	0·138
35	830	0·123	0·119
40	850	0·117	0·115

TABLE II

Concn. of invert sugar %	SO_2 found* p.p.m.	Ascorbic acid found % Direct titration	Separation method
1	750	0·200	0·181
5	830	0·199	0·194
10	770	0·183	0·178
20	810	0·184	0·180
30	810	0·184	0·180
40	840	0·184	0·182
50	840	0·193	0·190
60	900	0·200	0·199

* The amount of sulphur dioxide shown is that found in the sample at the time of the titration and does not represent the amount present in the solution made by diluting the weight of sample taken with metaphosphoric acid.

From these results it would appear that with about 800 p.p.m. of sulphur dioxide present the limiting concentration of sugar is 10% in glucose solutions and 5% in invert sugar solutions. It may be noted here that, in titrating solutions containing sulphur dioxide having a concentration of sugar below these limits, the end-points were very obscure, whereas in the solutions of higher concentration the end-points were quite sharp and reproducible by independent workers. Where the end-point is not definite therefore the sulphur dioxide is probably interfering with the titration.

(b) *Time factor.*—The time elapsing between addition of the sulphur dioxide to a sugar solution and the establishment of equilibrium was investigated. The method adopted for determining this was to add sulphur dioxide to a solution of sugar, citric acid, and ascorbic acid, and titrate at once and after certain intervals. The tests were made using an invert sugar solution containing 45% of sugars, this concentration being preferred as it is the lowest concentration of this sugar in which rapid loss of ascorbic acid does not occur. A carefully weighed amount of ascorbic acid was used for the addition but the separation method was used as a control. Two solutions were made up, each containing 0·200% of

[1] J.S.C.I., 1941, 60, 226.

ascorbic acid, one containing about 500 p.p.m. and the other about 1000 p.p.m. of sulphur dioxide. The results are shown in Table III.

TABLE III

Solution	Time (mins.)	SO_2 found p.p.m.	Ascorbic acid found % Direct titration	Ascorbic acid found % Separation method
1	0	—	0·200	—
	15	530	0·201	0·199
	30	—	0·201	—
2	0	—	0·206	—
	30	—	0·204	—
	66	1100	0·202	0·199
	120	—	0·202	—

With amounts of sulphur dioxide up to 500 p.p.m. equilibrium is established immediately; for amounts between 500 and 1000 p.p.m. an hour is necessary.

(c) *Orange juices.*—The results given by the sugar solutions indicate that the lowest concentrations of sugar which will prevent interference with the dye titration of sulphur dioxide in amounts normally found in citrus juices is of the order of 5 or 10%. Experiments have been made with commercial concentrated orange juices to determine the limits more exactly. The sugar concentration in the juice was obtained approximately by subtracting the citric acid, found by titration, from the total solids, as calculated from the refractive index and citric acid content by the method of Stevens and Baier.[2] The juice was then diluted with water to produce the required sugar concentration, sulphur dioxide was added to the diluted juice, and the analyses were made after at least an hour had elapsed. Two samples from different sources were used for the tests: no. 1 was a concentrated juice containing about 11% of citric acid, 60% of sugars, and a high ascorbic acid content, and no. 2 was a concentrated juice containing about 7% of citric acid, 60% of sugars, and about half as much ascorbic acid as no. 1. Both contained a small amount of sulphur dioxide. The results are given in Table IV.

TABLE IV

	Concn. of sugar %	SO_2 found p.p.m.	Ascorbic acid found % Direct titration	Ascorbic acid found % Separation method
No. 1	60	170	0·320	0·318
	30	1320	0·160	0·161
	20	1250	0·107	0·105
	15	1200	0·081	0·079
	15	700	0·079	0·078
	10	1010	0·060*	0·053
	10	540	0·055	0·054
No. 2	60	130	0·145	0·147
	60	1320	0·142	0·140
	15	1500	0·048*	0·036
	15	880	0·038	0·036
	10	420	0·023	0·022

Where the results are marked * the end-point was very obscure, and there is no doubt that the sulphur dioxide was interfering with the titration. In no. 2 the titration of the 10% sugar concentration was not good and although the results were reproducible, this was undoubtedly the lowest possible concentration for preventing interference by any appreciable amount of sulphur dioxide. In no. 1 it will be seen that there is interference at a sugar concentration of 10% by amounts of sulphur dioxide over 500 p.p.m., whilst in no. 2 there is interference at a sugar concentration of 15% when the sulphur dioxide exceeds about 900 p.p.m.

(d) *Sensitivity of direct titration method to sulphur dioxide.*—The concentration of sulphur dioxide in the sample necessary to interfere with direct titration by the method described, no sugar being present, was determined with aqueous solutions of sulphite. 1 ml. of the solution was diluted with 10 ml. of 2 w/v metaphosphoric acid, a drop of the dye solution added from a micro-burette, and the persistence of the colour noted. It was found that with solutions containing up to 300 p.p.m. of sulphur dioxide there was no obvious effect, decolorisation of the dye being very slow, but with 400 p.p.m. and more the rate of decolorisation increased rapidly. It would seem possible, therefore, that this method of direct titration can be applied to all materials irrespective of their containing fruit sugars, provided that the solution as titrated contains not more than 30 p.p.m. of sulphur dioxide. This might also find an application in the case of preparations of fruit juices in which the bulk of the sugar present is sucrose. Further work on the titration of sucrose solutions has shown that although the error introduced by sulphur dioxide may be small, the determining factor is the concentration of sulphur dioxide in the solution titrated and the principle stated above should be applied. There is no evidence of combination of sulphur dioxide occurring in sucrose solutions where a large amount of sulphur dioxide is present and no inversion has occurred. The separation method must be used for such preparations.

SUMMARY

(1) The effect of sulphur dioxide with varying sugar concentration on the direct titration of citrus fruit juices with dichlorophenol-indophenol has been studied.

(2) Sulphur dioxide in citrus juices in amounts up to 1300 p.p.m. does not affect the titration until the sugar concentration of the juice falls below 20%. With 15% of sugars, the critical amount of sulphur dioxide appears to be about 1000 p.p.m. and 10% about 500 p.p.m.

(3) Equilibrium between sugar and sulphur dioxide is reached in an hour.

(4) The method of direct titration is described, together with a modified separation method. The latter enables an accurate and rapid determination of the sulphur dioxide to be made in the same operation as the determination of the ascorbic acid by titration with the dye solution.

(5) The direct titration method described is not affected by sulphur dioxide in the titrated solution in amounts up to 30 p.p.m. and its possible application to materials containing little or no hexose sugar is suggested.

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[2] Ind. Eng. Chem. (Anal.), 1939, 11, 447.

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The Mechanism of the Virucidal Action of Ascorbic Acid. MORTON KLEIN (Dept. of Bacteriology, Univ. of Penna.). It has been reported that ascorbic acid is virucidal for the viruses of rabies, vaccinia, poliomyelitis, and influenza virus. Chemical studies of the oxidation of ascorbic acid have revealed that during the Cu catalyzed oxidation of ascorbic acid H_2O_2 is formed. It would seem reasonable to assume that the viru-

cidal action of ascorbic acid may be due to the H_2O_2 formed during the oxidation of the compound.

The PR8 strain of influenza A virus, grown in the allantoic sac of the developing chick embryo, was the test virus and intranasal mouse inoculation was used for indicating viral activity. It was found that 0.05 M ascorbic acid diluted in phosphate buffer, pH 7, inactivated 10 MLD of influenza A virus. Catalase, which is known to specifically inactivate H_2O_2, was found to neutralize completely the action of ascorbic acid. H_2O_2 (0.1%), which approximated the concentration of H_2O_2 produced under the conditions of our experiment by 0.05 M ascorbic acid, also inactivated 10 MLD of influenza virus and this inactivation was completely neutralized by catalase.

It is concluded that the virucidal action of ascorbic acid may be explained as being due to the production of H_2O_2 formed during the Cu catalyzed oxidation of ascorbic acid.

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THE RENAL THRESHOLD FOR ASCORBIC ACID

A MODIFIED METHOD FOR ITS ESTIMATION WITH RESULTS FOR TWELVE ADULT SUBJECTS [1]

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A method for estimating the renal threshold for ascorbic acid has been reported from this laboratory by Lewis, Storvick and Hauck ('43). A majority of their subjects were studied for 2-week periods, with daily determinations of fasting plasma ascorbic acid and corresponding 1-hour urinary excretion values. In later studies in this laboratory, such determinations were made daily for 6 weeks. With a large number of fasting values, a statistically significant difference was sometimes found between 2 means, both of which were below the renal threshold as estimated by inspection of the arrayed data and scatter diagrams. This paper presents a modified method for treating such data, with values for renal thresholds for ascorbic acid for 12 adult subjects, 1 of whom was previously studied by Lewis, Storvick and Hauck ('43).

EXPERIMENTAL PROCEDURE

Personal data concerning the subjects, the diets and experimental procedure are reported in connection with the studies

[1] Some of the data in this paper are taken from theses presented to the faculty of the Graduate School of Cornell University in fulfillment of the requirement for the M.S. degree, by Ann C. Moore, June 1944, and J. Estelle Haines, February 1946. Further details will be found in the theses.

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on tissue reserves of ascorbic acid at various intakes (Haines et al., '47).

Blood and urine samples to be used in estimating the renal threshold were obtained as described by Lewis, Storvick and Hauck ('43) for their second series of experiments, i.e., the blood sample was taken midway in the hour for which the urine specimen was collected. Some of these specimens were obtained under fasting conditions, and some after various sized doses of ascorbic acid.

METHOD OF ESTIMATING THE RENAL THRESHOLD

In our modified method, inspection of the means and standard deviations for hourly urinary excretion of ascorbic acid at various plasma levels is substituted for Student's ('25) "t" test for determining the mathematical significance of the differences between means. The first step was to array the data for plasma ascorbic acid values for each subject, with the corresponding hourly urinary excretion values. Scatter diagrams were also made from these data. From inspection the apparent renal threshold for ascorbic acid was noted for each subject. The mean value, with its standard deviation, for urinary excretion of ascorbic acid at the apparent renal threshold was then calculated. This was compared with the mean value and its standard deviation for urinary excretions at all plasma levels below the apparent renal threshold. Since by definition, the renal threshold for ascorbic acid is the plasma concentration at which a marked rise in urinary excretion of ascorbic acid occurs, if the threshold value obtained by inspection is correct, the excretion of ascorbic acid at this plasma value will be relatively large and variable. Below the renal threshold, the excretion is low and relatively constant (Friedman, Sherry and Ralli, '40). To test the validity of the apparent renal threshold, similar calculations were made for plasma values 0.1 mg % below and 0.1 mg % above the apparent renal threshold.

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RESULTS AND DISCUSSION

Renal threshold values for 12 subjects

Mean urinary excretion values for ascorbic acid corresponding to plasma values at, above and below the renal thresholds for 12 subjects are presented in table 1. For these subjects, the renal threshold values for plasma ascorbic acid as estimated by this method, ranged from 1.0 to 1.3 mg %. Mean excretion values for ascorbic acid at the threshold were all above 2 mg per hour, with most standard deviations considerably more than half the mean.

Of the renal thresholds for ascorbic acid reported in this paper and that of Lewis, Storvick and Hauck ('43), for 23 individuals, 21 were between 1.0 and 1.3 mg %. Inspection of the data for the entire series suggests that if a subject excretes 2 mg or more of ascorbic acid in 1 hour, his plasma ascorbic acid value has almost certainly exceeded his renal threshold during that hour, whereas if the excretion is 1.0 mg or less, it probably has not.

Comparison of the modified method for estimating renal threshold for ascorbic acid with that of Lewis, Storvick and Hauck ('43)

When the method described in this paper was applied to the data of Lewis, Storvick and Hauck ('43), the estimated renal thresholds were the same in all cases. When the method of Lewis, Storvick and Hauck was applied to data for the subjects of the present study, odds for significance for points below the apparent renal threshold were more comparable with those of Lewis, Storvick and Hauck when the fasting plasma values used in the calculations were limited to 2 weeks of the study. Thus the method presented here appears to have a wider usefulness than that previously devised in this laboratory.

Stability of the renal threshold for ascorbic acid

In the present study, 4 subjects, E.K., D.L., H.G. and A.M., were subjects for 2 successive years. Subject J.M. served

TABLE 1

Mean urinary excretion values for ascorbic acid corresponding to plasma values at, above, and below the renal threshold for ascorbic acid. (12 subjects.)[1]

SUBJECT	RENAL THRESHOLD	HOURLY EXCRETION AT THRESHOLD	HOURLY EXCRETION BELOW THRESHOLD	PLASMA VALUE BELOW THRESHOLD	HOURLY EXCRETION AROUND POINT TESTED	HOURLY EXCRETION BELOW POINT TESTED	PLASMA VALUE ABOVE THRESHOLD	HOURLY EXCRETION AROUND POINT TESTED	HOURLY EXCRETION BELOW POINT TESTED
	mg %	*mg*	*mg*	*mg %*	*mg*	*mg*	*mg %*	*mg*	*mg*
M. H.[2]	1.0	2.6 ± 1.3 (5)[3]	0.7 ± 0.4 (7)	0.9	1.4 ± 0.6 (2)	0.6 ± 0.2 (15)	1.1	2.4 ± 0.8 (5)	1.1 ± 1.1 (22)
J.L.	1.0	3.3 ± 1.9 (4)	1.1 ± 0.6 (38)	0.9	1.7 ± 0.7 (6)	1.0 ± 0.5 (32)	1.1	3.1 ± 1.9 (3)	1.2 ± 0.9 (41)
P.H.	1.05	5.7 ± 7.6 (4)	0.9 ± 0.4 (38)	0.95	1.3 ± 0.7 (5)	0.8 ± 0.3 (33)	1.15	7.8 ± 7.1 (4)	1.3 ± 2.5 (42)
E.K.	1.1	2.8 ± 2.0 (5)	1.4 ± 0.6 (46)	1.0	1.9 ± 0.8 (6)	1.3 ± 0.5 (40)	1.2	2.8 ± 1.5 (7)	1.3 ± 0.6 (46)
H.G.	1.1	2.1 ± 1.6 (7)	1.0 ± 0.3 (24)	0.95	1.3 ± 0.4 (3)	1.0 ± 0.2 (21)	1.25	3.4 ± 3.5 (6)	1.2 ± 0.9 (31)
J.M.[4]	1.1	3.2 ± 4.3 (9)	0.9 ± 0.5 (32)	1.0	0.9 ± 0.3 (6)	0.9 ± 0.5 (29)	1.2	8.3 ± 6.3 (5)	0.9 ± 0.4 (37)
D.L.[2]	1.15	4.2 ± 3.7 (5)	0.8 ± 0.3 (38)	1.05	1.3 ± 0.6 (3)	0.8 ± 0.2 (35)	1.25	2.7 (1)	1.2 ± 1.5 (43)
A.M.	1.2	7.7 ± 7.1 (3)	0.8 ± 0.4 (43)	1.1	1.2 ± 0.8 (6)	0.7 ± 0.2 (37)	1.3	4.8 ± 5.7 (6)	1.2 ± 2.3 (46)
L.G.	1.3	6.5 ± 4.3 (7)	1.0 ± 0.4 (27)	1.2	1.2 (1)	1.0 ± 0.4 (26)	1.4	6.3 ± 0.1 (2)	2.2 ± 3.0 (34)
J.E.S.[2]	1.3	2.8 ± 1.6 (3)	1.1 ± 0.5 (21)	1.2	1.9 ± 0.2 (2)	1.0 ± 0.4 (19)	1.4	2.5 ± 1.7 (6)	1.3 ± 0.9 (24)
E.H.	1.3	3.3 ± 2.3 (8)	1.0 ± 0.5 (28)	1.2	1.1 ± 0.4 (4)	1.0 ± 0.6 (24)	1.4	6.8 ± 1.2 (4)	1.5 ± 1.5 (36)
S.P.	1.3	2.2 (1)	0.9 ± 0.3 (30)	1.2	1.1 ± 0.2 (4)	0.8 ± 0.3 (26)	1.4	4.8 ± 0.2 (2)	0.9 ± 0.4 (41)

[1] When a subject was studied during 2 successive years, values for the larger series of the 2 are given.

[2] Some of the determinations used in calculating the renal threshold for these subjects were made by Margaret Delaney.

[3] Figures in parentheses represent number of values included in the mean.

[4] This subject also served for the experiments of Lewis, Storvick and Hauck ('43).

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for this experiment and also that of Lewis, Storvick and Hauck ('43). These authors also reported the renal threshold for 1 subject (J.S.) who served for 2 successive years. Whether values for these subjects were calculated for the separate years or combined, the estimated renal thresholds were the same. Evidence from these 6 subjects suggests, therefore, that the renal threshold for ascorbic acid is relatively stable in the normal adult from year to year, and, within the limits of this experiment, at various levels of intake.

SUMMARY

A modified method for estimating the renal threshold for ascorbic acid based on the comparison of means and standard deviations for urinary excretions at various plasma ascorbic acid levels is presented. This method is simpler and appears to have a wider usefulness than that previously reported from this laboratory.

The renal thresholds for 12 normal adults, as estimated by this method, were between 1.0 and 1.3 mg %.

Further evidence is presented that the renal threshold for ascorbic acid is relatively stable from year to year.

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BIOCHEMICAL STUDIES OF OLFACTORY TISSUE: RESPONSES OF ATPase ACTIVITIES TO OCTANOL AND ASCORBIC ACID

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SUMMARY

The responses of ATPase activities to octanol and ascorbic acid have been useful in showing variations in properties of these enzymes from different tissues and from different subcellular particles or structures from a single tissue.

The response of Na–K ATPase activity to low concentrations of octanol was quite different between olfactory tissue and brain subcellular particle preparations. At a concentration of approx. 0.4 m*M* octanol, the Na–K ATPase from olfactory tissue showed a definite stimulation of specific activity. For similar preparations from brain homogenates, the Na–K ATPase activity was inhibited at 0.4 m*M* octanol. At higher octanol concentrations the Na–K ATPase was strongly inhibited in preparations from both tissues. The Na–K ATPase activity was inhibited by ascorbic acid and the responses were similar for both olfactory tissue and brain preparations.

There were differences in response to octanol and ascorbic acid by Mg^{2+} ATPase. However, for this enzyme, the differences were between subcellular fractions; similar preparations from brain and olfactory tissue had similar responses of Mg^{2+} ATPase to octanol and ascorbic acid. Octanol caused stimulation of the mitochondrial but inhibition of nonmitochondrial Mg^{2+} ATPase activities. Ascorbic acid caused stimulation of nonmitochondrial but had little or no effect on mitochondrial Mg^{2+} ATPase.

The octanol stimulating effect on olfactory tissue Na–K ATPase was further enhanced by NaI treatment but eliminated by lubrol WX solubilization of subcellular

Abbreviations: DNP, 2,4-dinitrophenol; DTT, dithiothreitol; NEP, nerve ending particles; PK, pyruvate kinase; TCA, trichloroacetic acid.

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particle preparations. However, lubrol solubilization did not effect the ascorbic acid stimulation of the Mg^{2+} ATPase.

The use of oligomycin in conjunction with octanol and ascorbic acid on a liver mitochondrial preparation was useful in understanding the results obtained on olfactory tissue and brain preparations.

INTRODUCTION

In initiating a biochemical study of olfactory tissue for the purpose of understanding reactions occurring in the early stages of odor perception, fractionation of tissue homogenates was conducted essentially according to the procedures of ALBER *et al.*[1], DEROBERTIS[2] and WHITTAKER[3]. Their techniques were developed for isolation of nerve ending particles (NEP) from brain homogenates. Results of the olfactory tissue fractionation study[4] showed that the specific activity of Na–K ATPase was highly active in a plasma membrane fraction from a terminal swelling (NEP) concentrate. The high concentration of the Na–K ATPase activity in the plasma membrane of olfactory NEP caused an interest in the study of this enzyme in relation to its possible action in the initial phases of odor sensing.

In deciding on what odorants to test on the enzyme system, several straight-chained primary alcohols were investigated concerning their effects on enzyme activity. ISRAEL AND SALAZAR[5] observed that alcohols have an inhibitory action on ATPase activity from brain microsomes. Our studies on NEP from brain and olfactory tissue showed differences from those of ISRAEL AND SALAZAR, whereas results from brain microsomes were in agreement with their results.

Further studies on the Na–K ATPase activities and responses to alcohols were conducted to determine whether the effects observed on crude fractions could be enhanced. NAKAO and co-workers[6,7] reported that sodium iodide treatment inactivated Mg^{2+} ATPase activity while enhancing Na–K ATPase activity, using preparations from erythrocytes and various tissues from the rabbit. UESUGI *et al.*[8] and KAHLENBERG *et al.*[9] reported that lubrol WX treatment of NaI-treated preparations caused solubilization of Na–K ATPase activity. SHIRACHI *et al.*[10] recently reported that a lower lubrol concentration was more effective when used directly on membrane preparations. These techniques were used to prepare samples from olfactory tissue to further study the response of the ATPases to octanol. The purpose of this paper is to report the results of these studies. In addition, the effects of ascorbic acid on ATPase activities on similarly treated tissues are reported. Oligomycin as a specific inhibitor of Mg^{2+} ATPase and a liver mitochondrial preparation were used to delineate differences in ATPase responses to octanol and ascorbic acid.

MATERIALS AND METHODS

Materials and methods of homogenization and fractionation of rabbit brain cortex and the bipolar cell-rich fraction of olfactory tissue were the same as those

reported earlier[4]. Tissues were homogenized in ice-cold 0.32 *M* sucrose, 1 m*M* EDTA and 10 m*M* imidazole at pH 7.5. This solution was used for suspension and dilution of all fractions. Fractions were obtained by differential and density gradient centrifugation and designated as follows: crude NEP and mitochondria (B), NEP concentrate (B_2), NEP *plus* free mitochondria (B_3), plasma membrane concentrate from B_2 by osmotic shock (E_2), plasma membrane *plus* mitochondria fragments (E_3), and microsomes (C) (see ref. 4). In addition one study is reported in which fractions were prepared according to the procedure of SCHWARTZ *et al.*[11]. In their procedure the following fractions were recovered: mitochondrial (Mt_1), second mitochondrial (Mt_2) and microsomal (M_s). All fractions were divided into small aliquots, quick-frozen with liquid nitrogen and stored at −20°.

ATPase activities were measured by two types of determinations, chemical and enzymatic. The chemical or discontinuous method was essentially that of SKOU[12,13] as reported by KOCH[4]. A 850-μl reaction mixture contained 5 m*M* ATP, 5 m*M* Mg^{2+}, 20 m*M* K^+, 100 m*M* Na^+, 120 m*M* imidazole or Tris buffer (pH 7.5) and 25–100 μl homogenate fraction. Reaction time and temperature were 30 min and 37°, respectively. P_i was determined by the method of LOWRY AND LOPEZ[14]. Absorbance was read at 870 nm. The enzymatic or continuous method was essentially that described by PULLMAN *et al.*[15] and FRITZ AND HAMRICK[16]. A 3-ml reaction mixture contained: 4.3 m*M* ATP, 5 m*M* Mg^{2+}, 20 m*M* K^+, 100 m*M* Na^+, 120 m*M* Tris buffer (pH 7.5), 0.14 m*M* $NADH^+$, 0.5 m*M* phospho-enol-pyruvate, 0.02% bovine serum albumin, approx. 9 and 12 units of pyruvate kinase (PK) and lactic dehydrogenase (LDH), respectively, and 25–100 μl homogenate fraction. Absorbance was measured at 340 nm using a Beckman DU or a Cary-14 spectrophotometer at 37°. When used, ouabain was present in the reaction mixture at a concentration of 1 m*M*.

Protein determinations were made on each fraction by the method of LOWRY *et al.*[17].

ATPase nomenclature

Total ATPase activity was measured when Mg^{2+}, Na^+ and K^+ were present in the reaction mixture. Mg^{2+} ATPase activity was measured when ouabain was present in the reaction mixture containing Mg^{2+}, Na^+ and K^+. [Ouabain is a "cardiac" glycoside which specifically inhibits the Na-K ATPase[18].] Na-K ATPase activity was the total activity *minus* the Mg^{2+} ATPase activity.

Special additions

Where indicated special additions were made to the reaction mixture just prior to addition of ATP and the enzyme fraction for the discontinuous procedure, and just prior to the enzyme addition for the continuous method. Octanol additions were made as 1-μl aliquots from appropriate ethanol-octanol mixtures using a 1-μl Hamilton Syringe (Whittier, Calif.). The alcohol solution was injected below the surface of the reaction mixture while being rapidly stirred by a Vortex Jr. Mixer. Ascorbic acid solutions were prepared twice daily and held in an icebath until added to the reaction mixture. Special reagents were obtained from Sigma, all others were analytical reagent grade.

RESULTS

Contents and properties of the more generally used fractions are presented in Table I. Standard deviations indicate that in general reproducibility for different preparations was within 10%. Greater variations were observed in some preparations of olfactory tissue, which may be related to the difficulty in separating the bipolar cell-rich region from the respiratory cells of the tissue. However, reproducibility between duplicate and triplicate measurements on the same sample (including the response to additions) was better than 5%.

TABLE I

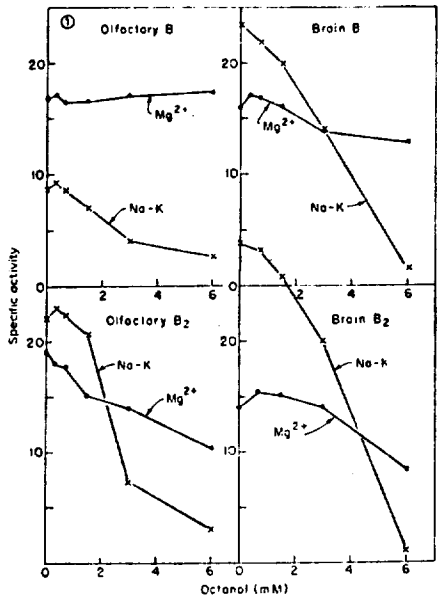
PROTEIN CONTENT, TOTAL ATPase SPECIFIC ACTIVITY AND OUABAIN SENSITIVITY OF VARIOUS FRACTIONS FROM RABBIT TISSUE HOMOGENATES

Fractions	*Protein (mg/ml)*	*Specific activity* [a] *(μmoles P_i mg^{-1} protein h^{-1})*	*Ouabain inhibition (%)*
Olfactory			
B	0.42	24.5 ± 0.8 (7)	33.9 ± 2.3 (7)
B_2	0.27	41.0 ± 0.9 (5)	52.7 ± 2.9 (6)
B_3	0.45	24.2 ± 1.3 (3)	20.8 ± 2.1 (3)
C	0.37	18.4 ± 3.0 (3)	24.8 ± 2.4 (3)
Brain			
B	0.26	39.4 ± 2.0 (8)	59.6 ± 5.5 (8)
B_2	0.26	42.8 ± 2.8 (6)	67.4 ± 4.6 (6)
B_3	0.34	32.2 ± 1.5 (4)	37.2 ± 2.0 (4)
E_2	0.15	71.4 ± 4.5 (3)	71.2 ± 0.1 (3)
E_3	0.19	56.0 ± 1.2 (4)	55.7 ± 2.7 (4)
C	0.40	26.0 ± 2.8 (8)	61.0 ± 4.5 (5)
Liver			
B	0.64	57.0 ± 4.1 (6)	0 (4)

[a] Specific activity and ouabain data are: mean values ± standard deviation; ( ), number of experiments. Fraction designation: B, crude mitochondria and NEP; B_2, NEP with entrapped mitochondria; B_3, NEP and free mitochondria; E_2, plasma membrane; E_3 plasma membrane and mitochondrial fragments; C, microsomes.

Responses of ATPase activities to octanol

Effects on Na–K ATPase. In initial studies on the effect of alcohols on ATPase activities using a series of primary alcohols, it was observed that Na–K ATPase from olfactory tissue preparations was far more sensitive to octanol than to lower-carbon-number alcohols. 50% inhibition of Na–K ATPase activity was produced by approx. 4 m*M* octanol, while 1.1 *M* (or over 200 times more) ethanol was required to produce the same amount of enzyme inhibition. In Fig. 1 the effect of octanol concentration on Na–K ATPase activity is shown for olfactory tissue and brain subcellular particle fractions. At the highest octanol level (6 m*M*) the Na–K ATPase activity was nearly completely inhibited in both tissues. However, at the lowest octanol concentration (about 0.4 m*M*) the olfactory tissue Na–K ATPase activity showed a small but consistent stimulation. No stimulation of brain Na–K ATPase was observed at any level of octanol (Fig. 1).



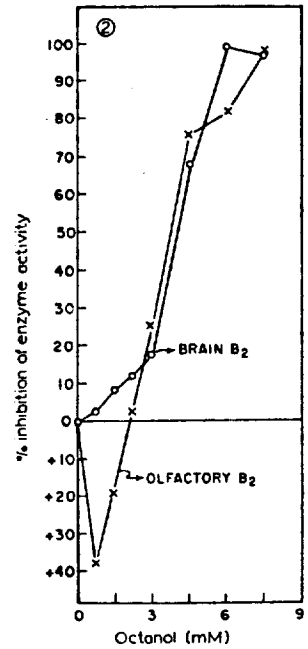


Fig. 1. Effect of concentration of octanol on the ATPase activities from brain and olfactory tissue crude NEP (B) and NEP concentrate (B_2) fractions. Na–K (Na–K ATPase), Mg^{2+} (Mg^{2+} ATPase). Discontinuous method with reaction mixture (850 μl total) containing: 5 mM ATP Na_2, 5 mM Mg^{2+}, 100 mM Na^+, 20 mM K^+, 120 mM imidazole buffer (pH 7.5) and 1 mM ouabain (where added). Activity measured at 37° for 30 min. Sample size 25–50 μl. Reaction stopped by addition of 150 μl ice-cold 30% TCA followed by determination of P_i by the method of LOWRY AND LOPEZ[14]

Fig. 2. Comparison of brain and olfactory tissue Na–K ATPase activity response to octanol at various concentrations. Stimulation is indicated by plus values. B_2, NEP concentrate fraction. Enzyme method and reaction conditions same as Fig. 1.

Several different rabbit olfactory tissue preparations (combination of tissue from 3–5 rabbits per preparation) were tested for stimulation of Na–K ATPase activity by low levels of octanol. There was a fairly wide variation in the stimulation from B_2 fractions. A number of factors may be responsible, since a range of ouabain sensitivity was also observed. One important factor was the lack of a means to determine whether the olfactory tissue was fully operative in odor sensing. Rabbits with obvious rhinitis were not used. In some olfactory preparations from a single rabbit B_2 fractions were obtained in which the stimulation of Na–K ATPase activity was between 35 and 40% (Fig. 2). Brain B_2 preparations from the same rabbit showed inhibition of Na–K ATPase activity at all levels of octanol tested (Fig. 2).

Studies were conducted to determine whether the octanol stimulation of olfactory Na–K ATPase could be enhanced. Crude NEP fractions from brain and olfactory tissue were tested with NaI, which caused specific inactivation of Mg^{2+} ATPase activity. The NaI-treated samples were solubilized by addition of lubrol WX. The procedures used and results of the above treatments are shown in Table II. Comparing

TABLE II

OCTANOL EFFECTS ON Na–K ATPase ACTIVITY OF NaI- AND LUBROL-TREATED RABBIT BRAIN AND OLFACTORY TISSUE Mt_1 FRACTIONS

Mt_1, crude mitochondrial and NEP fraction after SCHWARTZ *et al.*[11]. NaI and lubrol WX treatments after NAKAO and co-workers[6,7], UESUGI *et al.*[8] and KAHLENBERG *et al.*[9]. Fraction (4 mg protein per ml in 5 m*M* EDTA solution) treated with an equal volume of 4 *M* NaI, 50 m*M* β-mercaptoethanol, 5 m*M* $MgCl_2$, 3 m*M* ATP, and 5 m*M* EDTA for 30 min; then diluted to 0.8 *M* NaI and centrifuged at 20 000 *g* for 30 min. Sediment washed and suspended in 5 m*M* EDTA. NaI-treated sample (protein 0.5–2 mg/ml) diluted 10 : 11 with solution containing 12% lubrol WX, 0.1 *M* Tris (pH 7.5), 50 m*M* EDTA and 50 m*M* DTT; let stand 30 min at 4° and centrifuged at 100 000 *g* for 1 h; use supernatant. Reaction conditions, see MATERIALS AND METHODS under *continuous method*.

	Specific activity of Na–K ATPase[a]					
	Control		*NaI-treated*		*Lubrol + NaI-treated*	
	Olf.	*Br.*	*Olf.*	*Br.*	*Olf.*	*Br.*
Octanol (m*M*)						
0	11.9	30.3	11.7	49.1	10.8	15.4
0.45	13.6	26.7	15.1	38.2	4.7	14.3
4.5	10.9	23.5	8.6	22.5	4.0	10.7
9.0	5.4	5.8	3.2	—	—	—
Ouabain inhibition	18.5%	44.6%	58.2%	84.8%	72.0%	87.5%

[a] Specific activity = μmoles P_i mg^{-1} protein h^{-1}.

ouabain inhibition of the treated samples with control samples, it can be seen by the large increases in ouabain sensitivity that NaI treatment and lubrol solubilization caused specific inhibition of Mg^{2+} ATPase activity. As seen in Table II the Na ATPase activity of NaI-treated brain samples was even more sensitive to octanol inhibition than the control samples. However, the Na–K ATPase activity of the NaI-treated olfactory tissue sample showed a stimulation of 29% compared to only 16% stimulation for the olfactory tissue control sample at the low octanol concentration (0.45 m*M*). The amount of stimulation of Na–K ATPase activity at 0.45 m*M* octanol for different olfactory tissue preparations treated with NaI showed variations as did control samples as mentioned above. In one preparation from bovine olfactory tissue, NaI treatment caused an increase of 65% in the Na–K ATPase stimulation by 0.45 m*M* octanol compared to the enzyme activity from the control sample.

Also shown in Table II are the effects of increasing octanol concentration on the Na–K ATPase activity of lubrol-solubilized NaI-treated preparations of olfactory tissue and brain. The solubilized brain Na–K ATPase did not appear to be as sensitive to octanol, but compared with the NaI-treated samples solubilization caused a much greater loss of Na–K ATPase activity from brain than from olfactory tissue. It was of great interest to note that solubilization caused not only a loss of stimulation of olfactory tissue Na–K ATPase but that the enzyme was strongly inhibited by 0.45 m*M* octanol (Table II).

Effects on Mg^{2+} ATPase. The comparison of ATPase responses to octanol between olfactory tissue and brain B fractions in Fig. 1 shows that Mg^{2+} ATPase activity in both preparations was relatively insensitive to octanol. However, the Mg^{2+}

ATPase of B_2 (NEP concentrate) fractions (bottom Fig. 1) from both tissues was inhibited by octanol despite the fact that octanol had no effect on Mg^{2+} ATPase activity of the B fractions from which the B_2 fractions were obtained. The reason for this discrepancy can be seen in Fig. 3 (upper curves). The B_3 fractions from both tissues showed stimulation by octanol. Thus, the apparent lack of sensitivity of Mg^{2+} ATPase to octanol in the B fractions was the result of two opposite responses of this enzyme activity from different subcellular particle fractions. Since the B_3 fraction consisted of small NEP and free mitochondria, it was assumed that the mitochondrial Mg^{2+} ATPase was stimulated at 2–6 m*M* octanol concentration.

Fig. 3 also shows the response of Mg^{2+} ATPase to octanol in E_2 and E_3 fractions obtained by density gradient separation of an osmotically shocked B_2 fraction (see ref. 4). The entrapped mitochondria of the NEP were released and fragmented by osmotic shock and separated in E_3 from the plasma membrane (E_2). In Fig. 3 it can be seen that opposite responses were obtained from E_2 and E_3 subfractions. The plasma membrane concentrate (E_2) showed inhibition of Mg^{2+} ATPase, while the fraction with freed mitochondrial fragments showed stimulation of Mg^{2+} ATPase.

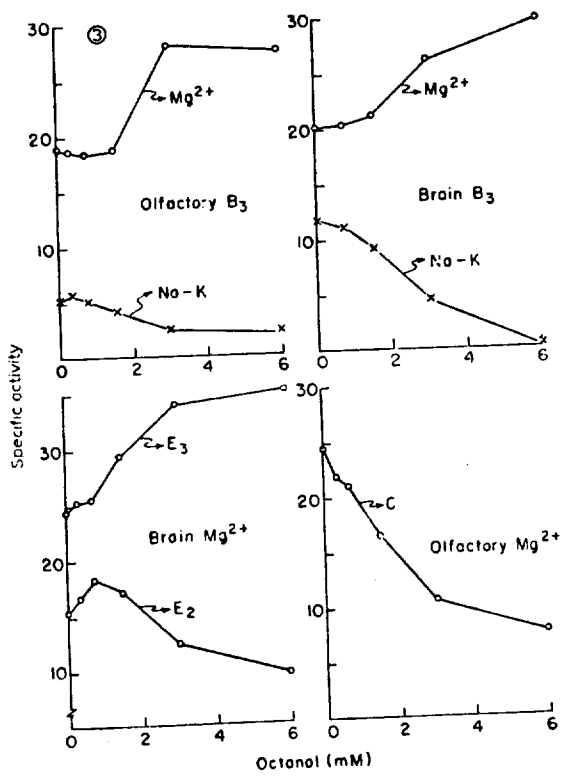


Fig. 3. Effect of concentration of octanol on the ATPase activities from various brain and olfactory tissue fractions: NEP + mitochondria (B_3), microsomes (C), plasma membranes (E_2), mixed mitochondria and nonmitochondrial membranes and subparticles (E_3). Enzyme method and reaction conditions same as Fig. 1. Na-K (Na-K ATPase), Mg^{2+} (Mg^{2+} ATPase).

Also the Mg^{2+} ATPase activity of the olfactory tissue microsomes (C fraction) showed inhibition by octanol (bottom right, Fig. 3). The above results strongly indicate that mitochondrial Mg^{2+} ATPase was stimulated and that nonmitochondrial (including plasma membrane) Mg^{2+} ATPase was inhibited by octanol.

Responses of ATPases to ascorbic acid

ASH[19] reported that ascorbic acid was a cofactor in a stimulant (linalool, a secondary alcohol) induced change at 267 nm in a preparation from olfactory epithelium. His results led to an investigation of the response of olfactory tissue ATPases to ascorbic acid. Because ascorbic acid rapidly oxidized to dehydroascorbic acid at pH 7.4 and 37 (bottom curve, Fig. 4), ascorbic acid solutions were prepared fresh twice daily and held in an icebath until added to the reaction mixture just prior to the addition of the enzyme. As little elapsed time as possible was allowed before taking the zero-time reading. As seen in Fig. 4, dithiothreitol (DTT) greatly retarded the oxidation of ascorbic acid to dehydroascorbic acid in buffer at pH 7.5.

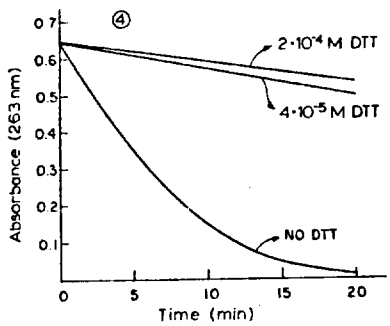


Fig. 4. Effect of indicated addition of DTT on the loss of absorbance at 263 nm due to autoxidation of ascorbic acid ($4 \cdot 10^{-5}$ *M*) in 0.2 *M* Tris buffer (pH 7.6) at 37°. Absorbance change measured with a Cary-14 automatic recording spectrophotometer.

It was observed that the addition of $4 \cdot 10^{-5}$ *M* ascorbic acid caused a transient stimulation of the total ATPase activity followed by partial inhibition. By the use of ouabain it was found that the complex response (as with octanol) was due to a combination of opposite responses by the Mg^{2+} and Na–K ATPases. Mg^{2+} ATPase activity showed an immediate stimulation which gradually decreased to the control level after about 30 min of reaction. Na–K ATPase was inhibited by ascorbic acid and the amount of initial inhibition was dependent on ascorbic acid concentration, while the total amount of inhibition was time dependent.

The ascorbic acid responses were studied using several different sources of tissue in which untreated and NaI- and lubrol-treated samples were compared (Table III). An overall comparison of the results showed that each of the two types of ATPase from the different tissues reacted similarly to ascorbic acid. The Na–K ATPase in an untreated fraction (left column under Na–K ATPase heading) showed greater sensitivity to ascorbic acid than the NaI- and lubrol-treated samples and the inhibition

TABLE III

EFFECT OF ASCORBIC ACID ON VARIOUS TREATED FRACTIONS FROM BRAIN AND OLFACTORY TISSUE HOMOGENATES

Br Mt_1 and Olf Mt_2, rabbit brain and olfactory tissue crude NEP fractions. Br M_s (Lamb), lamb brain microsomal preparation, NaI-treated, obtained from Dr. RONALD BARNETT, Chemistry Department, University of Minnesota. Specific activity = μmoles P_i mg^{-1} protein h^{-1} calculated from 20-min reaction time. Details of NaI and lubrol treatments given in Table II. ATPase activity measured by the continuous method (see MATERIALS AND METHODS). Buffered ascorbic acid (pH 7.5) was held at 4° until just prior to enzyme addition.

Ascorbic acid ($\times 10^5$ *M*)	*Specific activity* Mg^{2+} *ATPase*			*Na–K ATPase*		
	Br Mt_1	*NaI*	*+ Lubrol*	*Br* Mt_1	*NaI*	*+ Lubrol*
0	42.2	3.2	2.5	22.2	34.7	11.1
4	52.3	11.6	6.8	6.0	11.4	7.2
20	50.8	10.4	5.3	2.4	7.5	5.2
	Olf Mt_2	*NaI*	*+ Lubrol*	*Olf* Mt_2	*NaI*	*+ Lubrol*
0	27.0	8.7	3.5	10.0	14.0	8.8
4	33.1	11.6	5.8	5.7	7.2	9.1
20	31.0	11.4	5.2	3.6	5.6	6.9
	Br M_s *(Lamb)*	*NaI*	*+ Lubrol*	*Br* M_s *(Lamb)*	*NaI*	*+ Lubrol*
0	—	6.3	2.3	—	85.1	24.7
4	—	19.2	10.1	—	14.7	3.5
20	—	15.7	8.3	—	9.5	2.4

of Na–K ATPase was dependent on concentration of ascorbic acid (Table III). Mg^{2+} ATPase response to ascorbic acid was opposite that observed for Na–K ATPase (Table III). The actual increase in specific activity of Mg^{2+} ATPase was greatest for the untreated fractions, but the percentage stimulation of specific activity was much greater for the NaI- and lubrol-treated samples. Stimulation of Mg^{2+} ATPase activity showed an optimum below $20 \cdot 10^{-5}$ *M* ascorbic acid (Table III). These results indicated that a Mg^{2+} ATPase was present which was stimulated by ascorbic acid and resistant to inactivation by NaI and lubrol treatments.

Since DTT prevented the oxidation of ascorbic acid (Fig. 4), it was possible to determine the importance of the ascorbic acid to dehydroascorbic acid conversion in relation to the effects of ascorbic acid on ATPase activities. Table IV shows the results of various combinations of DTT and ascorbic acid on the ATPase activities of NaI-treated lamb brain microsomes. The inhibition of Na–K ATPase activity by ascorbic acid was reduced by DTT; the amount of reduction was related to the concentration of both compounds (Table IV). However, both compounds when used separately caused inhibition of the Na–K ATPase, but ascorbic acid was much more effective. The stimulation of Mg^{2+} ATPase by ascorbic acid was not affected by the presence of DTT, and when used alone, DTT had little or no effect on the Mg^{2+} ATPase activity (Table IV). When ascorbic acid was added prior to the addition of the enzyme, to an otherwise complete system and held at 37° for 10 min (a time sufficient for oxidation of ascorbate to dehydroascorbate), there was no effect on either ATPase activity.

TABLE IV

EFFECT OF ASCORBIC ACID AND DTT ON THE ATPase ACTIVITIES OF NaI-TREATED LAMB BRAIN MICROSOMES

Specific activity = μmoles P_i mg^{-1} protein h^{-1} calculated from 20-min reaction time. NaI-treated lamb brain microsomes (see footnote of Table III). Enzyme activity measured by continuous procedure (see MATERIALS AND METHODS). Ascorbic acid added just prior to enzyme addition (see Table III).

Ascorbic acid ($\times 10^5$ *M*)	*DTT* ($\times 10^5$ *M*)	*ATPase specific activity* Mg^{2+}	*Na–K*
0	0	11.9	84.8
4	0	19.7	15.5
4	4.4	19.2	63.7
4	22	21.7	71.2
20	0	18.7	5.0
20	4.4	17.0	44.6
20	22	17.3	82.5
0	4.4	13.5	76.2
0	22	12.5	59.9

Liver mitochondrial ATPase responses to octanol and ascorbic acid in the presence and absence of oligomycin

In order to obtain a better understanding of the observed differential effects of octanol and ascorbic acid and at the suggestion of Dr. R. W. ALBERS (personal communication), studies were conducted on a rabbit liver mitochondrial (B-type fraction) preparation using oligomycin. LARDY *et al.*[20], HUIJING AND SLATER[21], GLYNN[22], ASKARI AND KOYAL[23] and PALATINI AND BRUNI[24] have shown that Mg^{2+} ATPase from mitochondrial and submitochondrial particles was completely inhibited by oligomycin at concentrations as low as 10^{-7} *M*. They also found that Na–K ATPase was inhibited by oligomycin, but a much higher concentration (30–100 times) was required compared to Mg^{2+} ATPase inhibition. In addition, there was a residual oligomycin-insensitive Mg^{2+} ATPase activity.

The rabbit liver mitochondrial preparation showed no inhibition of ouabain (Table I), indicating the absence or a very low concentration of Na–K ATPase. Octanol (4.5 m*M*) stimulation of the Mg^{2+} ATPase activity was greater than 40% (Table V), while pentanol, at about the same concentration, caused inhibition of the enzyme activity. It was felt that octanol stimulation was unlikely to be caused by an uncoupling effect of oxidative phosphorylation because stimulation by octanol occurred in the presence of Mg^{2+} (ref. 25). In our preparation neither 0.1 m*M* DNP nor octanol had any effect on Mg^{2+} ATPase activity in the absence of Mg^{2+}. Also in the presence of Mg^{2+}, DNP had little or no effect on Mg^{2+} ATPase activity.

As seen in Table V, 0.1 μg oligomycin caused over 70% inhibition of the liver B fraction Mg^{2+} ATPase activity. Assuming a molecular weight of approx. 800 for oligomycin[26], $4.2 \cdot 10^{-8}$ *M* was sufficient for total inhibition of oligomycin-sensitive Mg^{2+} ATPase. 30 times the above concentration of oligomycin caused no further inhibition of the Mg^{2+} ATPase. The addition of octanol to the oligomycin-inhibited system

TABLE V

EFFECTS OF VARIOUS ADDITIVES ON LIVER MITOCHONDRIAL ATPase ACTIVITY

Protein content 16.5 μg/3 ml reaction mixture. ATPase measurement by continuous procedure (see MATERIALS AND METHODS). Oligomycin (15% A and 85% B) from Sigma. Liver mitochondria obtained as B fraction (13 000 g sediment) had 0% ouabain sensitivity.

Additive	*ATPase specific activity*[a]
None	52.8
Octanol (4.5 m*M*)	74.3
Pentanol (3 m*M*)	49.1
Pentanol (15 m*M*)	39.2
Oligomycin (0.1 μg)	14.9
Oligomycin (3.0 μg)	14.9
Then octanol (4.5 m*M*)	
from 0 to 3 min	14.9
from 5 to 10 min	42.0

[a] Initial reaction rates calculated from absorbance change from 1 to 6 min except where indicated. Specific activity calculated as μmoles P_i mg^{-1} protein h^{-1}.

caused no increase in the initial reaction rate of the Mg^{2+} ATPase activity (0–3 min). However, a gradual recovery of activity was observed with time (5–10 min) after addition of octanol (Table V). This recovery of oligomycin-inhibited ATPase activity was studied further and found to be affected by the length of time that octanol was vigorously stirred in the reaction mixture (Table VI — long "stir time" was about 45 sec, short time was a few seconds). It can be seen in Table VI that oligomycin inhibition can be reversed by octanol. However, the reversal was still time dependent (activity increased with reaction time even for the sample stirred for the longest time). These results show that the oligomycin-sensitive Mg^{2+} ATPase, the mitochondrial enzyme, was specifically stimulated by octanol.

The effect of ascorbic acid was also determined on the Mg^{2+} ATPase of liver B fraction in the presence and absence of oligomycin. Addition of ascorbic acid in the absence of oligomycin had little observable effect on ATPase activity. However, when ascorbic acid was added 20 min after addition of oligomycin (as was done in the octanol studies), a definite stimulation (over 40%) was observed (Table VI). The ascorbate stimulation occurred immediately and did not increase with time. Thus it appeared that the oligomycin-insensitive Mg^{2+} ATPase activity was specifically stimulated by ascorbic acid. The lack of observable effect of ascorbic acid on total Mg^{2+} ATPase activity was probably related to the fact that the oligomycin-insensitive activity was a relatively small percent of the total activity.

DISCUSSION

The results of these studies show that differences in properties of the Na–K and the Mg^{2+} ATPases can be observed by the use of octanol and ascorbic acid. Also in combination with oligomycin, it was possible to further show the specificity of action of these two compounds on mitochondrial and nonmitochondrial Mg^{2+} ATPase.

TABLE VI

TIME RESPONSE OF THE OCTANOL REVERSAL OF OLIGOMYCIN-INHIBITED LIVER MITOCHONDRIAL Mg^{2+} ATPase ACTIVITY AND EFFECT OF ASCORBIC ACID

Specific activity calculated as μmoles P_i mg^{-1} protein h^{-1} for each indicated reaction period. Enzyme activity measured by continuous procedure (see MATERIALS AND METHODS). Additions made with Hamilton syringe (1 or 5 μl) below the surface of rapidly stirred (Vortex mixer) solution.

Addition and sequence		*Specific activity (per indicated reaction period, min)*		
		0–5	*5–10*	*10–15*
None		49.6	46.4	42.5
Octanol (4.5 m*M*)		74.2	64.0	48.5
Oligomycin (3 μg)		13.9	10.0	8.9
Then octanol (4.5 m*M*)				
Stir time:	Short	8.1	6.6	11.3
	Medium	22.4	27.0	31.0
	Long	42.5	49.8	60.0
Oligomycin (3 μg)		16.3	12.1	10.0
Then ascorbate ($2 \cdot 10^{-4}$ *M*)		14.2	14.2	14.2

The use of octanol showed that there was a definite difference in reactivity of the Na–K ATPase between olfactory tissue and brain NEP preparations. At low levels of octanol (about 0.4 m*M*) Na–K ATPase from olfactory tissue showed definite stimulation of activity. The stimulation of Na–K ATPase from a particular preparation was further increased after treatment with NaI. The NaI treatment caused a specific inactivation of Mg^{2+} ATPase activity. In some instances we observed enhancement of the Na–K ATPase activity by NaI treatment. In most experiments, NaI treatment about doubled the octanol stimulation that was observed in the untreated preparations.

Lubrol solubilization of either the untreated or NaI-treated preparation caused a loss of enzyme stimulation by octanol. In fact, the lubrol-solubilized NaI-treated preparation showed a fairly strong inhibition at 0.4 m*M* octanol. Although treatment of control fractions (B and B_2) with a low concentration of lubrol WX (0.1% as suggested by SHIRACHI *et al.*[10]) caused an increase in specific activity of Na–K ATPase from olfactory tissue, the stimulation by low levels of octanol was still eliminated (unpublished data).

Thus it appears that the Na–K ATPase from rabbit olfactory tissue has properties significantly different from the same enzyme activity from brain. The fact that the stimulation of olfactory tissue Na–K ATPase by octanol was eliminated by lubrol solubilization indicated a possible importance of the "normal" lipoprotein structure in the stimulation process. This observation has led us to consider further studies on the relation of lipids to the enzyme stimulation. Initial efforts to restore octanol stimulation of Na–K ATPase activity by addition of soy, egg or brain phospholipids have been unsuccessful. However, KAGAWA AND RACKER[27] and PALATINI AND BRUNI[24] have shown that phospholipid was essential for oligomycin inhibition of ATPase activities. Studies are contemplated to examine whether olfactory tissue lipids may be selectively required for the octanol stimulation.

The results obtained from studies using ascorbic acid addition to systems con-

taining brain and olfactory tissue preparations showed that ATPase response was the same for both tissues. Na–K ATPase activity was very sensitive to ascorbic acid and we found that in order for it to be effective, reaction conditions had to be such that ascorbic acid could be readily oxidized to dehydroascorbic acid. The presence of DTT, which retarded ascorbate oxidation, also retarded Na–K ATPase inhibition by ascorbic acid (Fig. 4, Table IV). However, DTT by itself caused some inhibition of Na–K ATPase activity. It appears that the strong reducing action of ascorbic acid may be responsible for Na–K ATPase inhibition; why DTT prevents this action is not understood.

A further effect of ascorbic acid was observed with Mg^{2+} ATPase activity. By the use of a rabbit liver mitochondrial preparation and the specific mitochondrial Mg^{2+} ATPase inhibitor oligomycin, it was possible to show that ascorbic acid specifically stimulates the nonmitochondrial Mg^{2+} ATPase. The fact that NaI and lubrol treatments caused large increases in percent stimulation of residual Mg^{2+} ATPase activity by ascorbic acid showed that the mitochondrial Mg^{2+} ATPase activity was apparently more readily inactivated by these treatments. The stimulation of Mg^{2+} ATPase was apparently not due to oxidation of ascorbic acid, since stimulation occurred in the presence of DTT (Table IV). Neither DTT nor dehydroascorbic acid had any effect on Mg^{2+} ATPase activity. Contrary to octanol stimulation of olfactory tissue Na–K ATPase, lubrol solubilization did not eliminate the stimulation of Mg^{2+} ATPase caused by ascorbic acid. Further studies are required to determine the nature and specificity of ascorbic acid stimulation of nonmitochondrial Mg^{2+} ATPase.

Although the selective stimulation of olfactory tissue Na–K ATPase activity is only weak evidence for its possible participation in the initiation process of odor sensing, this along with other findings leads us to make such a speculation. We have observed in preliminary studies that the Na–K ATPase stimulation by low levels of octanol can be eliminated by the addition of odor counteractants (unpublished data). Complete inhibition of octanol stimulation can be obtained at significantly lower concentrations of the odor counteractant than octanol.

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Nitrite Toxicosis in the Ascorbic Acid-Deficient Guinea Pig[1–3]

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Nitrite Toxicosis in the Ascorbic Acid-Deficient Guinea Pig. KOCIBA, RICHARD J., and SLEIGHT, STUART D. (1970). *Toxicol. Appl. Pharmacol.* **16**, 424–429. Subcutaneous administration of 50 mg/kg $NaNO_2$ produced significantly higher levels of methemoglobin in ascorbic acid-deficient guinea pigs than in controls. This dosage was fatal to a high percentage of the deficient group, whereas all control guinea pigs survived. Pretreatment of deficient guinea pigs with 10 mg/kg methylene blue protected against high levels of methemoglobin formation following the administration of 50 mg/kg $NaNO_2$.

Abortions occurred in a high percentage of ascorbic acid-deficient female guinea pigs following the subcutaneous administration of 45 mg/kg $NaNO_2$. This same dosage caused no abortions in the control females. Ascorbic acid-deficient females not given $NaNO_2$ had normal litters.

Maternal blood levels of methemoglobin were significantly higher in ascorbic acid-deficient pregnant females following the subcutaneous administration of 40 mg/kg $NaNO_2$. The accompanying levels of methemoglobin in fetuses derived by laparohysterotomy were similar in both the deficient and control groups. These data indicated that the fetal deaths were related to higher levels of methemoglobin in maternal blood of ascorbic acid-deficient guinea pigs.

The use of food containing nitrate and nitrite compounds has continued to increase in recent years. Interest in the toxic properties of these compounds has correspondingly increased. Improperly processed meat products, vegetables and certain well waters may contain toxic levels of these compounds.

The most prominent feature of nitrite toxicosis is the conversion of hemoglobin to methemoglobin. Nitrate must undergo reduction to nitrite before it can cause the formation of excess levels of methemoglobin. Hölscher and Natzschka (1964) described methemoglobinemia in young infants following the ingestion of spinach. Apparently nitrate present in the spinach was reduced to nitrite during storage. The hazards of excess nitrites and nitrates in water supplies have been emphasized since Comly (1945) described cyanosis in human infants after ingestion of well waters containing nitrate

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[2] This study was supported in part by the National Institute of Child Health and Human Development, Grant HD01600-03, and was completed as part of the requirements for the Master of Science degree.

[3] Michigan Agricultural Experiment Station Journal Article No. 4702.

pparently the human infant is more susceptible than the adult to the action of met-emoglobin-forming agents. The higher gastric pH and greater relative fluid intake in the ormal infant facilitate the bacterial reduction of nitrate to nitrite.

Sleight and Atallah (1968) reviewed the effects of nitrate and nitrite on reproduction. In their work, reproductive disturbances were noted in female guinea pigs treated with KNO_3 and KNO_2. Abortion, fetal absorption and mummification were noted. Sinha (1968) recently reported that a minimal dosage of 60 mg kg $NaNO_2$ was capable of producing abortion in the guinea pig.

To properly evaluate the potential health hazards of nitrite compounds, various other factors that may modify the susceptibility to nitrite toxicosis must be considered. One variable factor common to both the human and animal populations is the nutritional status of the body. Relatively little experimental work has been done with respect to nitrite toxicosis during a period of nutritional deficiency.

The present experiments were attempts to determine the effects of nitrite toxicosis during a nutritional deficiency of ascorbic acid. Special emphasis was placed on the pregnant animal, in which nutritional deficiencies are more common.

The guinea pig was selected as the test animal because of its similarity to man in that both species are dependent on dietary ascorbic acid. Subcutaneous injections of $NaNO_2$ were used in all cases to eliminate experimental variables which would have been introduced by other methods. Levels of methemoglobin formed as a result of the oxidation of hemoglobin by nitrite were determined. The reproductive performance of ascorbic acid-deficient guinea pigs following nitrite toxicosis was also studied.

MATERIALS AND METHODS

Experiment A

Twelve guinea pigs were maintained on a commercial test diet[4] deficient in sources of ascorbic acid until the plasma concentration of ascorbic acid decreased to subnormal values (Schow, 1966). The diets of 6 control guinea pigs were supplemented with cabbage as a source of ascorbic acid. Guinea pigs of both groups were injected with 50 mg/kg $NaNO_2$ subcutaneously. Blood samples were collected 60, 90, and 120 min after injection of $NaNO_2$. Hemoglobin, packed cell volume (PCV), plasma ascorbic acid and methemoglobin values were determined by standard procedures. Six additional guinea pigs, 3 of which had subnormal plasma ascorbic acid levels, were pretreated with 10 mg/kg methylene blue ip 30 min prior to injection of 50 mg/kg $NaNO_2$ sc. Blood samples were collected and analyzed in the same manner outlined above.

Experiment B

Eleven pregnant guinea pigs with similar body weights and various plasma ascorbic acid concentrations were injected subcutaneously with 45 mg/kg $NaNO_2$ during the last week of gestation. Reproductive performance was recorded at the time of abortion or parturition. Three additional guinea pigs with subnormal plasma ascorbic acid levels were allowed to carry their fetuses through normal parturition with no additional treatment. All aborted and live fetuses were examined for the presence or absence of gross malformations.

[4] Nutritional Biochemicals Corp., Cleveland, Ohio.

Experiment C

Eight pregnant guinea pigs, 5 of which had subnormal plasma ascorbic acid levels, were injected during the last week of gestation with 40 mg/kg $NaNO_2$ sc. Fifty-five minutes after injection of $NaNO_2$, ether anesthesia was induced and laparohysterotomy was performed. Maternal and fetal blood samples were collected 60 min after injection for determination of hemoglobin, packed cell volume (PCV), and plasma ascorbic acid. Methemoglobin levels were also determined.

RESULTS

Experiment A

Administration of 50 mg/kg $NaNO_2$ produced significantly higher values of methemoglobin in ascorbic acid-deficient guinea pigs as compared to controls (Table 1). These higher levels of methemoglobin were noted in blood samples collected 60, 90, and 120 min after injection of $NaNO_2$. The F value obtained following the weighted square of means analysis was significant at the $P = 0.01$ level. Pretreatment with 10 mg/kg methylene blue prevented the formation of high levels of methemoglobin subsequent to the injection of 50 mg/kg $NaNO_2$ in deficient guinea pigs.

A high mortality rate (83.3%) occurred in the ascorbic acid-deficient guinea pigs injected with 50 mg/kg $NaNO_2$ alone. No deaths occurred in the ascorbic acid-deficient guinea pigs pretreated with 10 mg/kg methylene blue before injection of 50 mg/kg $NaNO_2$.

Experiment B

The injection of 45 mg/kg $NaNO_2$ caused abortion in the pregnant guinea pigs with plasma ascorbic acid levels below 0.40 mg/100 ml (Fig. 1). This same dosage of $NaNO_2$

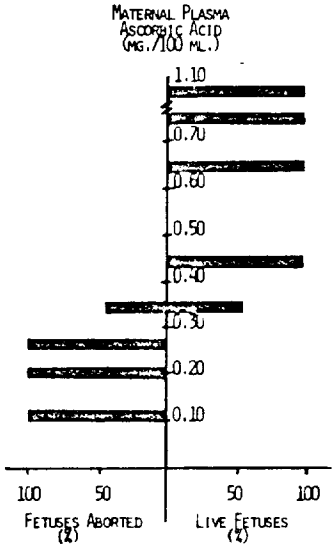

FIG. 1. Reproductive performance of pregnant guinea pigs after treatment with 45 mg/kg $NaNO_2$ during the final week of gestation.

TABLE 1

AVERAGE HEMATOLOGIC VALUES, METHEMOGLOBIN CONCENTRATIONS, AND MORTALITY RATES OF GUINEA PIGS USED IN EXPERIMENT A

Number of guinea pigs	Plasma ascorbic acid (mg/100 ml)	Hb (g/100 ml)	PCV (%)	$NaNO_2$ (mg/kg)	Methylene blue (mg/kg)	Methemoglobin[c] (%) Minutes 60	90	120	Mortality rate (%)
12[a]	0.34	11.1	36	50	0	60.0 ± 6.1[d]	57.1 ± 8.4[d]	29.4 ± 7.6[d]	83.3
6[b]	0.88	11.7	37	50	0	43.4 + 2.7	36.5 ± 0.6	17.6 ± 0.9	0
3[a]	0.37	10.1	35	50	10	8.6 ± 0.8	6.7 ± 0.7	3.1 ± 1.0	0
3[b]	0.97	12.5	41	50	10	5.4 ± 1.3	3.3 ± 1.1	1.4 ± 0.6	0

[a] Guinea pigs maintained on diet deficient in ascorbic acid.
[b] Control guinea pigs.
[c] Values expressed as mean ± standard error of the mean.
[d] Significantly different from appropriate control group, $P < 0.01$.

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did not cause abortion in guinea pigs with higher levels of plasma ascorbic acid. The ascorbic acid-deficient guinea pigs that were allowed to undergo normal parturition with no $NaNO_2$ treatment did not abort, and all gave birth to live fetuses. All fetuses were free of gross abnormalities.

TABLE 2

AVERAGE MATERNAL AND FETAL HEMATOLOGIC VALUES OF GUINEA PIGS USED IN EXPERIMENT C

Number of pregnant guinea pigs	Plasma ascorbic acid (mg/100 ml)	Hb (g/100 ml)	PCV (%)
5 Maternal	0.33	9.5	31
Fetal	0.57	11.0	40
3 Maternal	1.30	12.0	43
Fetal	1.65	11.7	41

Experiment C

Maternal hemoglobin, PCV, and plasma ascorbic acid values decreased to lower levels than those found in their fetuses during ascorbic acid deficiency (Table 2). Sixty minutes after injection of 40 mg/kg $NaNO_2$, the deficient dams had a mean methemoglobin level of 35.2% (Fig. 2). At this same time interval the mean methemoglobin level was 25.9% in the control dams. Analysis of these data by the Mann-Whitney U test showed significance at the $P = 0.05$ level. The mean methemoglobin levels in the fetuses of the deficient and control groups were 10.1% and 10.3%, respectively.

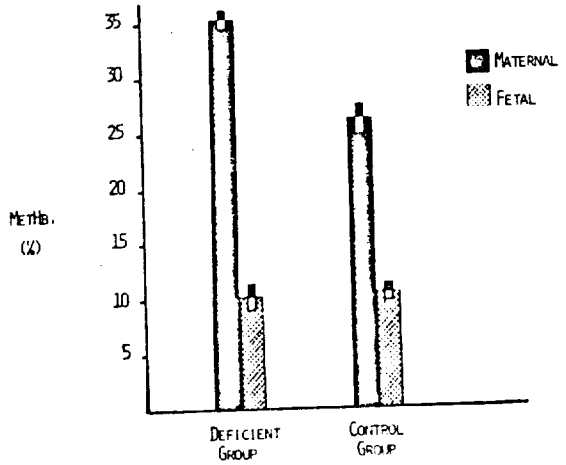

FIG. 2. Methemoglobin concentrations 60 min after injection of 40 mg/kg $NaNO_2$ in pregnant guinea pigs. Standard error is indicated at the top of each bar.

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DISCUSSION

The significantly higher methemoglobin concentrations noted in the ascorbic acid-deficient guinea pigs were probably the result of (1) a relative decrease in ascorbic acid available as a reducing substance, and (2) decreased hemoglobin and PCV values, which increased the ratio of nitrite per erythrocyte. The high mortality rate in the deficient group following nitrite toxicosis was most likely due to anoxia subsequent to high levels of methemoglobin. Pretreatment with methylene blue prior to $NaNO_2$ prevented the formation of high concentrations of methemoglobin by facilitating the enzymatic reduction of methemoglobin back to hemoglobin. The slightly higher levels of methemoglobin that persisted in the pretreated deficient group possibly could be explained by the lack of ascorbic acid as a reducing substance and lowered hemoglobin and PCV values.

Previous work (Sinha, 1968) had indicated a minimal dosage of 60 mg kg $NaNO_2$ was capable of causing abortion in the guinea pig. In this study pregnant guinea pigs with plasma ascorbic acid below 0.40 mg 100 ml aborted following the injection of 45 mg/kg. This indicated a subnormal plasma ascorbic acid level was associated with an increased susceptibility to $NaNO_2$ toxicity in the pregnant guinea pig.

The increased fetotoxic effects of $NaNO_2$ in the ascorbic acid-deficient guinea pig were associated with higher levels of methemoglobin in the dam. Apparently the partial placental barrier to $NaNO_2$ was not altered during ascorbic acid deficiency.

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XXVII. THE VITAMIN C CONTENT OF COW'S MILK

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(*Received 8 December 1936*)

It has been shown in a previous paper [Kon & Watson, 1936] that vitamin C may be present in milk in two forms, as ascorbic acid and as dehydroascorbic acid. Both are biologically active, but the latter does not react with the indophenol reagent unless treated with H_2S, is labile and undergoes spontaneous decomposition associated with loss of biological activity. Under the action of light the reduced form of ascorbic acid present in milk is reversibly oxidized to dehydroascorbic acid.

The present paper deals with the state in which vitamin C is secreted by the cow and presents estimations of the vitamin C content of herd milk at different times of the year under south of England conditions.

Experimental

A description of the methods used in estimating ascorbic and dehydroascorbic acids in milk will be found in the paper by Kon & Watson [1936].

(1) *The state in which vitamin C is secreted by the mammary gland of the cow*

For the purpose of this investigation it was necessary to obtain milk directly from the udder without any exposure to light. The rubber lining of a teat cup of a milking machine was slipped over the teat and its other end was placed in a milk bottle wrapped in black paper. The milk was then milked into the bottle and the vitamin C titration was carried out at once. All operations were done with the least exposure to light. The results of several estimations are given in Table I which shows that milk as it leaves the normal udder contains only the reduced form of ascorbic acid.

Table I. *The reduced (R) and total (T, reduced and reversibly oxidized) ascorbic acid of milk immediately after milking in the absence of light*

mg. of ascorbic acid in 100 ml.

Cow		Ascorbic acid	
Name	Breed	R	T
Flora 16	Shorthorn	2·30	2·37
Pippin	"	1·86	1·93
Flora 31	"	2·45	2·45
Campion 2	"	2·17	2·17
Rosey 8	Guernsey	2·23	2·31
Rosalie 8	"	2·02	2·02
	Average	2·17	2·21

(2) *The vitamin C content of milk at different times of the year*

For the purpose of this investigation "sale" milk[1] was obtained from the Institute's dairy immediately after passing over the cooler, shortly after the morning milking. The milk was from a mixed herd of Shorthorns and Guernseys, which during the time covered by the experiment consisted of 26 Shorthorn and 3 Guernsey cows. All samples were handled in a uniform way with the same amount of exposure to light. Estimations were carried out once or twice a week, but daily measurements were taken for a fortnight after the cows were turned out to grass in spring. The estimations of reduced and total ascorbic acid are given in Fig. 1. There is no evidence of any effect of pasture feeding on the level of

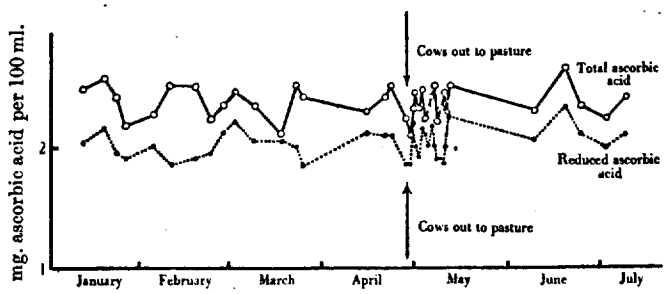

Fig. 1. The vitamin C content of herd milk at different times of the year.

vitamin C in milk. The average figures work out at 2·06 and 2·37 mg./100 ml. for milk from stall-fed cows and 2·06 and 2·35 mg./100 ml. for pasture milk respectively.

(3) *The vitamin C content of colostrum*

The relatively very high concentrations of carotene and vitamin A in colostrum as compared with milk [Dann, 1933; Semb *et al.* 1934] made it of interest to investigate the level of vitamin C in the colostral secretion.

Table II presents the results of such estimations in the case of 4 cows, while the average values are plotted in Fig. 2.

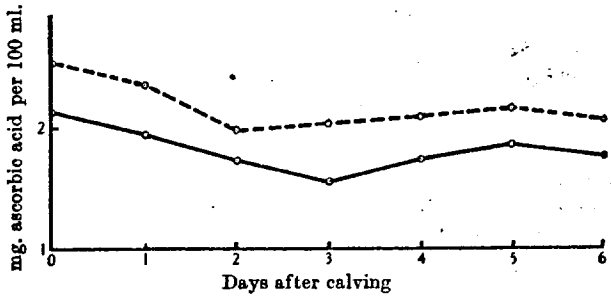

Fig. 2. The vitamin C content of colostrum, average curves for 4 cows.
--- Total ascorbic acid. —— Reduced ascorbic acid.

In three cases out of the four studied the vitamin C content of colostrum is higher than the average for the milk of the Institute's herd. The fourth colostrum contains definitely less vitamin C. The high values drop rather suddenly after

[1] Grade A (T.T.).

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Table II. *The vitamin C content of colostrum*

R, reduced; T, total ascorbic acid in mg. per 100 ml.

Days after calving	Lottie 7 Shorthorn		Lottie 9 Shorthorn		Winnie 6 Shorthorn		Valven 2 Guernsey	
	R	T	R	T	R	T	R	T
0	2·34	2·72	1·41	1·62	2·60	3·20	2·20	2·54
1	1·93	2·41	1·79	2·06	2·50	2·97	1·58	1·93
2	1·51	1·75	1·72	1·99	1·93	2·08	1·76	2·11
3	1·31	1·93	1·41	2·04	1·71	2·08	1·69	2·04
4	1·79	2·06	1·76	2·11	1·86	2·23	1·48	1·90
5	1·27	1·94	1·90	2·18	2·26	2·30	1·94	2·18
6	1·41	1·97	1·97	2·11	1·82	2·15	1·83	2·04

2 days at most to a level lower than the normal average. Lack of information does not allow us to say whether this is a transient depression or whether the milks of these particular cows contained permanently less vitamin C. It is evident that the difference between colostrum and milk is much smaller in the case of vitamin C than in the cases of vitamin A and carotene.

(4) *Effect of breed*

Judging from the figures in Tables I and II the vitamin C content of Guernsey milk is the same as that of Shorthorn milk. The few estimations can be taken only as an indication.

(5) *Vitamin C in mastitis*

A solitary estimation of the vitamin C content of the milk secreted by a normal and an infected quarter of the udder of a cow suffering from mastitis has given results sufficiently interesting to be included in this paper. The diseased quarter was found to give milk definitely poorer in vitamin C than that secreted by the normal quarter. The values were 2·19 mg./100 ml. of reduced and 2·48 mg./100 ml. of total ascorbic acid in the normal and 1·44 and 1·87 mg./100 ml. respectively in the infected quarter.

Discussion

The finding that the level of vitamin C in mixed milk is remarkably constant and independent of the season and the nutrition of the cow is in agreement with reports from other laboratories which have made use of the dichlorophenol-indophenol reagent [Harris & Ray, 1935; van Wijngaarden, 1935; Riddell *et al.* 1936].

It is true that Rasmussen *et al.* [1936], who also used the reagent, express the opinion that "after the first two months of lactation, the ascorbic acid content of milk is apparently dependent solely upon the ascorbic acid content of the diet of the cow". This opinion however is not based on experimental evidence, as throughout the experiment the diet of the cows remained unchanged. The authors have obviously not considered the possibility of a synthesis of vitamin C by the cow. They report values for the vitamin C content of milk definitely lower on the whole than those found by us and by van Wijngaarden [1935] and Riddell *et al.* [1936]. It seems probable that their observations have been complicated by exposure of the samples to light, especially as some of them were stated to have developed a cardboard flavour on standing. Marked seasonal variations have been reported by Stoerr [1936], who used the Bezssonoff reagent. These findings have not so far been confirmed.

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Summary

1. The healthy mammary gland of the cow secretes vitamin C only in the reduced form. No reversibly oxidized ascorbic acid is found in the milk as it leaves the udder.

2. Under south of England conditions, the season of the year and the nutrition of the cows have no effect on the vitamin C content of herd milk. The average figures for sale milk from a herd composed of about 26 Shorthorn and 3 Guernsey cows work out at 2·01 mg. of reduced and 2·37 mg. of total ascorbic acid per 100 ml. of milk during the stall-feeding period and 2·06 and 2·35 mg. 100 ml. respectively on early pasture.

3. The vitamin C content of colostrum is only slightly higher than that of milk.

Our best thanks are due to Miss D. V. Dearden for much help in obtaining the samples of milk.

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EFFECT OF SERUM-ASCORBIC-ACID LEVELS ON BABOON HEART-MUSCLE LIPOPROTEIN LIPASE

Baboon no.	Without ascorbic acid: Serum-ascorbic acid (mg. per 100 ml.)	Without ascorbic acid: Lipoprotein-lipase activity (neq. F.F.A. released per mg. dry mass per minute)	Baboon no.	With ascorbic acid: Serum-ascorbic acid (mg. per 100 ml.)	With ascorbic acid: Lipoprotein-lipase activity (neq. F.F.A. released per mg. dry mass per minute)
32	0·25	345	37	1·25	76
71	0·24	249	56	1·09	186
72	0·16	495	62	1·26	231
81	0·18	444	64	1·26	209
85	0·21	320	66	1·36	222
89	0·11	384	80	1·26	217
90	0·16	492	82	1·41	125
Average S.D.	0·19 ±0·050	389·9 ±92·4		1·27 ±0·10	180·9 ±58·4

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EFFECT OF ASCORBIC ACID ON LIPOPROTEIN-LIPASE ACTIVITY OF PRIMATE HEART MUSCLE

SIR,—The removal of lipoprotein triglycerides from the circulation system is facilitated by the enzyme lipoprotein lipase, and the control of lipoprotein-lipase activity in rat adipose tissue has been well summarised by Robinson and Wing.[1] Although heparin releases only a portion of rat-heart lipoprotein lipase, the fraction released is concerned with triglyceride/fatty-acid utilisation.[2,3] During fasting, increases in lipoprotein-lipase activity occur in the heart and skeletal muscle, and simultaneously the activity in adipose tissue declines.[3,4] Robinson[5] interpreted these changes as being concerned with the direction of plasma-triglyceride/free-fatty-acids (F.F.A.) away from storage in adipose tissue and towards oxidation by muscle during starvation.

Ascorbic acid has been implicated in hydroxylation reactions—i.e., the formation of hydroxyline[6]—and drug metabolism.[7] Lately there has been an upsurge of literature connecting ascorbic acid with hypocholesteræmic effects in guineapigs and man.[8-10] However, this finding has been questioned by Anderson et al.[11] Very little is known about the effect of ascorbic acid on lipoprotein-lipase activity.[1] Therefore, we set out to determine the effect of ascorbic-acid feeding on the lipoprotein-lipase activity of the heart muscle of 14 young baboons, *Papio ursinus*.

14 animals, with an average body mass of 11 kg. (range 7 to 13 kg.) were divided into two groups of 7, and all received a stiff porridge consisting of precooked maize meal, skimmed-milk powder, and a salt solution.[12] One group, however, received in addition about 34 mg. ascorbic acid per kg. body-mass per day. The experiment lasted 3 months. Animals were immobilised with 'Sernylan' (phencyclidine), 1 mg. per kg., for blood-sampling and subsequent ascorbic-acid determinations. Ascorbic acid was determined on serum samples according to the method of Roe and Kuether[13] as modified by Schaffert and Kingsley.[14] Baboon heart muscle was obtained from the heart apex after anæsthesia with sodium pentobarbitone. The samples were rinsed in cold Krebs-Ringer bicarbonate buffer, and the mass obtained was homogenised in three times its mass, of Krebs-Ringer bicarbonate buffer with an Ultra Turrax homogeniser for 1 minute at 2°C. In order to release the lipoprotein lipase, sodium heparin was added to Krebs-Ringer buffer to make 250 I.U. per ml. The homogenised samples were incubated under a constant flow of a gas mixture (CO_2/O_2; 5%/95%) for 30 minutes. Lipoprotein lipase was determined according to the method described by Huttunen et al.,[15] but, instead of radioactive labelled ^{14}C triolein, cold triolein was used, and the liberated fatty acids were determined according to the method of Novak.[16] Dry mass was determined by mass difference after drying of aliquots at 105°C for 24 hours.

Although the levels of serum-ascorbic-acid in the control animals (no dietary ascorbic acid) were very low and averaged 0·19 mg. per 100 ml., no clinical signs of scurvy could be detected (see table). This ascorbic-acid serum-level did, however, have a pronounced effect on the lipoprotein-lipase activity extractable from baboon heart muscle, because the average activities were 2·15 times that of the animals receiving ascorbic acid. This finding must be of metabolic significance, because Borenstajn and Robinson[3] reported that the extractable lipoprotein lipase is responsible for the removal of lipoprotein triglycerides from the blood. Furthermore, the diet deficient in ascorbic acid had an effect on heart-muscle lipoprotein-lipase activity similar to that of the fasting condition.

A diet deficient in ascorbic acid would take about 6 months to cause clinical signs of scurvy in man. It is therefore important to note that in apparently healthy animals in our experiment such a big difference in heart-muscle lipoprotein lipase was caused by ascorbic-acid

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feeding. The average serum-ascorbic-acid level of 1·27 mg. per 100 ml. in the animals receiving 34 mg. ascorbic acid per kg. per day was still lower than 1·56 mg. per 100 ml. found in 209 wild free-living baboons of the Kruger National Park.[4]

Further experiments are in progress on the control mechanism of ascorbic acid on primate lipoprotein-lipase activity, because to our knowledge this is the first report of the phenomenon.

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VITAMIN CONTENT OF CITRUS PRODUCTS[1]

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In view of the importance of citrus fruits in the human dietary, it is considered important to obtain as adequate information as is possible concerning the vitamin content of these fruits and the various products into which they are processed. Although considerable information is at hand concerning the thiamine, niacin, pantothenic acid, carotene, and ascorbic acid content of citrus fruits and juices, only scattered data have been published on their biotin, pryidoxine, folic acid, and inositol content. It is further important to have more extensive information on the effects of processing and storage on this latter group of vitamins.

The present study was initiated, therefore, for the purpose of obtaining comprehensive data on the biotin, pyridoxine, folic acid, and inositol content of various citrus fruits, and to evaluate the effects of processing and storage on these vitamins. In addition, total soluble solids (expressed as sucrose), total acid (expressed as anhydrous citric acid), and total and reduced ascorbic acid, were determined for purposes of reference to other published data on similar classes of material.

EXPERIMENTAL PROCEDURE

Materials Investigated: Homogeneous samples, both fresh and canned, of orange, grapefruit, and tangerine juices; comparable fresh and 42° Brix frozen concentrated orange juice, and 65° Brix pasteurized concentrated orange juice; canned orange sections, grapefruit sections, and citrus salad (mixture of orange and grapefruit sections) were examined. All citrus studied was produced and processed in Florida.

Sampling Procedure: The problem of obtaining a "representative sample" of a natural product always poses considerable difficulty, owing to the great variation that exists in the vitamin content within any species, and even variety of a species, of a natural product. One evidence of this variation can be seen from the data obtained on the vitamin C content of the various citrus products. Values obtained are in general below the accepted "average" vitamin C value, as a result of the fact that the samples analyzed were collected late in the packing season, at which time the vitamin C content of the fruit is lowest. It was decided, therefore, that the most reasonable sampling procedure would be one that would give a composite value under actual, practical processing circumstances. Seventeen Florida citrus processing plants cooperated in obtaining the samples, and the actual preparation and storage of the samples was carried out by the research personnel of the Florida Citrus Commission and the University of Florida Citrus Experiment Station, Lake Alfred, Florida.

[1] This study was made possible through a grant in aid provided by the Florida Citrus Commission, Lakeland, Florida.

The number of boxes of fruit processed during any sampling period was recorded, and at regular intervals during the processing day samples of the fresh juice were withdrawn from the holding tank prior to pasteurizing and filling, and canned samples taken immediately after the pasteurization and filling process. In this way homogeneous samples of fresh and canned products were obtained. The samples of fresh juice were placed in cans, sealed, and frozen as quickly as possible by immersion in a dry ice-alcohol bath. Samples so prepared were considered "fresh" and were stored and shipped in the frozen state and remained so until thawed and opened for analysis. For the most part, then, a "sample" consisted of at least 10 cans of juice, fresh or canned, collected at regular intervals during a processing day and, therefore, represented a composite sample of a large number of boxes of fruit. This system of sampling has further merit, in that it more clearly represents the average value of the citrus product as it might be obtained by the purchaser for consumption, and of course gives the most clear-cut comparison between the fresh and canned product. Type of fruit sampled and number of boxes represented in a sample are shown for the representative classes of citrus (Tables 2, 3, 4, and 5).

The samples were first stored at controlled storage temperatures at the Citrus Experiment Station, Lake Alfred, Florida, and then forwarded to the Yale Nutrition Laboratory, New Haven, Connecticut. The frozen "fresh" samples were packed in dry ice and shipped air express; all other samples were shipped by railway express. Temperatures and duration of storage before analyses are shown (Tables 1 to 5).

At the time of analyses, the 10 or more cans that represented the composite sample were opened and their contents pooled, after which suitable portions were withdrawn for the respective analyses. The frozen "fresh" samples and the 42° Brix frozen concentrated orange juice were thawed rapidly by placing in a 70 to 80°C.(158 to 176°F.) water bath until the juice was not quite all melted. The cans were then opened and their contents pooled for subsequent analyses. Since this method of treatment did not impair the stability of vitamins in fresh orange juice frozen and stored in the laboratory (see Table 1), it was considered satisfactory.

Samples of grapefruit sections and the citrus salad were prepared by opening the 10 or more cans of the composite sample, blending to a fine consistency in the Waring Blendor, then pooling the blended sample, from

TABLE 1

Effect of Storage at —10°C.(14°F.) on Fresh Orange Juice

Sample	Storage time	Total solids (Brix)	Total acid (Citric)	Ascorbic acid		Biotin	Folic acid	Pyridoxine	Inositol
				Reduced	Total				
		pct.	*pct.*	*mg./100 ml.*	*mg./100 ml.*	*µg./100 ml.*	*µg./100 ml.*	*µg./100 ml.*	*mg./100 ml.*
Fresh orange juice (prepared from Florida oranges, Valencia)	Fresh	12.2	1.12	55.8	56.2	0.80	2.4	23.2	178
	1 mo.	12.3	1.11	54.7	56.0	0.75	2.2	22.4	162
	2 mo.	12.2	1.12	55.0	56.7	0.72	2.0	24.6	164
	4 mo.	12.2	1.13	54.2	54.4	0.79	2.6	22.1	165
	6 mo.	12.2	1.12	54.6	55.1	0.75	2.7	24.2	170
	8 mo.	12.2	1.12	53.9	54.2	0.77	2.9	22.7	171

which portions were then taken for analyses. At the same time, a Number 2 enamel-lined can was filled with this pooled sample, sealed, and frozen immediately in a bath of dry ice and acetone mixture. These re-canned, pooled samples were kept stored at —10°C.(14°F.) and reserved in case any repetition of analysis was required. This procedure proved to be valuable, since in several instances it was desired to confirm first-run values for biotin and folic acid. From results obtained with this procedure for storing samples there was no reason to believe it was not satisfactory.

In order to evaluate the effect of frozen storage on the vitamin content of fresh citrus juice, and to verify that our field-collected fresh frozen samples could be considered "fresh" for the purposes of our study, fresh orange juice prepared in the laboratory from Florida oranges (Valencia) was analyzed immediately for the various vitamins in question, and a large portion of the same juice was at the same time frozen and stored at —10°C. (14°F.) in enamelled Number 2 cans. At intervals of one, two, four, six and eight months thereafter a can of this juice was opened for repeat analyses. Results of these analyses may be found (in Table 1).

METHODS OF ANALYSIS

Total soluble solids, as sucrose, were determined with a Brix Hydrometer, which had been checked in a salt solution against a Bureau of Standards Hydrometer. The spindle checked within 0.05 degree Brix. The 42 and 65° Brix concentrated orange juice were diluted with three and seven parts of water, respectively, before total solids were determined and portions taken for other analyses.

Total solids were determined for the grapefruit sections and the citrus salad by taking a suitable portion of the blended sample and determining actual dry weight by drying the sample in a drying oven at 110°C.(230°F.) to a constant weight.

Total acid, as anhydrous citric acid, was determined by titrating 25 ml. of the juice or blended sample of sections with standard NaOH, one ml. of which was equivalent to 20 mg. of anhydrous citric acid. Phenolphthalein was used as the indicator.

Reduced ascorbic acid was determined by the photometric method of the National Canners' Association (1944). An Evelyn Colorimeter, with a filter having a maximum transmission at 515 mμ., was used to determine the comparative decolorization of the dye 2,6-dichlorophenolindophenol, by the sample and standard, respectively.

Total ascorbic acid was determined by adapting the methods of Roe and Kuether (1943), and Lowry, Lopez, and Bessey (1945), to citrus samples. A five-ml. portion of pooled citrus sample was diluted to 100 ml. with distilled water. To two ml. of this solution in a test tube were added eight ml. of a five per cent suspension of activated norite A in five per cent trichloracetic acid. After shaking and allowing to stand five minutes, the mixture was filtered; four ml. of this clear filtrate were placed in an Evelyn tube, followed by one ml. of a solution which contained two gm. of 2,4-dinitrophenylhydrazine and 0.25 gm. of thiourea dissolved in 100 ml. of nine N H_2SO_4. The tube was then incubated for three hours at 37°C.

(99°F.), after which it was placed in an ice bath. After thorough cooling, five ml. of 85 per cent H_2SO_4 were added very slowly to prevent heating and charring. The tube was then allowed to stand for 30 minutes for full color development, after which the intensity of the developed color was determined with an Evelyn Colorimeter, fitted with a filter having maximum transmission at 540 mμ.

Microbiological methods were used for determination of biotin, folic acid, pyridoxine, and inositol. A thorough review of the methodology of microbiological assay procedures has been published by Snell (1948).

Biotin and Inositol Determination: Samples were prepared by autoclaving 25 ml. of citrus product (25 gm. in case of citrus sections) with 25 ml. of two N H_2SO_4 for two hours at 121°C.(250°F.). Although somewhat more drastic treatment of the sample has been suggested for liberation of biotin and inositol, Snell (1948), longer periods of autoclaving with more concentrated acids did not increase significantly the values for biotin and inositol, and it was concluded that the treatment employed provided maximum liberation of the respective vitamins.

After autoclaving, the solutions were cooled, neutralized to pH 5, and diluted to such a volume that at least three levels of the diluted sample would fall within the assay range of the organism using the pure vitamin to establish a standard curve.

Inositol was determined with the yeast *Saccharomyces cerevisiae* as the test organism. The medium used was that employed for the yeast *Saccharomyces carlsbergensis* by Atkin, Schultz, Williams, and Frey (1943), modified by the addition of pyridoxine and the removal of inositol. Growth was measured by turbidity, as determined with the Evelyn Photoelectric Colorimeter, using a filter with maximum transmission at 620 mμ.

The lactic acid bacterium, *Lactobacillus arabinosus,* was the test organism used for the determination of biotin, using the biotin free medium of Krehl and Fruton (1948). The graded production of lactic acid, in response to the biotin present in the sample, was compared to the pure vitamin and used as the end-point for the assay.

For the determination of folic acid, 25 ml. of the citrus sample (25 gm. of citrus sections) were neutralized to pH 7.2 with one N NaOH, 25 ml. of distilled water added, and the solution autoclaved for one hour at 121°C. (250°F.). This solution was cooled and the volume adjusted so as to fall within the assay range of the test organism *Lactobacillus casei,* using pure folic acid as a standard. The medium employed was the folic acid free medium of Krehl and Fruton (1948). The treatment of samples with hog kidney conjugase, as suggested by Olson, Fager, Burris, and Elvehjem (1948), did not reveal a significantly greater content of folic acid in the citrus products tested and was, therefore, abandoned, since it is considerably more cumbersome. Whether or not the procedure employed is completely adequate for liberation of bound folic acid in citrus products is debatable; however, since amounts of this vitamin found are of such a small order of magnitude, extensive study on this aspect of the problem did not seem justified.

Pyridoxine samples were prepared by adding 25 ml. of 0.1 N H_2SO_4 to 25 ml. of citrus product, after which the solution was autoclaved at

121°C. for one hour. After cooling, the pH of the solution was adjusted to five and the sample diluted to such a volume that three levels of the diluted sample fell within the assay range of the test organism *S. carlsbergensis,* when pure pyridoxine was used as the standard. The medium proposed by Atkin, Schultz, Williams, and Frey (1943) was used, and growth of the yeast as turbidity was measured as in the inositol assay. It should be noted that this organism measures all three forms of the vitamin, pyridoxine (i.e., pyridoxal, pyridoxamine, and pyridoxine).

Although longer periods of autoclaving have been recommended for the liberation of vitamin B_6 from certain types of material, Rabinowitz and Snell (1947), the sample treatment outlined above gave maximum pyridoxine values for the citrus products tested.

RESULTS AND DISCUSSION

It is apparent (Table 1) that it is reasonable to consider the test samples collected and frozen immediately as "fresh" for the practical purposes of the present study, since no significant losses of the vitamins in question were detected in the fresh juice prepared and stored under laboratory conditions. The differences that do exist are for the most part within the range of experimental error of the methods employed. The fact that the ascorbic acid content of the fresh juice prepared in the laboratory is considerably higher than that found in the large scale sampling procedure, has no great significance and, in fact, emphasizes the importance of a sampling procedure that represents not a single box of fruit or an isolated sample but many boxes of fruit, composited in such a way as to cover the natural wide range of ascorbic acid values. Since our laboratory sample was prepared from Valencia oranges at early maturity, and most of the field samples were collected in late season, one should expect a difference in ascorbic acid content. It has been shown, for example, that a gradual decrease in mg. of ascorbic acid per ml. of juice has been associated with the ripening of Valencia oranges, Harding, Winston, and Fisher (1940).

That the ascorbic acid values of the fresh juice are higher than those of the comparable canned products is revealed by an examination of Table 2. This is about what would be expected as a result of the reasonably long storage, i.e., six to eight months at 27°C.(81°F.) and 6°C.(43°F.) for two to four months, Moore, Wiederhold, and Atkins (1944). It is interesting to note, however, that the folic acid content of the canned products is, in general, higher than of the fresh. No explanation of this difference is available, as yet. The fact that the fresh orange juice contains more peel oil than the canned product does not seem to be an important factor in influencing the growth of the microorganisms used in the assay, since the folic acid content of a canned juice sample to which peel oil had been added in quantities to simulate the fresh product, remained essentially the same. Whether or not the canning and pasteurization process results in a greater release of bound folic acid has not been established. It should be noted, however, that preparation of samples for folic acid assay involves autoclaving at 121°C.(250°F.) for one hour, conditions far more rigorous than the customary processing procedure. The three types of citrus ex-

TABLE 2

Vitamin Content of Fresh and Canned Orange Juice

Orange juice (unsweetened)	F[1]/C[2]	Total solids (Brix)	Total acid (Citric)	Ascorbic acid		Biotin	Folic acid	Pyridoxine	Inositol	No. field boxes sampled	Type of fruit
				Reduced	Total						
		pct.	*pct.*	*mg./100 ml.*	*mg./100 ml.*	*µg./100 ml.*	*µg./100 ml.*	*µg./100 ml.*	*mg./100 ml.*		
Plant A	F	11.0	0.74	41.0	42.8	0.42	1.6	23.2	164	1,500	Valencia
	C	11.6	0.78	37.1	39.5	0.50	2.0	26.6	146		
Plant B	F	10.4	0.75	35.0	37.3	0.64	1.2	18.4	128	4,500	Valencia
	C	11.6	0.83	32.2	35.7	0.70	1.5	19.4	118		
Plant C	F	10.5	0.79	32.4	34.8	0.69	1.3	17.8	152	2,400	Valencia
	C	11.0	0.82	31.0	33.5	0.70	2.1	17.6	128		
Plant D	F	10.8	0.69	36.0	38.5	0.76	1.4	23.0	136	1,800	Valencia
	C	12.5	0.75	34.1	36.8	0.90	2.1	20.2	104		
Plant E	F	11.2	0.73	38.1	39.8	0.89	1.6	17.4	170	3,000	Valencia
	C	12.3	0.84	34.6	35.5	0.85	2.8	16.0	158		
Plant F	F	11.0	0.69	38.0	41.4	0.65	1.8	27.8	164	1,800	Valencia
	C	13.5	0.81	43.1	44.0	0.94	2.6	31.4	178		
Plant G	F	12.4	0.85	39.7	42.8	0.79	1.5	24.4	160	3,000	Valencia
	C	12.8	0.81	37.5	38.8	0.75	1.7	23.2	136		
Plant H	F	11.6	0.89	42.0	44.3	0.88	1.5	21.0	140	2,400	Valencia
	C	12.0	0.90	37.1	39.3	0.70	2.1	18.2	132		
Plant I	F	13.1	0.82	43.1	45.3	1.14	1.6	28.8	148	250	Valencia
	C	12.6	0.89	37.0	39.8	0.78	2.3	24.0	140		
Plant J	F	13.9	0.84	41.2	43.4	1.5	1.9	32.4	146	2,000	Valencia
	C	14.2	0.86	37.1	41.8	1.1	3.2	30.4	153		
Plant K	F	10.5	0.79	39.5	41.8	0.63	1.5	24.6	128	2,143	Valencia
	C	11.5	0.84	34.5	36.3	0.75	1.5	28.4	118		

[1] F=fresh. Samples stored at −10 to −12°C.(14 to 10°F.) for four to six mo. before analyses. [2] C=canned. Samples stored at 27°C.(81°F.) for six to eight mo., then at 6°C.(43°F.) for two to four mo. before analyses.

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amined, i.e., orange, grapefruit, and tangerine, had about the same range of values for their folic acid content.

A part of the apparent increase of folic acid in the canned products is, of course, explainable by the fact that the total solids of the canned citrus is uniformly higher and, therefore, represents a concentration. This explanation also applies to certain other cases where an increase in the vitamin content of the canned product seems evident. It must be remembered, however, that the assay methods for folic acid, pyridoxine, and inositol have not reached as high a degree of accuracy as certain other microbiological methods, i.e., niacin. This, of course, does not mean that the present methods cannot be conducted with reasonable accuracy and precision; it simply means that the stage of our knowledge concerning the determination of the particular vitamins studied has not advanced as far.

The level of pyridoxine in orange juice (Table 2) seems to be on the whole higher than in grapefruit juice (Table 3), and the same general conclusion can be applied to inositol. The pyridoxine and inositol content of tangerine juice, on the other hand, compares favorably with orange juice.

Of interest was the excellent retention of ascorbic acid in the 42° Brix frozen concentrated orange juice (see Table 4). Although three individual plants supplied composite samples of this product, excellent uniformity of juice was observed, particularly with respect to total soluble solids, total acid, and ascorbic acid content. The level of the other vitamins in the 42° Brix frozen concentrated orange juice was in the same general range as observed in the previous fresh and canned samples (see Table 2). Although no analogous fresh juice was available to compare with the 65° Brix pasteurized concentrated orange juice, it is evident (see Table 4) that the samples of this product examined had uniformly excellent ascorbic acid levels and about the same general range of values for the other vitamins as fresh orange juice (Table 2).

The canned citrus sections examined, i.e., orange sections, orange and grapefruit sections mixed, and grapefruit sections, were sweetened, and as might be expected had a higher total solids content than the citrus juices, and on the average their ascorbic acid content was somewhat below that of the respective citrus juices. On the other hand, the levels of the other vitamins were not strikingly different.

In reference to the ascorbic acid levels, it is worthy of note that the total ascorbic acid values, which represent both the oxidized and reduced form of ascorbic acid, are higher than values for the reduced ascorbic acid. Since good recoveries of ascorbic acid were obtained with both methods when applied to citrus products, it is evident that some of the oxidized (biologically available) ascorbic acid is present in citrus products.

The justification for careful and extensive sampling is made clear (in Table 6), where all of the data have been summarized and vitamin levels presented as a range of values. This is to emphasize the futility of expressing a definite value for a substance in a natural product, since too many factors—both environmental and genetic—are brought to bear in deciding this "amount." To illustrate—more ascorbic acid is found in oranges from outside branches well exposed to sunlight than from inside branches. Certainly, the somewhat low average ascorbic acid values found

TABLE 3

Vitamin Content of Fresh and Canned Grapefruit Juice and Tangerine Juice

Grapefruit juice (unsweetened)	F[1]/C[2]	Total solids (Brix)	Total acid (Citric)	Ascorbic acid		Biotin	Folic acid	Pyridoxine	Inositol	No. field boxes sampled	Type of fruit
				Reduced	Total						
		pct.	*pct.*	*mg./100 ml.*	*mg./100 ml.*	*µg./100 ml.*	*µg./100 ml.*	*µg./100 ml.*	*mg./100 ml.*		
Plant E	F	10.2	1.31	34.2	38.8	0.36	1.8	9.6	112	3,600	Duncan
	C	10.2	1.31	32.0	33.8	0.55	2.2	8.0	104		
Plant L	F	9.5	1.24	38.0	40.8	0.75	0.8	8.4	98	2,500	20 pct. Marsh Seedless
	C	10.7	1.33	37.2	39.0	0.80	1.9	8.8	112		80 pct. Duncan
Plant B	F	9.5	0.99	30.2	36.3	0.71	0.8	10.4	96	4,500	50 pct. Marsh Seedless
	C	10.1	1.15	30.1	36.8	0.60	1.5	8.8	92		50 pct. Duncan
Plant F	F	9.4	0.86	35.1	38.3	0.97	0.9	11.4	102	900	50 pct. Marsh Seedless
	C	9.9	0.91	30.2	38.8	0.92	1.7	12.2	92		50 pct. Duncan
Plant J	F	9.6	1.07	37.4	39.7	0.83	0.8	14.2	100	4,500	67 pct. Duncan
	C	9.8	1.14	36.2	38.3	0.80	1.5	18.2	104		33 pct. Marsh Seedless
Plant H	F	9.9	1.02	37.8	40.0	0.4	0.9	18.0	88	1,800	Seeded
	C	10.6	1.11	40.0	39.3	0.7	1.7	15.2	101		
Tangerine juice (sweetened)											
Plant A	F	12.5	0.69	30.4	30.8	0.45	1.2	22.6	135	700	Tangerine
	C	12.8	0.70	27.2	28.3	0.46	1.8	33.4	147		

[1] F=fresh. Samples stored at −10 to −12°C.(14 to 10°F.) for two to four mo. before analyses. [2] C=canned. Samples stored at 27°C.(81°F.) for six to eight mo., then at 6°C.(43°F.) for two to four mo. before analyses.

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TABLE 4

Vitamin Content of Orange Juice Concentrates[3]

Type of sample	F/C[2]	Total solids (Brix)	Total acid (Citric)	Ascorbic acid		Biotin	Folic acid	Pyridoxine	Inositol	No. field boxes sampled	Type of fruit
				Reduced	Total						
65° Brix—Conc. orange juice[1] (unsweetened)		*pct.*	*pct.*	*mg./100 ml.*	*mg./100 ml.*	*µg./100 ml.*	*µg./100 ml.*	*µg./100 ml.*	*mg./100 ml.*		
Plant M	C	10.5	.74	47.5	48.1	0.58	2.8	32.0	136	900	Midseason
	C	10.5	.74	35.9	36.3	0.52	2.2	22.1	124	900	Valencia
Plant J	C	10.3	.79	48.0	50.8	0.68	2.4	24.7	184	4,300	Pineapple 39 pct.
	C	10.4	.75	37.1	40.0	0.81	1.8	22.3	146		Seedlings 19 pct. Valencia 42 pct.
Plant B	C	10.6	.95	45.0	49.0	0.63	2.2	22.4	148	1,440	Pineapple
42° Brix frozen conc. orange juice (1)—Including both fresh raw juice and concentrates (all unsweetened)											
Plant N	F	11.5	.74	38.5	41.3	0.61	2.2	22.2	156	2,500	40 pct. Indian River
	C	11.9	.83	40.1	42.5	0.44	1.7	21.6	140		60 pct. Valencia
	F	11.1	.72	37.5	40.8	0.58	2.3	24.6	152	1,000	33 pct. seedlings
	C	12.0	.80	42.0	42.6	0.37	2.0	23.4	142		67 pct. Valencia
	F	11.4	.73	40.4	44.2	0.58	2.1	26.4	154	1,128	33 pct. seedlings
	C	12.0	.81	42.0	46.3	0.38	2.0	23.4	146		67 pct. Valencia
Plant B	F	11.0	.79	42.2	46.1	0.34	1.8	16.0	112	2,979	15 pct. Pineapple
	C	11.9	.85	46.0	45.8	0.37	1.9	19.8	124		85 pct. Valencia
	F	10.7	.81	46.0	47.8	0.38	1.6	18.6	108	2,977	2 pct. Pineapple
	C	12.2	.97	45.2	46.8	0.42	1.7	17.2	114		98 pct. Valencia
Plant M	F	10.9	.85	44.1	49.5	0.49	1.5	17.0	146	900	Valencia
	C	11.2	.88	43.2	45.0	0.51	1.2	26.0	162		

[1] 65 and 42° Brix concentrate samples were diluted according to label directions and then samples taken for the various analyses. [2] F=fresh. Samples stored at −10 to −12°C.(14 to 10°F.) for two to four mo. before analyses. C=canned. [3] 65° Brix orange juice concentrates stored at 6°C.(43°F.); 42° Brix stored at −10°C.(14°F.) for eight to 10 mo. before analyses.

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TABLE 5

Vitamin Content of Canned Citrus Sections[1]

Type of sample	Total solids (dry wt.)	Total acid (citric)	Ascorbic acid		Biotin	Folic acid	Pyridoxine	Inositol	No. field boxes sampled	Type of fruit
			Reduced	Total						
	pct.	*pct.*	*mg./100 gm.*	*mg./100 gm.*	*µg./100 gm.*	*µg./100 gm.*	*µg./100 gm.*	*mg./100 gm.*		
Orange sections (sweetened)										
Plant K	15.5	0.61	36.0	37.5	1.5	3.2	31.0	152	308	Valencia
Salad (orange and grapefruit sections mixed, sweetened)										
Plant K	18.6	0.67	27.5	28.8	1.6	2.2	22.4	140	342	Valencia 42 pct. Duncan 58 pct.
Plant F	17.5	0.60	22.0	23.8	1.7	1.8	22.1	156	1,200	Duncan Seeded 67 pct. Valencia oranges 33 pct.
	16.0	0.62	23.0	25.0	2.2	1.9	26.2	168		
	17.9	0.65	22.1	23.5	2.5	1.5	20.0	140		
Grapefruit sections (sweetened)										
Plant O	15.0	1.01	26.3	27.3	1.9	0.9	24.4	132	1,000	Duncan
	15.8	0.89	25.2	26.3	1.0	1.1	22.1	144		
Plant H	16.9	0.81	29.1	31.8	1.3	0.6	23.4	76	750	Seeded
	17.1	0.85	29.3	31.3	1.1	0.9	24.2	72		
	18.3	0.72	29.0	30.8	1.4	0.8	18.1	77		
Plant P	15.5	0.77	31.5	33.8	0.91	0.6	22.0	80	600	Duncan
Plant B	16.9	0.94	29.4	31.5	0.70	0.8	20.1	124	600	Duncan
	15.7	0.89	27.0	29.3	0.81	0.9	19.0	116		
Plant Q	14.3	0.91	32.5	34.2	0.91	1.1	16.7	108	252	Duncan
	15.5	0.62	34.0	35.5	1.1	1.3	19.2	118	297	Duncan

[1] Stored at 27°C.(81°F.) for six to eight mo., then at 6°C.(43°F.) for two to four mo. prior to analyses.

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TABLE 6

Range of Vitamin Content of Citrus Products

Type of product	No. samples	Total solids	Total acid (Citric)	Ascorbic acid		Biotin	Folic acid	Pyridoxine	Inositol	No. field boxes sampled
				Reduced	Total					
		pct.	*pct.*	*mg./100 ml.*	*mg./100 ml.*	*µg./100 ml.*	*µg./100 ml.*	*µg./100 ml.*	*mg./100 ml.*	
Orange juice (unsweetened)										
Fresh	11	10.4-13.9	0.69-0.89	32.4-43.1	34.8-45.3	0.42-1.5	1.2-1.9	17.8-32.4	128-170	24,793
Canned	11	11.0-14.2	0.75-0.90	31.0-43.1	33.5-44.0	0.5 -1.1	1.5-3.2	16.0-31.4	104-178	
Grapefruit juice (unsweetened)										
Fresh	6	9.4-10.2	0.86-1.31	30.2-38.0	36.3-40.8	0.36-9.97	0.8-1.8	8.4-18.0	88-112	17,800
Canned	6	9.9-10.7	0.91-1.33	30.1-40.0	33.8-39.3	0.55-0.92	1.5-2.2	8.0-18.2	92-112	
Tangerine juice (sweetened)										
Fresh	1	.....[1]	.....[1]	.....[1]	.....[1]	.....[1]	.....[1]	.....[1]	.....[1]	
Canned	1	.....[1]	.....[1]	.....[1]	.....[1]	.....[1]	.....[1]	.....[1]	.....[1]	
65° Brix—Orange juice conc. (unsweetened)	5	10.3-10.6	0.74-0.95	35.9-47.5	36.3-50.8	0.52-0.81	1.8-2.8	22.1-32.0	124-184	7,540
42° Brix—Orange juice (unsweetened)										11,484
Fresh raw juice	6	10.7-11.5	0.73-0.85	37.5-46.0	40.8-49.5	0.34-0.61	1.5-2.3	16.0-26.4	108-156	
After conc. and canning	6	11.2-12.2	0.80-0.97	40.1-46.0	42.5-46.3	0.37-0.51	1.2-2.0	17.2-26.0	114-162	
Orange sections (sweetened)	1	.....[2]	.....[2]	.....[2]	.....[2]		.....[2]	.....[2]	.....[2]	
				mg./100 gm.	*mg./100 gm.*	*γ/100 gm.*	*γ/100 gm.*	*γ/100 gm.*	*mg./100 gm.*	
Citrus salad (sweetened), orange and grapefruit sections mixed	4	15.5-18.6	0.60-0.67	22.0-36.0	23.5-37.5	1.5 -2.5	1.5-3.2	20.0-31.0	140-168	1,542
Grapefruit sections (sweetened)	10	14.3-18.3	0.62-1.01	25.2-34.0	26.3-35.5	0.81-1.9	0.6-1.3	16.7-24.4	72-144	3,499

[1] See Table 3—no range of values possible—only one plant in sample. [2] See Table 5—no range of values possible—only one plant in sample.

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in the citrus products in the present study are explainable by the fact that more ascorbic acid is generally found in immature (early season) than ripe (late season) fruit, Harding *et al.* (1940). In the present study every effort was made, therefore, to obtain a sample from a "pool" that would have represented in it as many of the variables that might conceivably contribute to variation in the vitamin content of the fruit. Even after such composites of representative samples a considerable range of values is observed, as might be expected, since, again, these samples were contributed from 17 different citrus processing plants over a wide area of the Florida "citrus belt."

Examination of the data in all the tables makes it obvious that of all vitamins studied only ascorbic acid and inositol appear in appreciable amounts. Although it is of interest to know the amount and range of biotin, folic acid, and pyridoxine in citrus products, they are present in such small quantities that the consumption of citrus in the usual amounts would not be likely to contribute an amount of these vitamins that would be significant in meeting the vitamin requirement of the organism.

On the other hand, it is clear that citrus fruits remain as one of the most practical sources of ascorbic acid in the nutritional economy. It is further evident that citrus products contain an abundance of inositol and are, in fact, one of the richest natural sources of this vitamin. Although the nutritional significance of inositol has not as yet been elucidated, the occurrence of inositol as an integral part of a phosphatide, Woolley (1944), and of an important enzyme, pancreatic amylase, Williams, Schlenk, and Eppright (1944), makes it clear this compound is a potentially important dietary factor. It is of theoretical interest to note that inositol and ascorbic acid, both six-carbon "carbohydrate-like" compounds, exist in the same material in relatively large amounts. Whether inositol may be the reserve six-carbon compound that serves as a precursor for ascorbic acid for the plant, remains to be shown by experimental investigation.

SUMMARY

Samples of fresh and processed citrus juices, as nearly homogeneous as possible, were withdrawn from a pooled composite sample representing a large number of boxes of citrus fruit in 17 Florida citrus processing plants.

These fresh and processed samples were assayed for their content of ascorbic acid, biotin, folic acid, pyridoxine, and inositol. Total solids and total acid were also determined.

Although measurable quantities of biotin, folic acid, and pyridoxine were noted, these vitamins were present in only limited amounts in terms of practical nutritional significance.

Ascorbic acid, as might be expected, was present in significant amounts and, in addition, the large amount of inositol present in the citrus products examined makes them a rich natural source of this vitamin.

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The Determination of Ascorbic Acid in Blackcurrant and Other Coloured Fruit-juice Syrups

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A simple method, based on the quantitative reduction of mercuric chloride to mercurous chloride by ascorbic acid, has been developed for determining ascorbic acid in blackcurrant and other coloured fruit-juice syrups. The insoluble mercurous chloride is separated by centrifugation, dissolved in standard iodine solution, the excess of which is titrated with standard sodium thiosulphate solution with starch as the indicator. The results obtained by this method compare favourably with those found by the potentiometric-titration method. Sodium sulphite, citric acid, glucose, sucrose and substances normally present in such syrups do not interfere.

The addition of sulphite to fruit-juice syrups as a preservative does not prevent the loss of ascorbic acid, particularly in specimens that have been exposed to sunlight or stored for considerable periods. Routine checks of such syrups are therefore necessary so that it is desirable to develop a rapid and suitable method for this purpose.

The usual colour indicator titration methods for ascorbic acid are unsuitable because such syrups are intensely pigmented. Evered[1] titrated the ascorbic acid in blackcurrant juice with *N*-bromosuccinimide, the end-point being detected by the liberation of iodine, which was extracted by an organic solvent. This end-point, which is a brown colour in the organic layer, is difficult to establish when viewed against a red-coloured aqueous layer. The use of diazotised 4-methoxy-2-nitroaniline, which gives a blue colour with ascorbic acid in an alkaline medium, has been proposed by Schmall, Pifer and Wollish[2] and they have reported the use of this method for determining ascorbic acid in various fruit juices such as orange, grapefruit and tomato. However, Johannesen[3] and Bergeret[4] found it necessary to separate the ascorbic acid by paper chromatography before this colorimetric method could be applied to rose-hip and blackcurrant extracts.

A currently used method is based on the potentiometric-titration technique first described by Foulk and Bawden[5] in 1926. Liebman and Ayres[6] adapted this technique for determining ascorbic acid by titration with 2,6-dichlorophenolindophenol. This method has precision, but is laborious. The standard dye solution is unstable and requires standardisation each time before use.

The quantitative reduction of mercuric chloride to mercurous chloride by ascorbic acid has been investigated by Suryanarayana Rao, Veereswara Rao and Gopala Rao.[7] This procedure appeared to be suitable for determining ascorbic acid in coloured solutions and was therefore adapted as a basis for the determination of ascorbic acid in fruit-juice syrups.

Method

Reagents—

Mercuric chloride—Prepare a saturated aqueous solution.
Iodine, 0·01 N—Standardise the solution against arsenous oxide.
Sodium thiosulphate, 0·01 N—Standardise the solution against 0·01 N standard iodine.
Potassium iodide solution, 10 *per cent. w/v, aqueous.*
Starch solution, 1 *per cent. w/v, aqueous.*
Acetone—Analytical-reagent grade.
Acetic acid, 10 *per cent. w/v, aqueous.*

Procedure

Transfer an accurately measured volume or a weighed amount of the syrup containing about 5 to 10 mg of ascorbic acid to a 50-ml centrifuge tube containing 5 ml of saturated mercuric chloride solution and 10 ml of acetone. Stir the solution with a glass rod and wash the rod with water. After the solution has been set aside for 30 minutes, spin it in a centrifuge for 10 minutes at 2500 r.p.m. at a radius of 15·0 cm. Carefully remove the supernatant liquid with a pipette, wash the precipitated mercurous chloride with 20 ml of hot 10 per cent.

acetic acid and spin the solution in a centrifuge again for a further 10 minutes. Again remove the supernatant liquid with a pipette and transfer the mercurous chloride quantitatively into a 250-ml conical flask with water. Dissolve the mercurous chloride by adding 25 ml of 0·01 N standard iodine and 5 ml of 10 per cent. potassium iodide solution. Titrate the excess of iodine with 0·01 N sodium thiosulphate with starch as the indicator.

1 ml of 0·01 N iodine 0·88 mg of ascorbic acid.

Results and discussion

Comparison of results—

Nineteen commercial samples of various fruit-juice syrups were analysed by the proposed method and by the method of Liebman and Ayres[6] with 2,6-dichlorophenolindophenol as the titrant. The results are shown in Table I. It can be seen that in general the proposed method gives results that are in good agreement with those obtained by the potentiometric-titration method. With old samples that are very low in ascorbic acid, greater differences were observed between the values given by the two methods. It is possible that certain substances present in such samples may have interfered with the proposed method, thus giving higher results. It must be mentioned that difficulties were encountered in obtaining the end-point during the electrometric titration of these samples. Extremely low ascorbic acid figures seldom come up for consideration and a difference of 3 mg of ascorbic acid per fluid ounce of syrup in such samples is of little practical significance.

Table I

Comparison of results

Number of determinations by proposed method*	Ascorbic acid, mg per oz of syrup, found by proposed method: Range	Mean	potentiometric method; mean of two determinations
Blackcurrant-juice syrup—			
10	58·3 to 60·2	59·4	58·6
10	43·7 to 45·5	44·8	44·8
10	25·8 to 27·2	26·5	26·8
6	37·8 to 39·1	38·6	38·2
6	38·6 to 40·3	39·7	39·7
4	41·8 to 43·0	42·6	42·6
4	11·6 to 13·1	12·3	11·2
4	15·7 to 16·2	15·9	15·6
4	9·6 to 10·2	9·8	9·6
4	8·1 to 9·0	8·3	5·1
3	49·9 to 50·4	50·2	50·4
3	41·5 to 42·8	42·1	40·7
Guava and grape juice syrup—			
4	34·7 to 36·1	35·0	35·4
4	30·7 to 31·8	31·3	30·9
4	11·2 to 11·8	11·4	11·7
3	21·8 to 22·2	22·0	22·1
3	5·1 to 5·8	5·3	2·6
Other types of syrups			
4	33·0 to 34·3	33·6	33·6
4	36·7 to 37·5	37·0	36·7

* Each horizontal line of results represents a different sample.

Table II

Determination of ascorbic acid in blackcurrant-juice syrups by the proposed method

Specimen	Number of determinations	Ascorbic acid, mg per oz of syrup: Range	Mean	Standard deviation
1	10	58·3 to 60·2	59·4	0·57
2	10	43·7 to 45·5	44·8	0·54
3	10	25·8 to 27·2	26·5	0·46

PRECISION OF THE METHOD—

A study was made of the precision of the method. Typical standard deviations are shown in Table II and are quite satisfactory for most purposes. It is recommended that wash-out pipettes should be used or that the sample should be taken by weight rather than by volume as the use of ordinary pipettes for the measurement of viscous liquids such as fruit-juice syrups is usually less satisfactory for accurate work.

TABLE III

RECOVERY OF ASCORBIC ACID FROM BLACKCURRANT-JUICE SYRUP BY THE PROPOSED METHOD

Ascorbic acid, mg—				
Added to 3 ml of syrup	Found	Mean	Recovered	Recovery, per cent.
0	4·53 4·46	4·50	—	—
1	5·49 5·46	5·48	0·98	98·0
2	6·39 6·57	6·48	1·98	99·0
3	7·48 7·52	7·50	3·00	100·0
4	8·44 8·49	8·47	3·97	99·25
5	9·50 9·54	9·52	5·02	100·4

RECOVERY OF ADDED ASCORBIC ACID—

Known amounts of ascorbic acid were added to 3 ml of blackcurrant-juice syrup and the recovery results are shown in Table III. It can be seen that amounts of added ascorbic acid ranging from 1 mg to 5 mg can be recovered from 3 ml of blackcurrant-juice syrup with an error of ± 2 per cent.

EFFECTS OF pH—

Suryanarayana Rao, Veereswara Rao and Gopala Rao[7] reported that the optimum range in which ascorbic acid reduces mercuric chloride quantitatively to mercurous chloride is between pH 2·0 and 8·0. The pH of the sixteen samples studied ranged from 2·2 to 3·0. The addition of acetone and mercuric chloride solution did not alter the pH of any of these specimens by more than 0·5 pH units. Since the pH of all the solutions studied was within the optimum range of 2·0 to 8·0, no buffers were used.

ORDER OF ADDITION OF REAGENTS—

It is important that the syrup be added to the mercuric chloride solution and not the reverse. This is to ensure that there is always an excess of mercuric chloride to prevent the mercurous chloride formed from being further reduced to mercury by an excess of ascorbic acid. Further, it is essential that iodine be added to the mercurous chloride before the addition of potassium iodide solution. If the reverse order is followed low recoveries will sometimes result because of the precipitation of mercury according to the reactions—

$$Hg_2Cl_2 + 2KI \rightarrow Hg_2I_2 + 2KCl \quad \ldots \quad (1)$$

$$Hg_2I_2 + 2KI \rightarrow K_2HgI_4 + Hg \quad \ldots \quad (2)$$

The correct order of addition of reagents must be followed for the attainment of accurate and reproducible results.

INTERFERENCE OF OTHER SUBSTANCES—

Citric acid, glucose and sucrose, which are substances normally present in fruit-juice syrups, were found not to interfere with the proposed method. An excess of acetic acid from the washing and mercuric chloride also do not interfere. Because of the general use of sulphite as a preservative, the actual effect of this radical was studied and it was found that up to 16 mg of sulphite, as SO_2, approximately 5 times the normal amount used as preservative, when added to 5 ml of syrup gave no interference. Trace metals such as iron[11],

zinc and lead do not interfere, but oxidising agents such as cupric and ferric ions destroy the ascorbic acid. Because of the effect of oxidising conditions, interference from such oxidising ions is of little practical consequence.

The good agreement between the results found by the proposed method and the potentiometric method reflect the specificity of the reagent used under the conditions described. The possibility of substances other than ascorbic acid, which might reduce mercuric chloride under these conditions, is not excluded, particularly with old samples. Examination of specimens that have been treated with ascorbic acid oxidase by the proposed method may permit this point to be settled.

SEPARATION OF MERCUROUS CHLORIDE FROM THE SOLUTION--

It is recommended that the precipitated mercurous chloride be separated by centrifugation. The precipitate is heavy and the separation can be easily effected by this means. Attempts to separate it quantitatively by filtration through filter-paper were unsuccessful because of the viscosity of the solutions and the small particle size of the precipitated mercurous chloride.

ADVANTAGES OF THE METHOD--

The proposed method can be adapted for routine use and it is simple and reliable. The precipitation and centrifugation can be done in batches and require no special technique or constant attention. No special equipment or reagents are required. The method can be applied to the determination of ascorbic acid in blackcurrant-, guava- and grapefruit-juice syrups and other types of synthetic syrups. It is possible for one analyst to complete the analysis of 20 to 25 samples in a 6½-hour day.

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DANGERS OF MASSIVE VITAMIN C INTAKE

To the Editor: If present sales of vitamin C around Burlington, Vermont, are any indication there must be a tremendous increase in the consumption of vitamin C or ascorbic acid throughout the country in response to recommendations made by Nobel Prize winner Dr. Linus Pauling in his recent popular book, Vitamin C and the Common Cold. What is worrisome is that the amounts recommended represent massive doses of ascorbic acid (1 to 15 g), of the order of 10 to 100 times the usual dietary intake of vitamin C. Furthermore, one gets the impression from this book that these amounts and even 40, 100 or 350 g a day are completely innocuous except for a laxative action when taken without food. Although ascorbic acid has the respectable reputation of being innocuous in relatively large amounts, there is good reason to be concerned about massive ingestion particularly for long periods. This is emphasized in the 312th issue of the Medical Letter and quoted in the New York Times of Sunday January 3, 1971, which points out possible harm in the management of diabetes since the increased urinary ascorbic acid could give false-positive tests for sugar. Furthermore, the Medical Letter indicates that acidification of the urine by large doses of ascorbic acid can favor precipitation of urate or cystine stones in patients with a tendency to gout or cystinuria. I should like to point out other ways in which massive ingestion of ascorbic acid may prove harmful unless demonstrated otherwise:

Lamden and Chrystowski[1] have shown that the daily ingestion by man of 4 to 9 g of ascorbic acid significantly increases the excretion of urinary oxalate, a known product of ascorbic acid metabolism. Elevated oxaluria favors the possibility of kidney and bladder damage from oxalate stones. This work will be extended to include some of the higher levels being consumed today.

Neuweiler[2] and Mouriquand and Edel[3] found that massive doses of vitamin C affected the reproductive system of guinea pigs. They noted dead fetuses, less possibility of conception and other signs of interference with reproduction. To my knowledge these findings have never been refuted, and no information is available on this subject in man or primates.

Patterson[4] reported that dehydroascorbic acid, a metabolic product of ascorbic acid, when injected into rats, produced an alloxan-type diabetes. Whether massive ingestion of ascorbic acid by man would ever result in a sufficiently high concentration of dehydroascorbic acid in pancreatic cells to be diabetogenic is not known.

Thornton[5] has observed that vitamin C injected into chicks (in amounts that would be considered massive in man) promoted bone salt mobilization and increased excretion of phosphorus over a four-day period. The implications in this work for man are a matter of conjecture.

Thus, although ascorbic acid is a highly valuable and useful vitamin and drug, it is also an exceptionally reactive substance biochemically and should be treated with respect. As long as there is reasonable doubt about the harmlessness of massive ingestion of ascorbic acid, such use by anyone not under the close scrutiny of a physician should be emphatically discouraged.

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SHORT COMMUNICATION

Large and Small Doses of Ascorbic Acid in the Absorption of Ferrous Iron

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IT IS widely stated that ascorbic acid increases the absorption of iron,[1] and the literature contains several studies in support of this. Most of the papers use small numbers of subjects[2] and do not analyze their results statistically.[3-7] Studies that have adequate numbers and statistical analyses are open to criticism on other grounds, such as the use of animals[8] or applicability only to food-incorporated iron[9] or to ferric iron.[10]

We set out to demonstrate the effect of ascorbic acid in 50-mg. and 1000-mg. dosages on the absorption of 200 mg. of ferrous sulfate administered simultaneously, using "iron absorption curves", because of the apparent hiatus in the literature concerning the effect of ascorbic acid on the absorption of ferrous sulfate administered therapeutically.

METHOD

Forty patients in a general medical department were studied. None were under treatment for iron deficiency and none had hemoglobins below 75% (11.0 g./100 ml.) The following procedures were done on each:

Procedure 1:

(a) 8.00 a.m. Blood taken for serum iron. Patient fasting (zero reading).

(b) Followed immediately by the administration of 200 mg. of ferrous sulfate.

(c) 9.00 a.m. Blood taken for serum iron.

(d) 10.00 a.m. Blood taken for serum iron.

(e) 10.00 a.m. Patient now allowed breakfast.

(f) 11.00 a.m. Blood taken for serum iron.

(g) 12.00 a.m. Blood taken for serum iron.

Procedure 2:

The same procedure was followed except that 50 mg. of ascorbic acid was administered in addition to the 200 mg. of ferrous sulfate.

Procedure 3:

The same procedure was followed except that 1 g. of ascorbic acid was added to the 200 mg. of ferrous sulfate.

The different procedures were carried out at intervals of at least four days. The sequence was randomized to nullify the effect of initial tests on subsequent ones in the overall results. The person carrying out the serum iron estimations (D.C.S.) did not know which test was being carried out.

The method used for serum iron estimations was that described by Ramsay.[11] The estimations were carried out within 24 hours of the serum being withdrawn; all glassware was boiled for 15 minutes in dilute HCl. Tests on samples of the tubes used for collection of blood using distilled water drawn up with samples of the needles and the syringes used for venipuncture showed no trace of iron.

RESULTS

The results were analyzed as follows. For each subject the average of the first, second, third and fourth levels of serum iron was estimated, and from this figure the zero reading was subtracted to give an average rise in serum iron for each procedure. Thus, three values were established for each subject: (1) the average rise in serum iron when ferrous sulfate was administered alone; (2) the average rise in serum iron when ferrous sulfate was administered with 50 mg. of ascorbic acid; (3) the average rise in serum iron when ferrous sulfate was administered with 1 g. of ascorbic acid.

Assuming that ascorbic acid had no effect on the absorption of ferrous sulfate, the average

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TABLE I.—COMPARISON OF PROCEDURE 1 WITH PROCEDURE 3

Sex	Age	Diagnosis	Hb. (%)	Patient No.	$FeSO_4$ alone: average rise in serum iron µg./100 ml.) 1	$FeSO_4$ and 1 g. ascorbic acid; average rise in serum iron (µg./100 ml.) 2	Difference (2 minus 1) D	D^2
M	49	Myocardial infarction	109	1	59	62	3	9
M	68	Myocardial infarction	98	2	20	76	56	3136
F	75	Hemiplegia	94	3	69	165	96	9216
M	74	Chronic bronchitis	135	4	136	104	−32	1024
M	64	Myocardial infarction	100	5	127	140	13	169
F	55	Chronic bronchitis	106	6	98	71	−27	729
M	20	Lobar pneumonia	90	7	36	45	9	81
F	47	Mitral stenosis	94	8	76	97	21	441
M	59	Rheumatoid arthritis	80	9	57	56	− 1	1
F	38	Chronic bronchitis	78	10	235	227	− 8	64
M	57	Myocardial infarction	99	11	58	78	20	400
M	28	Acute nephritis	86	12	30	117	87	7569
M	58	Myocardial infarction	114	13	9	56	47	2209
F	52	Myocardial infarction	101	14	0	32	32	1024
M	49	Myocardial infarction	95	15	26	53	27	729
M	59	Congestive heart failure	96	16	2	113	111	12,321
F	70	Myocardial ischemia	104	17	−15	21	36	1296
M	74	Myocardial infarction	99	18	− 2	54	56	3136
F	75	Osteoarthritis	86	19	− 2	59	61	3721
F	64	Peripheral neuropathy	113	20	− 7	13	20	400
M	52	Myocardial infarction	96	21	12	41	29	841
M	68	Hemiplegia	90	22	11	−21	−32	1024
F	44	Rheumatoid arthritis	80	23	89	85	− 4	16
F	63	Rheumatoid arthritis	82	24	34	1	−33	1089
F	72	Rheumatoid arthritis	75	25	33	9	−24	576
M	57	Myocardial infarction	95	26	100	94	− 6	36
F	60	Rheumatoid arthritis	86	27	138	186	48	2304
M	41	Myocardial infarction	96	28	107	70	−37	1369
M	69	Myocardial infarction	96	29	29	31	2	4
M	80	Left ventricular failure	93	30	12	11	− 1	1
F	19	Disseminated lupus erythematosus	62	31	−43	21	64	4096
M	40	Myocardial infarction	96	32	19	36	17	289
F	64	Myocardial infarction	94	33	23	44	21	441
M	74	Cerebral arteriosclerosis	60	34	−26	33	59	3481
F	64	Rheumatoid arthritis	73	35	18	43	25	625
F	74	Mitral stenosis	92	36	15	−40	−55	3025
F	59	Disseminated lupus erythematosus	87	37	0	8	8	64
M	64	Myocardial infarction	101	38	− 4	−11	− 7	49
M	64	Myocardial infarction	95	39	18	60	42	1764
F	74	Cirrhosis of liver	78	40	23	82	− 59	3481
							$\bar{D}$ 19.5	ΣD^2 72,250

"T" test:

$$S^2 = \frac{\Sigma D^2}{N} - \bar{D}^2 = \frac{72250}{40} - 380 = 1426$$

$$t = \frac{\bar{D}\sqrt{n-1}}{S} = \frac{19.5\sqrt{39}}{\sqrt{1426}} = 3.224$$

$t = 3.224$ When $n = 40$ $P < .005$

rise of serum iron with each procedure should be the same for each subject. The difference between the average rise in serum iron between any two procedures should not differ statistically from zero for all of the 40 subjects.

There was no significant difference between procedure 1, where ferrous sulfate was given alone, and procedure 2, where ferrous sulfate was given with 50 mg. of ascorbic acid.

A significant difference was found between procedure 1 and procedure 3, where ferrous sulfate was given with 1 g. of ascorbic acid; the results are presented in Tables I and II. We believe that the difference was not due to more rapid absorption of an equal amount of ferrous iron in procedure 2 because the maximum rise in serum iron occurred at the same time in each curve (Fig. 1). Serum iron values are all in µg./100 ml.

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TABLE II.

Detailed results of procedures 1 and 3

Sex	Age	Diagnosis	Hb. (%)	Patient No.	Procedure 1: $FeSO_4$ alone — Iron absorption curve: Zero	1 Hr.	2 Hr.	3 Hr.	4 Hr.	Average Rise	Procedure 3: $FeSO_4$ with 1 g. of ascorbic acid — Iron absorption curve: Zero	1 Hr.	2 Hr.	3 Hr.	4 Hr.	Average Rise
M	49	Myocardial infarction	109	1	156	240	210	184	224	59	130	176	180	192	220	62
M	68	Myocardial infarction	98	2	120	90	124	192	152	20	106	106	180	200	240	76
F	75	Hemiplegia	94	3	100	108	150	176	240	69	100	160	240	300	360	165
M	74	Chronic bronchitis	135	4	140	240	320	272	272	136	160	200	216	320	320	104
M	64	Myocardial infarction	100	5	130	160	176	290	400	127	148	180	280	312	380	140
F	55	Chronic bronchitis	106	6	210	210	293	348	382	98	188	228	228	290	290	71
M	20	Lobar pneumonia	90	7	80	80	124	124	136	36	90	108	148	144	140	45
F	47	Mitral stenosis	94	8	140	180	192	284	208	76	140	220	230	250	248	97
M	59	Rheumatoid arthritis	80	9	102	148	164	162	162	57	152	268	200	188	176	56
F	38	Chronic bronchitis	78	10	108	196	360	396	420	235	92	206	292	380	398	227
M	57	Myocardial infarction	99	11	110	140	168	162	200	58	120	190	200	200	200	78
M	28	Acute nephritis	86	12	116	100	166	158	158	30	92	156	230	228	220	117
M	58	Myocardial infarction	114	13	140	140	144	148	164	9	108	128	164	164	200	56
F	52	Myocardial infarction	101	14	100	100	100	100	100	0	112	108	150	160	156	32
M	49	Myocardial infarction	95	15	88	96	120	120	120	26	120	188	188	160	156	53
M	59	Congestive heart failure	96	16	136	108	160	144	140	2	146	160	260	328	288	113
F	70	Myocardial ischemia	104	17	160	130	160	160	130	−15	120	120	132	160	152	21
M	74	Myocardial infarction	99	18	140	140	132	148	132	−2	100	132	172	152	160	54
F	75	Osteoarthritis	86	19	54	50	50	54	54	−2	90	88	150	190	168	59
F	64	Peripheral neuropathy	113	20	150	140	140	150	140	−7	180	176	172	260	164	13
M	52	Myocardial infarction	96	21	106	108	108	140	114	12	120	148	178	160	156	41
M	68	Hemiplegia	90	22	114	112	128	130	128	11	190	164	176	168	166	−21
F	44	Rheumatoid arthritis	80	23	64	104	158	188	160	89	88	88	130	236	236	85
F	63	Rheumatoid arthritis	82	24	50	72	76	100	88	34	64	60	64	76	60	1
F	72	Rheumatoid arthritis	75	25	40	60	160	40	30	33	34	34	34	52	52	9
M	57	Myocardial infarction	95	26	76	152	186	182	182	100	108	128	216	244	220	94
F	60	Rheumatoid arthritis	86	27	30	80	170	220	200	138	30	88	232	272	272	186
M	41	Myocardial infarction	96	28	92	124	140	276	256	107	62	98	132	148	148	70
M	69	Myocardial infarction	96	29	100	108	142	130	134	29	48	50	110	88	68	31
M	80	Left ventricular failure	93	30	108	120	120	120	120	12	136	130	152	152	152	11
F	19	Disseminated lupus erythematosus	62	31	88	36	64	36	44	−43	44	60	74	72	54	21
M	40	Myocardial infarction	96	32	80	94	100	100	100	19	96	96	110	140	180	36
F	64	Myocardial infarction	94	33	116	116	144	148	148	23	60	104	104	104	104	44
M	74	Cerebral atherosclerosis	60	34	120	100	100	94	80	−26	54	76	116	92	62	33
F	64	Rheumatoid arthritis	73	35	70	80	76	108	88	18	108	132	150	168	152	43
F	74	Mitral stenosis	92	36	80	90	110	100	80	15	115	92	96	64	46	−40
F	59	Disseminated lupus erythematosus	87	37	84	68	68	120	80	0	26	16	26	26	66	8
M	64	Myocardial infarction	101	38	104	104	104	104	88	−4	84	84	80	76	50	−11
M	64	Myocardial infarction	95	39	76	116	80	80	100	18	48	88	106	118	120	60
F	74	Cirrhosis of liver	78	40	70	80	94	100	96	23	76	92	152	190	196	82
Mean curves					104	119	145	160	156		102	128	162	181	180	

CONCLUSION

This paper has shown that when ferrous sulfate was administered to fasting non-anemic subjects in a dose of 200 mg. the iron absorption curve was significantly higher when 1 g. of ascorbic acid was added. We were unable to demonstrate any difference between procedure 1, where ferrous sulfate was given alone and procedure 2, where ferrous sulfate was administered with 50 mg. of ascorbic acid.

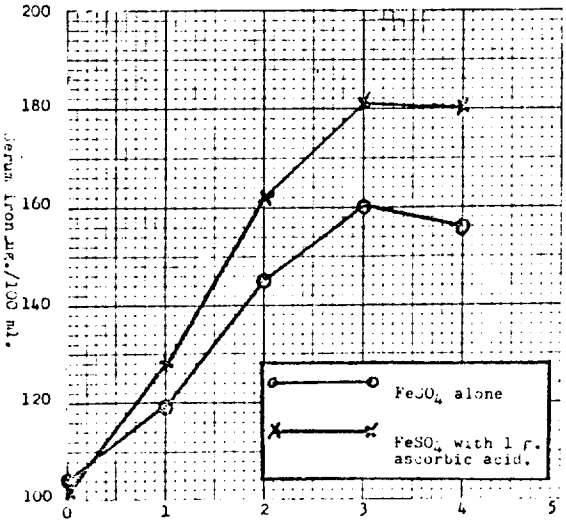

Fig. 1.—Mean iron absorption curves comparing procedure 1 with procedure 3.

The most reasonable explanation of the higher absorption curve when ferrous sulfate is administered with 1 g. of ascorbic acid is enhanced absorption of the ferrous iron. The alternative explanation that ascorbic acid mobilizes body iron stores is unlikely in view of the failure of intravenous ascorbic acid to affect the serum iron.[12]

This paper suggests that if ascorbic acid is used to enhance iron absorption therapeutically, it should be used in doses of the order of 1 g. rather than in doses of the order of 50 mg. Further work is required to demonstrate (a) the effect of intermediate dosages and (b) that the enhanced iron absorption shown in this paper has therapeutic significance. Pregnant women, for example, have a relatively high incidence of intolerance to oral iron; they may benefit from the addition of large doses of ascorbic acid because the dose of iron might then be reduced.

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Summary The study reported in this article was carried out on 40 fasting, non-anemic subjects. Procedure 1: Following the taking of blood for an initial serum iron estimation, oral ferrous sulfate, 200 mg., was given; then four serum iron estimations using the Ramsay method were made at hourly intervals. The method of study used in the other procedures was similar to that used in the first, except that in 2, 50 mg. of ascorbic acid was given with the ferrous sulfate and in 3, 1 g. of ascorbic acid was given with the ferrous sulfate. The procedures were carried out at four-day intervals in each subject, and their sequence was randomized. Higher rises in serum iron were found when ferrous sulfate was administered with 1 g. of ascorbic acid, suggesting that this dose, but not a dose of 50 mg., significantly enhances the absorption of ferrous sulfate. The results were analyzed statistically.

Résumé La présente étude a été entreprise en trois phases sur 40 sujets à jeun, non anémiques. Dans la phase un: après une prise de sang faite pour évaluer la première concentration de fer dans le sérum, on a donné *per os* 200 mg de sulfate ferreux; puis, à une heure d'intervalle, on a évalué le fer sérique par la méthode Ramsay. La méthode utilisée au cours des autres phases était semblable à celle de la première et n'en différait que par la dose d'acide ascorbique qui était de 50 mg dans la phase 2 et de 1 g dans la phase 3. Ces opérations se sont déroulées, chez chaque sujet, à intervalle de quatre jours. Toutefois leur succession se faisait au hasard selon le sujet. Comme les plus fortes augmentations de fer sérique ont été trouvées chez les sujets qui avaient ingéré la dose de 1 g d'acide ascorbique, l'auteur en conclut que c'est avec la dose de 1 g et non pas avec celle de 50 mg que l'acide ascorbique augmente de façon importante l'absorption de fer ferreux. Ces résultats ont été analysés au point de vue statistique.

We wish to thank the following: Roche Products Limited, for supplying 1-g. ascorbic acid tablets in the form of "Redoxon"; Drs. J. A. Bruce and J. G. Sclater, physicians at the Eastern General Hospital, Edinburgh, for permission to study patients under their care; Mr. Lutz of the Usher Institution, Edinburgh, for his advice with the statistics; and the patients for kindly agreeing to participate in the trial.

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The Vitamin C Status of Alcoholics[1]

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This study is designed to compare the vitamin C status of alcoholics to that of non-alcoholics, as assessed by the urinary excretion of oral, test doses of vitamin C. Jolliffe ('42) found vitamin deficiency diseases to be associated with alcoholism and Taverna ('54) found significantly low vitamin C levels in the blood of 30 alcoholic patients. The present study provides additional evidence to demonstrate that the vitamin C level of hospitalized alcoholics is substantially and significantly lower than that of non-alcoholics; the magnitude of this deficiency has been determined, establishing the basis for a rational program of nutritional therapy in these patients.

Low levels of vitamin C have also been observed to occur with significant frequency in the non-alcoholic population.

EXPERIMENTAL PROCEDURE

Vitamin C was administered to post-absorptive individuals in an oral, test dose of 500 mg. The bladder was emptied immediately prior to the administration of the test dose and the subsequent 4-hour excretion of urine collected, oxalic acid added to a concentration of 5% and preserved under refrigeration; the urine was analyzed for vitamin C by the method of Roe and Keuther ('43). The 4-hour period was chosen to facilitate the study; the shortness of the period produced no failures in urine collection.

The alcoholic population consisted of 85 male patients, 30 to 55 years old, who were tested on the day of admission to the Blue Hills Hospital of the Connecticut Commission on Alcoholism. The patients were either intoxicated or suffering from subsequent effects of having drunk liquor over three to 10 days. Intake of vitamin C preceding hospital admission was trivial. Ten of these patients were given 250 mg of vitamin C supplementation on the second through the 6th day and test doses of 500 mg on the first and 7th days of their hospital stay. Thirty patients were given 500 mg of supplementation daily and tested on the 7th day. Eight patients were given 1000 mg of supplementation daily and tested on the 7th day (supplementation was reduced to 500 mg on the 6th day in order to avoid possible residual vitamin C excretion on the test day; it had been determined that a 500 mg test dose could be excreted in 24 hours: a similar determination was not made for double this dose). The remaining 37 patients left the hospital against medical advice before a final test dose could be administered.

The control group consisted of 29 apparently healthy non-alcoholic subjects, 7 females and 22 males, aged 20 to 55 years, comprised of laboratory staff members and college undergraduates. Initially, 23 subjects were tested and then received daily vitamin C supplementation of 500 mg; 7 of these were tested on the 4th and 7th days, with 14 tested on the 7th day; two subjects did not complete the test. Another group of 6 non-alcoholic subjects was given 1000 mg of supplementation on the first, second and 5th days and 500 mg on the third, 4th, 6th and 7th days, and their vitamin C excretion was tested on the 4th and 7th days.

Neither alcoholic nor non-alcoholic individuals were allowed extraneous sources of high vitamin C content, such as citrus juices and vitamin tablets, on the days of testing.

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[1] This research was supported by a grant from the Miles Laboratories, Inc., Elkhart, Indiana.

RESULTS

The urinary excretion of vitamin C is a function of the amount ingested and its level in the body. Johnson and Zilva ('34) found that a more or less constant level of urinary excretion was noted when the store of ascorbic acid was complete. Hafkesbring and Freeman ('52) also noted in saturation studies that the amount of vitamin C in the urine is in proportion to its blood plasma level and that at saturation the percentage excretion of a test dose of vitamin C is relatively constant. The level of vitamin C in the body thus determines that proportion of an administered dose which will be excreted. If the amount of vitamin C excreted equals the intake, less any loss by other routes, then the body is considered to be saturated with respect to this vitamin. Accordingly, the minimal daily requirement is that amount of vitamin C below which the percentage of a test dose excreted will diminish; the minimal daily requirement serves to maintain the tissue reserves of vitamin C at a maximal level (Storvick and Hauck, '42).

The results of this study are shown in table 1. From observations of the 21 non-alcoholic subjects (column C), receiving daily vitamin C supplementation of 500 mg for a week, we conclude that under our test conditions the mean excretion of vitamin C in 4 hours for an individual at saturation is 34.9 ± 12.3%; any individual who excretes less than 10.3%, which represents two standard deviations below the mean, is by this criterion unsaturated or deficient in vitamin C. The P_D values in table 1 express the proportion of individuals in each group who were deficient on this basis.

Prior to supplementation (column A), alcoholic patients excreted a lower percentage of the test dose of vitamin C than non-alcoholic subjects (4.5% vs. 15.7%). Deficiency was observed in 85% of the alcoholic patients and 39% of the non-alcoholic subjects. The difference between these proportions (0.85 and 0.39) was significant at the 99% confidence level.

After a week of vitamin C supplementation of 250 mg daily, the excretion of 10 alcoholic patients (column B) had increased from 3.6 to 8.3%; 70% of these individuals still showed a deficiency at the end of the week. The 30 alcoholic patients who had received 500 mg of vitamin C supplementation daily (column C) increased their excretion from 4.8 to 22.4%; only 13% of these individuals were deficient at the end of the week. Neither the change from 3.6 to 8.3% nor the fall in the proportion deficient (column B) was significant, whereas that from 4.8 to 22.4%, as well as the fall in the proportion deficient (column C) was found to be highly significant. The results indicate that daily supplementation of 250 mg of vitamin C for a week is insufficient to correct the initial deficiency and that at least 500 mg daily is necessary.

In comparing the excretion of the 21 non-alcoholic subjects (column C) with that of the 30 alcoholic patients (column C), receiving the same supplementation, the difference in the mean excretions (34.9 vs. 22.4%) was found to be not significant, although the proportion (0.13) of deficient alcoholic patients differed significantly from zero. This indicates, we feel, that some importance should be attached to the lower mean level of excretion.

The data for the 8 alcoholic patients given 1000 mg of supplementation daily (column E) show the effect of larger amounts of supplementation. The mean of their excretion increased significantly from 2.6 to 29.8%, with only one patient deficient at the end of the week. Although a significant difference was not found between the mean of this group and that of the 30 alcoholic patients (column C), the fact that this mean is higher (29.8 vs. 22.4%) shows that amounts of supplementation larger than 500 mg tend to increase the mean excretion.

The increase from 15.4 to 34.9% in the mean excretion of the 21 non-alcoholic subjects (column C) was highly significant and shows that the non-alcoholic population was not initially at saturation. The value of 34.9% is only an approximation of the true saturation value even though it is not contradicted by the results noted for the 6 non-alcoholic subjects who received 1000 mg of supplementation (column E): no statistically significant difference is found between their mean excretions (45.0

TABLE 1

Urinary excretion of vitamin C

Day	Pre-supplementation (a)			Daily supplementation											
				250 mg (b)			500 mg (c)			500 mg (d)			1000 mg (e)		
	Mean excretion[1]	No. subjects	Proportion deficient[2]	Mean excretion	No. subjects	Proportion deficient	Mean excretion	No. subjects	Proportion deficient	Mean excretion	No. subjects	Proportion deficient	Mean excretion	No. subjects	Proportion deficient
							Alcoholic subjects								
1	4.5 ± 6.2[3]	85	0.85	3.6 ± 5.0	10	0.80	4.8 ± 7.0	30	0.83						
7				8.3 ± 7.4	10	0.70	22.4 ± 8.8	30	0.13				2.6 ± 1.1	8	1.00
													29.8 ± 20.1	8	0.13
							Non-alcoholic subjects								
1	15.7 ± 13.5	23	0.39				15.4 ± 13.0	21	0.38	19.5 ± 12.8	7	0.29			
4										30.4 ± 8.6	7	0.00	34.7 ± 10.1	6	0.00
7							34.9 ± 12.3	21	0.00	37.3 ± 11.8	7	0.00	45.0 ± 12.4	6	0.00

[1] Four-hour mean per cent excretion of 500 mg oral test dose of vitamin C.
[2] Percentage of individuals deficient in each group, excreting less than 10.3% of test dose.
[3] Standard deviation.

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vs. 34.9%). There are also indications that those 6 non-alcoholic subjects who had received 1000 mg supplementation (column E) and the 7 non-alcoholic subjects who had received 500 mg of supplementation (column D) had reached a plateau of excretion, because no significant difference was found between mean excretions on the 4th and 7th days (45.0 vs. 34.7% and 37.3 vs. 30.4%). The fact that some increase was noted in each case, however, suggests that the true value for saturation is somewhat higher than 34.9%. The values for the 24-hour excretion of the 6 non-alcoholic subjects showed a mean of 95.9% of the 500 mg administered, which corresponds closely to the definition of saturation given; therefore, the 4-hour excretion value of 45.0% would appear to be closer to the true saturation value than that of 34.9%, even in the absence of data for the 24-hour period for the 21 subjects.

No evidence was noted that vitamin C excretion was affected by differences in age or sex.

DISCUSSION

In the absence of contrary evidence and criteria for optimal functioning of the organism, the vitamin C saturated state is assumed to be desirable. It appears best to err on the side of safety and set standards for non-deficiency that are unassailable: if more than 10% of a 500 mg test dose of ascorbic acid is excreted in 4 hours, then the individual tested is deemed non-deficient in C. Although the excretion of less than 10% is consonant with an apparent state of well-being, an objective test which is more sensitive than any set of clinical signs is desirable. By the standards of this study, then, a significant number of individuals in the non-alcoholic population were found to be deficient in vitamin C prior to supplementation; this condition was corrected by a vitamin C supplementation of 500 mg daily for 7 days, and, in fact, evidence indicates that it was corrected by the 4th day. J. Sigurjonsson ('51) found similar low results with 5 male subjects; using oral test doses of 10 mg/kg of body weight, he found that during the first 4 hours after an initial test dose, subjects excreted a mean of 0.6%; after 4 days of supplementation their mean excretion had increased to 20.6%. Based on 24-hour collection periods, Youmans et al. ('36) found comparable results.

The great variability (15.7 ± 13.5%) of the excretion data for the non-alcoholics, pre-supplementation, shows clearly that no specific value can be set as the normal excretion of vitamin C and this confirms the conclusion of Johnson and Zilva ('34) that "The urinary excretion of ascorbic acid under normal conditions of existence is variable."

The present results establish a reliable, and yet simple, method for determining the vitamin C status of individuals. The use of a 4-hour urine collection and an oral test dose of 500 mg lend themselves conveniently to practical application. Twenty-four-hour collection periods and intravenous test doses eliminate most sources of error and produce more accurate and complete results, as Wright, Lilienfeld and MacLenathan ('37) argue; however, the shorter collection period considerably reduces the probability of subject errors in carrying out the determinations.

Our data show clearly that the vitamin C status of the alcoholic upon hospital admission differs considerably from that of the non-alcoholic. This is clear from a comparison of the pre-supplementation tests alone. Supplementation of 250 mg of vitamin C for a week is inadequate to correct the deficiency in alcoholic patients; at least 500 mg daily is necessary to correct the initial deficiency, and because of the severity of the deficiency, even this amount does not raise the level in alcoholic patients to that of non-alcoholic subjects. An increase to 1000 mg daily raises the level somewhat. Because alcoholic patients tend to leave the hospital soon after admission, and considering the evidence from this study, we recommend that such individuals receive at least 500 mg of vitamin C supplementation daily for a week or more, after which possibly 150 mg daily might be used.

SUMMARY

Prior to supplementation, the percentage excretion of a 500 mg oral test dose of vitamin C was significantly lower for

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85 alcoholic patients upon hospital admission than for 23 non-alcoholic subjects. Significantly more alcoholic patients than non-alcoholic were deficient in vitamin C, although a significant proportion (0.39) of non-alcoholics also were deficient.

The level of vitamin C excretion of the non-alcoholic subjects was raised to saturation value by administering 500 mg of vitamin C daily for a week; 34.9% excretion of an oral, test dose in 4 hours approximates the true saturation value.

Daily supplementation with 250 mg was inadequate to correct the initial deficiency in the alcoholic patients; at least 500 mg of vitamin C daily for a week is required in such patients before placing them upon a maintenance regime.

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ASCORBIC ACID in Treatment of the Canine Distemper Complex

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A CLINICAL investigation of ascorbic acid as a therapeutic agent in treatment of canine distemper complex was initiated in the author's practice early in 1967. This move was prompted by reading a report that vitamin C had been used clinically, with notable success, in treating 12 cases of distemper complex (canine and feline) in one practice.[1]

Ten years of practice had led me to view skeptically all reports of the type cited. However, experience during those same years had made me aware that the recovery rate among my patients showing signs of CNS disturbance, and treated with the generally accepted therapeutic regimen, was a dismal 5% to 10%. With many of these patients, the prognosis appeared to be hopeless from the first examination. Many others progressed rapidly from showing signs of the distemper complex to a state of chorea followed by death.

With this background in mind, intravenous injection of ascorbic acid (250 mg./cc.), Scorbate® Injection (Burns Pharmaceuticals) was added to the course of treatment given for canine distemper in our practice.

About a year after the investigation was started, John E. Reinert, M.D., a local neurologist and neurosurgeon, became interested in the work and thereafter was associated with the study. Dr. Reinert examined many of the dogs for neurologic impairment and observed their progress after treatment. After assessing the results in dogs, he began using ascorbic acid to

treat some of his own patients, with favorable results.

During the 22 months before this paper was prepared, 67 dogs in which canine distemper had been diagnosed were treated with ascorbic acid and a running summary of their histories was kept.* The following case histories are typical examples.

Case Histories

Case No. 1

This 2-year-old male Miniature Poodle with typical signs of distemper had been under treatment for 10 days. On the eleventh day, convulsions began to occur almost continuously. Within 24 hours, the animal was semicomatose, unable to stand, and stricken with chomping and foaming seizures. During the next five days, while the dog remained in the same condition and failed to respond to treatment, the owner refused permission for euthanasia to be performed.

On the morning of the sixth day following the onset of convulsions, 1,500 mg. of ascorbic acid was given intravenously. Late that afternoon, although mildly incoordinated, the dog was standing, walking in the cage and drinking water.

By the following morning, there were no signs of incoordination and the temperature had dropped from 103 F. to 101.8 F. After a second 1,500-mg. dose of ascorbic acid was injected, the condition continued to improve. The dog drank water and ate several meals of solid food during the day. A third dose of 1,500 mg. ascorbic acid was given the next day, although by that time no signs of distemper were present.

Five days after the beginning of treatment with ascorbic acid, the dog was discharged. Weekly checkups for the next three weeks indicated a complete return to clinical normalcy. When last examined, one and a half years later, the patient was physically sound and in apparent good health.

*A tabular summary showing clinical signs, daily temperatures, dosages of ascorbic acid, adjunctive therapy and results for each patient, is available upon request to the editors.

Case No. 22

A 2½-year-old male Shetland Sheepdog had been treated elsewhere for one month. Throughout that time, this dog's temperature had remained within a range of 103 F. to 104 F. The general condition of the animal upon presentation at our hospital was classified as poor.

In addition to our standard treatment for distemper, a 2,000-mg. intravenous dose of ascorbic acid was given daily for three days. By the second day, the temperature had dropped to 102 F. from 104 F.; on the third day it was 101.6 F.

The patient was discharged on the fifth day. Recovery was uneventful.

Case No. 43

Clinical signs in this 9-month-old male Poodle were convulsions, tremors over the entire body, incoordination, and a temperature of 106.4 F.

Treatment was immediately started with 2,000 mg. ascorbic acid in conjunction with Dilantin® Suspension (Parke-Davis), Sparine® (Wyeth), atropine, and phenobarbital. Within 24 hours, the convulsions had ceased. The temperature was 101 F., and it remained normal throughout the rest of the treatment period.

By the third day, the tremors had disappeared and all medication but ascorbic acid was discontinued. After the fifth day of treatment with ascorbic acid, the patient was discharged, giving every indication of being completely normal.

Case No. 65

When presented, this 2½-year-old male Poodle had been exhibiting signs of hard-pad distemper for six weeks. A slight posterior paralysis and mild incoordination were present. The temperature was 103.6 F.

After two daily doses of 2,000 mg. as-

TABLE 1: Recovery Rates among Dogs Treated with Ascorbic Acid* for Canine Distemper Complex

Patient Group	No. Treated	No. Recovered	Recovery Rate
All dogs treated	67	48	71.64%
Cases showing CNS disturbance	16	7	43.75%
Atypical cases with CNS disturbance but no convulsions	4	3	75.00%
Typical cases with convulsions	12	4	33.33%
Cases without CNS disturbance	51	41	80.39%
Typical cases with convulsions and given 3 or fewer doses of ascorbic acid	7	1	14.29%
Typical cases with convulsions and given more than 3 doses of ascorbic acid	5	3	60.00%
Typical cases without convulsions and given more than 3 doses of ascorbic acid	14	11	78.57%

*Scorbate® Injection (Burns Pharmaceuticals)

TABLE 2: Dogs Given Massive Doses of Ascorbic Acid over a Three-Day Period

Breed	Sex	Age	Weight	Total Dose*
Poodle—X	M	1 Yr.	16.5 Lb.	45,000 mg.
Terrier—X	F	8 Mo.	13 Lb.	45,000 mg.
Shepherd—X	F	4 Mo.	25 Lb.	45,000 mg.

*5,000 mg. ascorbic acid, Scorbate® Injection (Burns Pharmaceuticals) given intravenously three times a day for three days

corbic acid, the temperature was reduced to 101.4 F. After four more days of treatment with ascorbic acid, the patient was discharged.

Two and a half weeks later, the owner requested euthanasia because of a recurrence of the paresis and incoordination which were becoming progressively worse.

Discussion

RECOVERY RATES observed during the investigation are shown in Table 1. As might be expected, treatment beginning at the onset of clinical signs gave more favorable results than treatment delayed until the condition was in an advanced stage. Although relatively few animals exhibited convulsions in conjunction with the typical signs of distemper, the recovery rate for those in this group that were given more than three doses of ascorbic acid was much higher than that for those given fewer doses (60% as compared to 14%).

Temperatures were elevated in most of the 67 dogs at the time of the first examination, but in almost all cases were within normal limits at 24 or 48 hours after treatment was started. During the latter part of the investigation, when hourly temperature charts were kept, many temperatures were found to be normal within 2 to 6 hours

after the first injection of ascorbic acid.

In all instances, the ascorbic acid was administered intravenously at a rapid rate. Some drowsiness, which lasted only a few minutes, was seen in 2 dogs immediately after injection of the vitamin. However, there were no other visible side effects and no toxicity attributable to treatment. To help establish dosage and determine the possible consequence of giving large doses of ascorbic acid, 3 dogs were obtained from a shelter and given 5,000 mg. ascorbic acid three times daily for three days *(Table 2)*. No side effects were seen in any of these dogs. All three were placed in homes, and are doing well to date.

Conclusion

FROM THE results observed in 67 clinical cases of canine distemper complex, it appears that a daily dose of 1,000 mg. to 2,500 mg. of ascorbic acid given intravenously for at least three days is beneficial in the treatment of canine distemper, and that the recovery rate can be markedly improved by including ascorbic acid in the treatment regimen.

During this investigation, ascorbic acid produced a rapid drop in temperature. The recovery rate during a 22-month period was 71.64%. When more than three doses were given, the rate rose to 78.57% for dogs that did not have convulsions. When more than three doses were given to dogs that exhibited convulsions, the recovery rate rose from 14.29% to 60%.

Fully recognizing that this investigation did not constitute a controlled study, but encouraged by the results, the author has presented these observations in the hope that they will be of help to other practitioners and perhaps stimulate additional work in this area. Certainly, more basic research is needed to define the mechanisms involved and to validate the observations reported here.

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Electrometric Determination of Ascorbic Acid

By H. LIEBMANN and A. D. AYRES

(*Read at the Meeting of the Society on April* 4, 1945)

In coloured extracts the direct visual titration of ascorbic acid with 2, 6-dichlorophenol-indophenol is impossible, and some other means has to be adopted to locate the end-point of the determination. Electrometric methods have been employed by a number of workers,[1,2,3] and excellent results may be obtained by a procedure suggested by Harris, Mapson and Wang. These authors use a mercury-coated platinum electrode and measure its potential against a calomel electrode as the titration proceeds. The e.m.f. remains steady during the titration and rises rapidly as soon as excess of the dye is present in the soln. under test. For routine analyses Harris's method has, however, a certain number of disadvantages. Potentials have to be determined after each addition of dye and a certain time has to be allowed for establishment of equilibrium at the electrode. Furthermore, the platinum electrodes must be freshly coated with mercury at frequent intervals.

It is well-known that equilibrium is rapidly attained with polarised electrode systems and it was thought that the "dead stop" method proposed by Foulk and Bawden[4] for iodimetric titration might be applicable to the titration of ascorbic acid with 2,6-dichlorophenol-indophenol. Apart from a possible increase in speed of titration a simplification of the experimental arrangement would be the main advantage, as neither special preparation of the electrodes nor the use of a standard reference cell and a potentiometer is necessary.

Two bright platinum wires are dipped into the acid extract under test and a small e.m.f., approximately 15 m.v., is applied. Polarisation occurs, setting up a small voltage of opposite sign, and the external e.m.f. can be adjusted so that no current flows in the system. A galvanometer in the circuit will show no deflection. These conditions remain unchanged during the course of the titration until an excess of the dye depolarises the electrodes, allowing a current to flow under the influence of the external voltage. The end-point of the titration

is, therefore, indicated by a sudden permanent excursion of the galvanometer from its zero position.

In our experiments, a 4-oz. wide-mouthed bottle served as a titration cell. This was closed with a cork stopper carrying the electrodes—bright platinum wires fused into glass tubes—and a leading tube for carbon dioxide. The tip of a 2-ml burette was inserted through a hole. A 2-volt accumulator with a 1,000 Ω fixed resistance and a 10 Ω potentiometer in series supplied the polarising voltage. A Cambridge "Spotlight" galvanometer of a sensitivity of 23 divisions/micro-amp. was used as current indicator.

The solution under test is pipetted into the cell and dye is added initially at the rate of 1 drop per second. No movement of the galvanometer occurs till the end-point is nearly reached, when the galvanometer is displaced, but returns to its zero position in a few seconds. The end-point is registered by a permanent deflection. During the titration the solution is vigorously stirred by a stream of carbon dioxide.

No difficulties are experienced in obtaining reproducible results if bright platinum wire electrodes are used. These are cleaned in chromic acid and washed with distilled water at the beginning of the experiments. Between the titrations they are stored immersed in water.

Some typical results are given in the following tables. The first set of data shows a comparison of visual and electrometric titration of solutions of ascorbic acid in trichloroacetic acid. The consistency of electrometric titrations can be judged by the first four figures in Table I, which represent single determinations on the same solution. All other results are the means of duplicate determinations.

TABLE I

TITRATION OF ASCORBIC ACID SOLUTIONS

Results are expressed as ml of dye solution

Electrometric ..	1·73	1·69	1·68	1·68	1·98	1·87	1·67	1·29	1·69	1·79*
Visual	—	—	—	—	1·99	1·92	1·80	1·30	1·63	1·78*

* Ascorbic acid in 8% acetic acid.

Extracts of brussels sprouts and broccoli leaves were prepared in the usual manner. Unless otherwise stated, a mixture of trichloroacetic acid and metaphosphoric acid was used as extracting medium. The results of electrometric and visual titrations are given in Table II. Here again, the figures represent the means of two determinations.

TABLE II

ASCORBIC ACID CONTENT OF VEGETABLES

	Sprouts						Broccoli	
Electrometric ..	31·4	53·6	58·9	92·5*	25·4*	36·1†	.. ..	188·0 mg/100 g
Visual	35·0	53·5	60·5	89·6*	25·4*	36·9†	.. ..	188·0 ,,

* Trichloroacetic acid as extracting medium.
† ,, ,, and citric acid as extracting medium.

In order to test whether naturally occurring pigments would interfere with the depolarisation of the electrodes, ascorbic acid determinations were carried out on samples of blackcurrant juice and extracts from beetroot. The colour of these solutions was very similar to that of unreduced dichlorophenolindophenol and no reliable results could be obtained by visual titration even when chloroform extraction, as suggested by McHenry and Graham,[5] was used. Known amounts of ascorbic acid were therefore added to the solutions and their recoveries were determined by the electrometric method. The results are given in Table III.

TABLE III

TITRATION OF ASCORBIC ACID IN COLOURED EXTRACTS.

Product	Extract taken ml	Ascorbic acid: Added mg	Total found mg	From extract mg	Recovered mg	Recovery %
Blackcurrant ..	10	0·0	0·544	0·544	—	—
	5	0·231	0·509	0·272	0·237	102·5
	2	0·370	0·477	0·109	0·368	99·5
Beetroot	10	0·0	0·036	0·036	—	—
	10	0·231	0·264	0·036	0·228	98·7
	10	0·463	0·492	0·036	0·456	98·6

It appears that the "dead stop" method devised by Foulk and Bawden is well suited for the electrometric determination of ascorbic acid and that it has certain advantages over the procedures hitherto described.

The application of Foulk and Bawden's method to the determination of ascorbic acid was first suggested by Dr. C. G. Sumner, working in this Department, but wartime conditions made it impossible for him to test the practicability of this idea. We are grateful for his consent to the publication of our results. We are also indebted to the Metal Box Co., Ltd., and Mr. R. K. Sanders, Head of the Research Department, for permission to communicate this paper.

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Discussion

The President thanked the authors for their interesting paper and asked if in their method the condition of the surface of the electrodes was an important factor.

Dr. J. E. Page asked for particulars of the method used to stir the solution during titration.

Miss M. Olliver said that a modification of the McHenry and Graham visual method for estimating ascorbic acid in coloured solutions had been found exceedingly useful for routine control work; it was very rapid and, with experience, good agreement could be reached with results obtained by the Harris, Mapson and Wang electrometric method. Dr. Liebmann had stated that a pronounced change in E.M.F. took place when excess of dye was present; it appeared, therefore, that the described method would not have the advantage of the Harris, Mapson and Wang method, in which the absolute end-point could be determined. The results in Table I would be more conclusive if comparison had been made with the theoretical values instead of those obtained by visual titration. In Table II, one would have expected better agreement with sprouts extracts, being uncoloured, if both methods were applied to the same extracts. Finally, in view of Dr. Liebmann's remarks on the effect of iodide ions on the potential, Miss Olliver suggested that the possibility of interference of halide ions and other substances with the proposed method would need to be carefully investigated.

Dr. Liebmann, replying to the President, said that the magnitude of the polarisation E.M.F. set up at the electrodes, and hence the state of the surface of the electrodes, was not critical. By operation of the potentiometer the external voltage could be adjusted at will to balance the polarisation E.M.F. and to establish the condition of no current flowing at the beginning of the titration. In reply to Dr. Page, he said that the solutions were stirred by a vigorous stream of carbon dioxide, but mechanical stirring would be equally suitable. In reply to Miss Olliver, he said that neither the method of Harris, Mapson and Wang nor that now described could claim to yield "absolute" end-points. They both relied on the perception of an excess of the reagent by observation of a physico-chemical change. While Harris and his collaborators observed a rise in E.M.F. of an electrode in presence of an excess of dye, the end-point in the proposed method was indicated by the sudden flow of a current due to the depolarisation of an electrode by unreduced dye. Both were therefore indirect methods, and no advantage could be claimed for either on fundamental grounds. In both instances it was possible to determine the end-point by graphical interpolation. Iodide or other halide ions would not interfere with the titration. The decisive change at the end-point was the depolarisation of the cathode by excess dye. Throughout the titration the anode remained depolarised, probably first by the ascorbic acid and later by the reduced dye. Halide ions would also act as anode depolarisers and would therefore bring about no change at the already depolarised anode.

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HOLGER EHLERS

ANNIS LXX FELICITER EXACTIS DEDICATUM DIE XXX MAII MCMLXIX

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THE PRESSURE LOWERING EFFECT OF ASCORBIC ACID IN OCULAR HYPERTENSION

BY

ERIK LINNÉR

In previous studies I reported that ascorbic acid produced a fall in the intraocular pressure of about 2 mm Hg[4, 5, 6, 7]. The pressure-lowering effect was obtained after oral administration and also after topical application. The fall in pressure took place about two days following the administration of ascorbic acid. So far normal human eyes and a few glaucomatous eyes have been studied.

In the first study of normal human eyes there was found to be a slight numerical increase in the facility of outflow of about 15 per cent. The reduction in the outflow pressure of about 50 per cent could hardly be entirely explained by these tonographic changes. Other possible mechanisms were therefore discussed. A reduction in the rate of aqueous flow was considered to be the most likely explanation. In further studies the results obtained by means of the suction cup technique indicated a diminished increase in pressure and volume during treatment with ascorbic acid. These results supported the explanation that the pressure fall was caused by a reduction in the rate of aqueous flow. This is however true only under the assumption that no other pathways exist by which the aqueous humour can leave the eye. A bulk drainage by way of uveo-scleral routes in addition to the conventional routes as reported by Bill[2] cannot be excluded although such pathways have not been demonstrated in human eyes. According to this suggestion an increase in the drainage through the posterior part of the eye might occur if the hyaluronic acid in the posterior part of the eye was depolymerized by the high concentration of ascorbic acid.

The purpose of the present study was to investigate the effect of ascorbic acid on a group of human beings with ocular hypertension. In these cases the intraocular pressure was 20-25 mm Hg and the outflow pressure about 10-15 mm Hg instead of about 5 mm Hg at normal pressure levels.

Material and Methods

The individuals in this study belonged to a group in whom moderate ocular hypertension was detected. They have been kept under observation for five years without treatment. Two reports concerning this follow-up were published[8,9].

One group of 25 subjects with an average age of 63 years was first examined by tonography without treatment. A new tonographic examination was then carried out after administration of ascorbic acid by mouth, 0.5 gm four times a day for six days. The mean of the two eyes was used in all calculations as a representative value for each individual. The difference between the values during and before treatment was calculated for each individual.

In a separate group of 19 subjects with an average age of 63 years the intraocular pressure was measured by means of the same electronic tonometer and recorder. A 10 per cent aqueous solution of ascorbic acid was given topically in one eye three times a day for 3 days and the other eye was used as a control.* The right and the left eye were treated in alternate subjects. The effect of the treatment was calculated for each individual as the change in the difference between the test eye and the control eye following topical application of ascorbic acid. The differences are greater in ocular hypertension than in eyes with normal pressure.

A certified Schwarzer's electronic tonometer and a Philips recorder were used. The right eye was always examined before the left one. The facility of outflow was calculated on the first four minutes of the tracing using Friedenwald's table of 1955. Benoxinate 0.4 per cent (Novesin, Wander, Berne) was used as a local anaesthetic.

Results

Following oral treatment with ascorbic acid a significant fall in the intraocular pressure of 1.10 mm Hg was found, but no significant change in the facility of outflow was observed (Table 1).

* These drops were kindly prepared by AB Hässle, Göteborg, Sweden.

Table 1.

Effe of ascorbic acid on intraocular pressure (Schiötz) (P_o) and facility of outflow (C) in 2 subjects with ocular hypertension. 0.5 gm ascorbic acid was given four times a day by mouth for 6 days.

		Before ascordic acid I	During ascorbic acid II	Difference II - I
P_o	$\bar{x}$	23.3	22.2	÷ 1.10
	$s_{\bar{x}}$	0.71	0.69	0.299
C	$\bar{x}$	0.23	0.20	÷ 0.024
	$s_{\bar{x}}$	0.015	0.014	0.0098

In comparison to the difference between the two eyes before treatment the pressure value was significantly lower in the test eye than in the control eye during treatment with eye drops of ascorbic acid, the change being 1.19 mm Hg (Table 2).

The pressure lowering effect was of the same order of magnitude following systemic and topical treatment with ascorbic acid.

Discussion

The fall in pressure previously demonstrated in normal human eyes was also found in the subjects with ocular hypertension in this study. The effect was of

Table 2.

ect of 10 % ascorbic acid given topically 3 times daily during 3 days on the intraocular pressure (Schiötz) in 19 individuals with ocular hypertension.

	Before ascorbic acid		Ascorbic acid		Difference
	Test eye I	Control eye II	Test eye III	Control eye IV	(III - IV) - (I - II)
$\bar{x}$	23.7	23.1	22.8	23.4	1.19
$s_{\bar{x}}$	0.97	1.02	0.97	1.05	0.346

the same order of magnitude in spite of a marked difference in outflow pressure. The tonographic values of outflow facility were not significantly changed. These results are further evidence in support of the view that the fall in pressure could not be explained by a change in the outflow facility.

The findings in this study confirm the conclusion that the fall in pressure could be caused by a reduction in the rate of aqueous flow, or as another possibility which cannot be excluded by bulk drainage by way of posterior uveo-scleral routes. At the moment there is no way of distinguishing between a change in a pressure-independent uveo-scleral drainage and a change in the rate of aqueous flow entering the anterior chamber in human eyes.

In a paper by Gnädinger and Willome[3] the effect of topical application of a 10 per cent solution of ascorbic acid was examined. According to their figures there was a difference of 1.5 mm Hg in the mean value before and during application of ascorbic acid: in the control eye this difference was 0.57 mm. The authors reported that the treated and the untreated eye did not differ significantly, but they did not present the differences within each individual. The mean value of the fall in pressure was about 1 mm Hg greater in the treated eye than in the control eye and this effect is of the same order of magnitude as the results in the present study.

In a review Bietti[1] reported different studies concerning the pressure reducing effect of ascorbic acid. Doses as high as 0.1 to 0.5 gram per kilogram body weight were given by mouth or 0.4 to 1.0 gram per kilogram intravenously. A marked fall of the intraocular pressure was observed within a few hours. This result was considered to be caused by an osmotic effect at least in rabbits. Other possible pressure-reducing mechanisms were also investigated by using tonography. In most of the cases a diminished formation of aqueous humour rather than a decrease in the outflow resistance was considered to play a role although the possibility of the resistance being reduced was not completely excluded in some cases. Their conclusion that the tonographic evidence speaks in favour of a decrease in the rate of aqueous flow rather than a change in outflow facility is in agreement with my findings.

Summary

The effect of ascorbic acid on human beings with ocular hypertension was examined.

Following oral treatment with 2 gram of ascorbic acid daily for 6 days the intraocular pressure decreased significantly 1.10 mm Hg but no significant change in the facility of outflow was observed. Following topical administration

of : orbic acid the pressure in the test eye was significantly lower than the pre :re in the control eye in comparison to the difference between the two eyes bef : treatment.

T : findings in this study confirm the conclusion that the fall in pressure :ou be explained by a reduction in the rate of aqueous flow or as another pos :ility which cannot be excluded by bulk drainage by way of posterior uve:-scleral routes.

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ACTA VITAMINOLOGICA

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CIVIL HOSPITAL -P. COSMA - CAMPOSAMPIERO (PADOVA)

DEPARTMENT OF PATHLOGICAL ANATOMY AND OF CLINICAL RESEARCHES
(HEAD PHYSICIAN : PROF. U. LIPPI)

LYSOSOMAL LESIONS DUE TO LARGE DOSES OF ASCORBIC ACID

U. LIPPI - E. PULIDO - G. GUIDI

The administration of ascorbic acid in high doses causes in the liver of the guinea pigs definite changes of quantity and of distribution of certain cellular constituents. Burlina and Lippi (I) had means of observing, with the help of histochemical techniques, a significant increase of the cystoplasma of the RNA in the hepatocytes of guinea pigs which have been treated even for a short time with massive doses of ascorbic acid.

The participation of ascorbic acid in the activation of protein synthesis has been demonstrated by Vilter and Coll.(2), by Burlina and Lippi (I) and by Lippi and Coll.(3) in various experimental situations. Nevertheless it has not been possible to attribute to the hystochemical evidence a precise functional significance nor to establish with certainty the presence and the value of hepatic cellular damage blamed on ascorbic acid dosage.

Purpose of the present research has been that of studying the structural and distributive behavicur of lysosomes, the modifications of which are accompanied by situations of cellular damage also and only of functional type. To this end we made use of a histochemical technique in order to make evident the acid phosphatasic activity, particularly suitable accord-

~~ingto to De Duve (4), Holt (5) and Bitensky (6) to~~

ing to De Duve (4), and Bitensky (6) to --mark-- in their morphology the lysosomial structure.

The histochemical quantity regarding the behaviour of the lysosomes and the presence of eventual cytoplasmatic lesions have been controlled with ultramicroscopic observation of hepatic cells of animals treated with massive doses of ascorbic acid.

MATERIALS AND TECHNIQUES

Fifteen male rats of Wistar stock and of average weight of 200-300 grams have been used. Ten animals have been treated for five days with ascorbic acid dissolved in physiological solution and injected intramuscularly at the rate of 2.5 milligrams per gram per day. Other five animals have been treated for control with only the physiological solution. At the end of the treatment, all the animals were disposed of by decapitation or by bleeding. Pieces of liver removed from various places of the organ were rapidly frozen in isopentane refrigerated at minus 70 degrees centigrade and dissected in cryostat at -18 , -20 C° in series of sections of five microns thickness. The sections obtained in such a manner were fixed at -4 C° in anhydrous acetone for 30 minutes to incubate in the solution substratum containing naphtol AS-BI and the pararosaniline at pH 5 according to Barka and Anderson (7).

Other pieces of liver were rapidly fixed in gluteraldehyde solution 3%, tamponed at pH 7.2 and then postfixed in OSO_4 and inclosed in Epon 812.

HISTOCHEMICAL AND ULTRAMICROSCOPICAL EVIDENCE

In the livers of the animals treated with ascorbic acid the lysosomial structures appear, with respect to the controls, to be remarkably increased in number and in volume. In many hepatocytes the lysosomial phosphatasic granulations are uniformly distributed throughout the cytoplasmatic range; in other cells the lysosomial structures show a polar justanuclear disposition. The numerical and volumetric increase of the lysosomes ~~of the~~ is again more evident at the level of the hepatocytes which appear involved by voluminous cytoplasmatic vacuities of round form and with regular borders (Fig. I and 2).

Under ultramicroscopic scrutiny of the livers of the animals treated with ascorbic acid, the numerical increase of the lysosomial structures irregularly distributed in the cytoplasma or crowded in justanuclear layer. is confirmed. Yet the lysosomial formations do not appear modified in their ultramicroscopic morphology, there being no evidence of membrane lesions or of structural modifications (Fig 3).

DISCUSSION AND CONCLUSIONS

The administration of high doses of ascorbic acid causes a profound and significant alteration of the lysosomial structures in the liver of rats.

The lysosomes, -- marked -- with the histochemical evidence of the phosphatasic acid activity and observed under the electron microscope, appear increased in number and in volume along with other cytoplasmatic changes at the expense especially of the ribosomes and with lesions of vacuous type.

Lysosomial lesions very similar to those just now described have been

found by Dianzani (8) in various frameworks of cellular pathology, independently of the pathogenic agent used in the way of their determination.

For cellular damage of diverse type, the lysosomes would actively react in functional and morphologic sense, but in a monomorphic manner and totally nonspecific, showing an index very much precocious and significant of cellular suffering. In each case, nevertheless, co-exist structural and distributive modifications of the cytomembranes and quantitative changes of the proteic turnover, which, in the treatment with high doses of ascorbic acid, are also shown by a significant increase of the ribosomic RNA.

Fig. I(at left);Distribution of the lysosomial acidophosphatasic structures in the normal rat liver.

Fig.2 (at right): In the liver of rats with massive doses of ascorbic acid the lysosomes appear histochemically increased in number and volume

((Naphtol AS-BI phosphate ; pararosaniline; pH - 5)

Fig.3 - The lysosomes appear very numerous and voluminous in the complex. The endocytoplasmatic cisterns are enlarged with breaks in many points of the cytomembrane. The granules of the reticulum appear voluminous and intensely electro-opaque.(Electronic enlargement I5,000x).

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ACTA
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OSPEDALE CIVILE « P. COSMA » - CAMPOSAMPIERO (PADOVA)
REPARTO DI ANATOMIA PATOLOGICA E DI RICERCHE CLINICHE
(Primario: *Prof. U. Lippi*)

Lesioni lisosomiali da dosi elevate di acido ascorbico

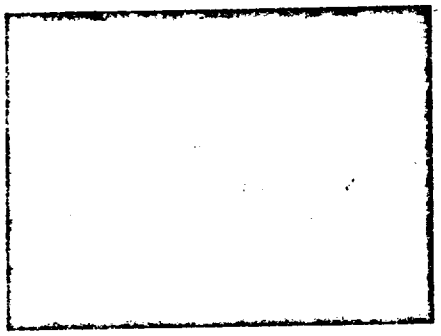
U. LIPPI - E. PULIDO - G. GUIDI

La somministrazione di acido ascorbico a forti dosi determina nel fegato della cavia sensibili modificazioni quantitative e distributive di alcuni costituenti cellulari. Burlina e Lippi (1) hanno avuto modo di osservare, con l'ausilio di tecniche istochimiche, un significativo aumento dell'ARN citoplasmatico negli epatociti di cavie trattate anche per breve tempo con dosi massive di acido ascorbico.

La partecipazione dell'acido ascorbico nell'attivazione delle sintesi proteiche è stata dimostrata da Vilter e Coll. (2), da Burlina e Lippi (1) e da Lippi e Coll. (3) in diverse situazioni sperimentali. Non si è pertanto potuto attribuire al reperto istochimico un preciso significato funzionale nè stabilire con certezza la presenza e l'entità di un danno cellulare epatico da carico di acido ascorbico.

Scopo della presente ricerca è stato quello di studiare il comportamento strutturale e distributivo dei lisosomi, le cui modificazioni si accompagnano a situazioni di patimento cellulare anche e solo di tipo funzionale. Ci siamo serviti a tale fine di una tecnica istochimica in grado di evidenziare l'attività fosfatasica acida, particolarmente adatta sec. De Duve (4), sec. Holt (5) e sec. Bitensky (6) a « marcare » nella loro morfologia le strutture lisosomiali.

Il dato istochimico riguardante il comportamento dei lisosomi e la presenza di eventuali lesioni citoplasmatiche sono stati controllati con l'osservazione ultramicroscopica di cellule epatiche di animali trattati con dosi massive di acido ascorbico.



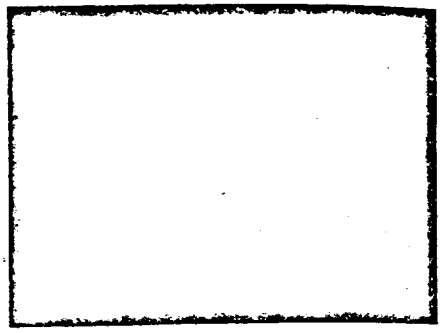


Fig. 1 (a sinistra): Distribuzione delle strutture lisosomiali acidofosfatasiche nel fegato normale di ratto.
Fig. 2 (a destra): Nel fegato di ratto con dosi massive di acido ascorbico i lisosomi appaiono istochimicamente aumentati di numero e di volume.

(Naftolo AS-BI fosfato; pararosanilina; pH = 5)

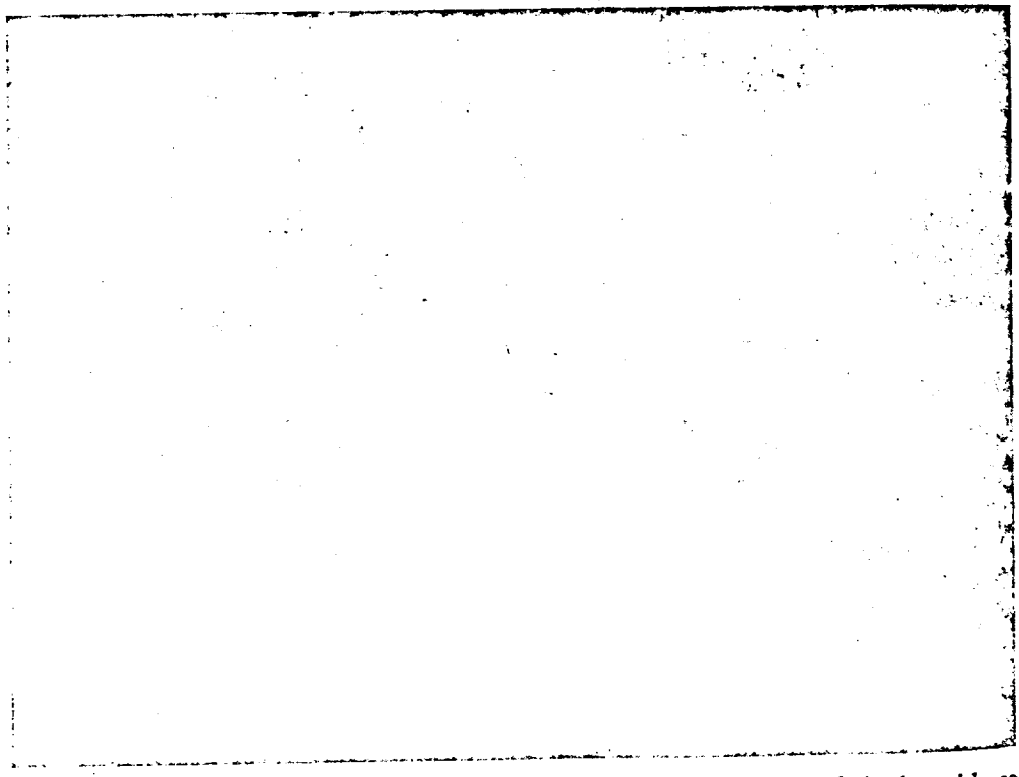


Fig. 3 - I lisosomi appaiono assai numerosi e nel complesso voluminosi. Le cisterne endocitoplasmatiche sono dilatate con interruzione in molti punti delle citomembrane. I granuli del reticolo appaiono voluminosi ed intensamente elettropachi. (Ingrandimento elettronico 15.000x).

MATERIALE E TECNICHE

Sono stati usati 15 ratti di ceppo Wistar, di sesso maschile e del peso medio di 200-250 g. Dieci animali sono stati trattati per 5 giorni con acido ascorbico disciolto in soluzione fisiologica ed iniettato intramuscolo in ragione di 2,5 mg/g/die. Altri 5 animali sono stati trattati, per controllo, con la sola soluzione fisiologica. Al termine del trattamento, tutti gli animali sono stati sacrificati per decapitazione e dissanguamento. Frammenti di fegato prelevati da punti diversi del viscere sono stati rapidamente congelati in isopentano refrigerato a —70 °C e quindi sezionati in criostato a —18°, —20 °C in sezioni seriate di 5 microns di spessore. Le sezioni ottenute in tale modo sono state fissate a +4 °C in acetone anidro per 30' e quindi poste ad incubare per 60' nella soluzione substrato contenente il naftolo AS-BI e la pararosanilina a pH 5 sec. Barka e Anderson (7).

Altri frammenti di fegato sono stati rapidamente fissati in gluteraldeide soluzione 3% tamponata a pH 7,2 e quindi postfissati in OSO_4 ed inclusi in Epon 812.

REPERTI ISTOCHIMICI ED ULTRAMICROSCOPICI

Nei fegati degli animali trattati con acido ascorbico le strutture lisosomiali appaiono, rispetto ai controlli, notevolmente aumentate di numero e di volume. In molti epatociti le granulazioni fosfatasiche lisosomiali sono uniformemente distribuite in tutto l'ambito citoplasmatico; in altre cellule le strutture lisosomiali presentano una disposizione polare iuxtanucleare. L'aumento numerico e volumetrico dei lisosomi è ancor più evidente a livello di epatociti che appaiono interessati da voluminose vacuolizzazioni citoplasmatiche di forma rotondeggiante ed a margini regolari (figure 1 e 2).

All'esame ultramicroscopico dei fegati degli animali trattati con acido ascorbico viene confermato l'aumento numerico delle strutture lisosomiali irregolarmente distribuite nel citoplasma od addensate in sede iuxtanucleare. Le formazioni lisosomiali non appaiono tuttavia modificate nella loro morfologia ultramicroscopica, non essendosi messe in evidenza lesioni di membrana o modificazioni strutturali endolisosomiali (figura 3).

DISCUSSIONE E CONCLUSIONI

La somministrazione di acido ascorbico a forti dosi determina un profondo e significativo rimaneggiamento delle strutture lisosomiali nel fegato di ratto.

I lisosomi, « marcati » con la evidenziazione istochimica della attività fosfatasica acida ed osservati al microscopio elettronico, appaiono aumentati di numero e di volume in concomitanza ad altre modificazioni citoplasmatiche a carico sopratutto dei ribosomi e con lesioni di tipo vacuolare.

Lesioni lisosomiali assai simili a quelle testè descritte sono state riscontrate da Dianzani (8) in quadri diversi della patologia cellulare, indipendentemente dall'agente patogeno in grado di determinarle.

A danni cellulari di tipo diverso, i lisosomi reagirebbero attivamente in senso funzionale e morfologico, ma in modo monomorfo e del tutto aspecifico, rappresentando un indice assai precoce e significativo di patimento cellulare. In ogni caso, pertanto, coesistono modificazioni strutturali e distributive delle citomembrane e variazioni quantitative del tournover proteico, che, nel trattamento con dosi elevate di acido ascorbico, sono anche dimostrate da un significativo aumento dell'ARN ribosomico.

RIASSUNTO

La somministrazione di acido ascorbico a forti dosi determina negli epatociti del ratto un notevole aumento numerico delle strutture lisosomiali. Anche in questo caso, le modificazioni strutturali e distributive dei lisosomi si accompagnano a lesioni citoplasmatiche delle citomembrane e ad una significativa attivazione delle sintesi proteiche con modificazioni quantitative e distributive dell'ARN ribosomico.

RESUME

Lésions des lysosomes provoquées par des doses élevées d'acide ascorbique

L'administration d'acide ascorbique à doses élevées détermine dans le cellules hépatiques du rat une augmentation remarquable numérique des lysosomes. En pareil cas, les modifications de la structure et de la distribution des lysosomes sont accompagnées de lésions cytoplasmatiques des cytomembranes et d'une activation significative des synthèses protéiques avec modifications de la quantité et de la distribution de l'ARN ribosomique.

SUMMARY

Lysosomal damage due to high doses of ascorbic acid

Administration of high doses of ascorbic acid in the rat induces a marked increase in the number of liver cell lysosomes. Also in this case the changes in the structure and distribution of the lysosomes are associated with lesions in the cellular membranes and with a significant increase in protein synthesis, as well as changes in the amount and distribution of ribosomal RNA.

ZUSAMMENFASSUNG

Lysosome Schädigungen durch hohe Dosen von Askorbinsäure

Die Verabreichung von Askorbinsäure zu hohen Dosen ruft in den Hepatozyten der Ratte eine deutliche zahlenmässige Zunahme der Lysosomen-Strukturen hervor. Die Struktur- und Verteilungsveränderungen der Lysosome führen auch bei diesem Falle zu zytoplasmatischen Schädigungen der Zytomembrane und zu einer signifikanten Aktivierung der Proteinsynthesen mit Mengen- und Verteilungsveränderungen der RNS.

RESUMEN

Lesiones lisosomiales debidas a dosis elevadas de ácido ascórbico

La administración de ácido ascórbico a fuertes dosis determina en los hepatocitos de rata un notable aumento numérico de las estructuras lisosomiales. También en este caso, las modificaciones estructurales y distributivas de los lisosomas presetan lesiones citoplasmáticas de las citomembranas y una significativa activación de las síntesis proteicas con modificaciones cuantitativas y distributivas del ARN ribosómico.

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The Role of Ascorbic Acid in the Metabolism of Storage Iron

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Summary. The role of ascorbic acid in the metabolism of storage iron was investigated in guinea-pigs. Ascorbic acid deprivation increased the total non-haem iron concentration in the spleen and reduced it in the liver, and in both organs ferritin was diminished and haemosiderin increased. Replacing the ascorbic acid restored the normal distribution of iron between the two storage compounds, and in the spleen the total storage iron concentration returned to control levels within 24 hr. Evidence was obtained in experiments with ^{59}Fe that the accumulation of iron in the spleen was due to a diminished release from reticulo-endothelial cells. When ^{59}Fe-labelled haemoglobin in denatured red cells was injected, release of the isotope was inhibited in scorbutic animals. In contrast, after injecting labelled transferrin, ^{59}Fe in the liver parenchymal cells was released to a greater extent than in normals. These observations may explain certain ferrokinetic peculiarities in patients with scurvy, and possibly also the predominantly reticulo-endothelial localization of the iron in Bantu subjects with siderosis.

There is reason to believe that the metabolism of ascorbic acid and iron are interrelated. For example, scurvy is common in the Bantu people of Southern Africa, but it is almost never seen in individuals who are not heavily siderotic (Seftel *et al*, 1966). Evidence has been obtained that irreversible oxidation of a proportion of the dietary ascorbic acid occurs, probably mediated by the massive tissue deposits of ferric iron (Lynch *et al*, 1967). The severity of the resultant ascorbic acid deficiency depends upon the amount of ascorbic acid in the diet, and consequently the incidence of clinical scurvy varies with the seasons.

Other observations, both clinical and experimental, suggest that deficiency of ascorbic acid may, in its turn, affect the metabolism of iron. In Bantu individuals with severe siderosis the plasma iron concentration is typically elevated, but if the tissue levels of ascorbic acid are depleted, the plasma iron is usually low (Bothwell *et al*, 1964). The administration of ascorbic acid to such subjects results in the prompt return of the plasma iron concentration to elevated levels. This suggests that the release of iron to the plasma may be inhibited by deficiency of the vitamin. Finally, there are certain experimental observations which strengthen the suspicion that the metabolism of storage iron is abnormal in the presence of ascorbic acid deficiency. It has been reported that deposits of haemosiderin are greater than normal in the tissues of scorbutic guinea-pigs, while the soluble fraction of liver iron is reduced (Banerjee & Chakrabarty, 1965).

These various findings are not altogether surprising since the results of studies *in vitro*

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suggest that ascorbic acid may be necessary, not only for the incorporation of transferrin-bound iron into hepatic ferritin (Mazur *et al*, 1960), but also for the release of iron from ferritin (Mazur *et al*, 1955). The present investigation was undertaken to obtain more information on the role of ascorbic acid in the uptake and release of iron from stores.

MATERIALS AND METHODS

Adult male guinea-pigs of the Onderstepoort strain were used. The diet consisted of ascorbic acid-free guinea-pig/rabbit production pellets (Delmas Milling Company, Randfontein, South Africa), hay which had been heated to 80°C for 1 hr, and tap water. This diet produced frank scurvy in 28–32 days. Control animals were fed the same diet except that ascorbic acid was added to the water (140 mg/l). The experiments were performed after 21 days, at which time the mean liver ascorbic acid concentrations (± SD mean) by the method of Roe (1954) were 0.6 (± 0.5) mg/100 g in the scorbutic group and 24 (± 5) mg/100 g in the control group. The mean haemoglobin concentrations (± SD mean) were 13.4 (± 2.1) g/100 ml and 14.1 (± 2.2) g/100 ml respectively, and the reticulocyte counts were normal in all animals studied. The mean weights (± SD mean) of the scorbutic and control groups were 589 (± 157) g and 629 (± 123) g respectively.

In kinetic studies radioiron was given to the experimental animals in one of three ways. Radioiron bound to transferrin was prepared by incubating guinea-pig plasma with tracer amounts of $^{59}FeCl_3$ for 30 min at 37°C. In another experiment 1000 μg iron as ferrous sulphate labelled with $^{59}FeCl_3$ was injected intravenously. No immediate effects were observed, and within 15 min 70–80% of the radioactivity was present in the liver. Finally, erythrocytes containing haemoglobin labelled with ^{59}Fe were prepared by incubating guinea-pig reticulocytes *in vitro* with ^{59}Fe of high specific activity, bound to transferrin. (Fractionation of such cells by the method of Gale *et al* (1963) revealed that more than 70% of the radioactivity was in the haemoglobin residue.) Prior to injection the cells were rendered non-viable in the following way. After washing, the cells were suspended in eight times their volume of a 1 vol to 1 vol mixture of 0.9% NaCl solution and ACD (5 g citric acid, 13.8 g sodium citrate, 13.9 g dextrose per litre of solution) and heated in a water bath at 49°C for 4 min. When erythrocytes which had been treated in this way were injected intravenously into normal guinea-pigs, almost all the radioactivity was present in the liver and spleen after 45 min.

In all studies the animals were killed by bleeding from the aorta under ether anaesthesia. The portal vein was cannulated and 30 ml 0.9% NaCl solution was perfused through the liver to wash out the blood. The liver and spleen were removed, cooled rapidly on ice, and were then stored at −20°C prior to processing. This was done by thawing and homogenizing the organs in four times their weight of distilled water. The relevant chemical and isotopic estimations were then carried out on separate aliquots. Ferritin iron was measured by the method of Drysdale & Munro (1965) and total non-haem iron by the method of Torrance & Bothwell (1968). 'Haemosiderin', or insoluble iron, was then obtained by difference. In the isotopic experiments total body radioactivity was measured in an Armac Counter. This was also used for measuring the localization of radioiron in the liver and spleen. The insoluble fraction of radioactivity was obtained by heating an aliquot to 80°C as described by Drysdale

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& Munro (1965). The radioactivity in the washed precipitate was then measured in a well-type counter, while ammonium sulphate was added to the supernatant solution to obtain a ferritin fraction. In those studies in which non-viable erythrocytes were administered, the radioactivity present in the haemoglobin fraction was determined after precipitation by the method of Gale *et al* (1963), and washing with distilled water. In such studies, 'haemosiderin' iron was obtained by subtracting the figure for haemoglobin iron from the heat-insoluble fraction of the ferritin method.

RESULTS

Non-Haem Iron and Ferritin Iron Concentrations in Livers and Spleens of Normal and Scorbutic Guinea-Pigs

The total non-haem iron and ferritin iron concentrations were measured in the livers of 18 scorbutic guinea-pigs, 18 control animals, and 11 animals which had been maintained on

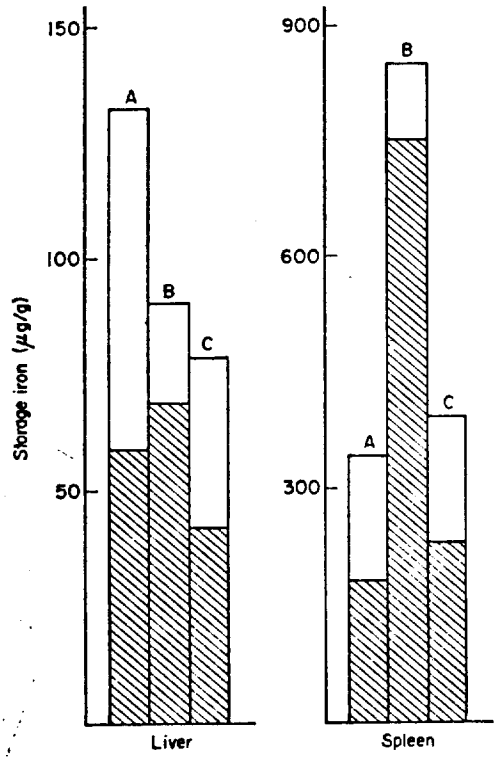

FIG 1. Total non-haem iron concentrations in livers and spleens of control animals (A), scorbutic animals (B) and scorbutic animals given ascorbic acid 24 hr before analysis (C). Unhatched = ferritin.

the ascorbic-acid-free diet, but which had received an intraperitoneal injection of 40 mg ascorbic acid 24 hr before being killed. Similar determinations were performed on the spleens of 10 animals from each group.

The effects of ascorbic acid deprivation were different in the two organs (Fig 1). In the

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liver, the total non-haem iron concentration was significantly lower in the scorbutic animals than in the controls, mean figures (± SD mean) being 90 (± 30) μg/g and 130 (± 36) μg/g respectively (t_{34}, 3.67; $0.001 > P$). In the spleens, on the other hand, the concentration of non-haem iron was significantly *higher* in the scorbutic animals, the mean figure (± SD mean) being 833 (± 195) μg/g compared with only 339 (± 90) μg/g in the control group (t_{18}, 7.28; $0.001 > P$). In both livers and spleens the proportion of non-haem iron present as ferritin was much lower in the scorbutic than in the control animals. In the livers, a mean of 23 (SD mean ± 11)% was present as ferritin in the scorbutic guinea-pigs compared with 55 (SD mean ± 8)% in the normal animals (t_{34}, 10.0; $0.001 > P$). In the group of scorbutic animals given an injection of ascorbic acid 24 hr before death, the mean non-haem iron concentration in the livers was similar to that in the scorbutic animals (78 μg/g, SD mean ± 23), but the percentage of ferritin had risen to a mean of 51 (SD mean ± 2)%. In the spleens of the scorbutic animals, ferritin formed a mean of only 9 (SD mean ± 2)% of the total non-haem iron, compared with a mean of 47 (SD mean ± 5)% in the normal animals. In the scorbutic guinea-pigs given the injection of ascorbic acid 24 hr before death, the splenic non-haem iron concentration was much lower than that in the untreated scorbutic group (mean 383 μg/g, SD mean ± 162) (t_{18}, 5.62; $0.001 > P$), and the percentage present as ferritin had risen to a mean of 42 (SD mean ± 16)%.

Uptake and Release of Heat-Damaged Erythrocytes Containing ^{59}Fe-Labelled Haemoglobin

Twelve control and 12 scorbutic animals received intravenous injections of heat-damaged erythrocytes containing 75 μg haemoglobin. Three guinea-pigs from each group were killed respectively at 45 min and 3, 24 and 48 hr. The spleen and liver of each animal were homogenized together and the total radioactivity as well as the distribution of the ^{59}Fe between the various fractions determined (Fig 2).

In the control animals a mean (range) of 96 (90–99)% of the injected radioactivity was present in the liver and spleen at 45 min, but by 3 hr the figure had fallen to a mean of 48 (47–49)% and by 24 hr to 36 (35–40)%. In the scorbutic animals, less of the injected ^{59}Fe was located in the livers and spleens at 45 min (mean and range, 70 (69–83)%), but the major difference from the control group was that there was little change in this figure during the course of the experiment. The subsequent means and ranges were: at 3 hr, 73 (70–79)%; at 24 hr, 63 (61–65)%; and at 48 hr, 64 (62–65)%.

The rate of disappearance of ^{59}Fe-labelled haemoglobin was somewhat more rapid in the control animals. Although the initial uptake of denatured cells was slightly greater in the control group, by 3 hr only a mean (range) of 17 (15–18)% of the injected radioactivity was still in the form of haemoglobin, compared with 31 (30–35)% in the scorbutic guinea-pigs. By 24 hr, however, it had all been processed in both groups. The mean percentage (range) of injected radioactivity present as ferritin increased slightly in the control animals from 13 (11–15)% at 45 min to 24 (19–27)% at 3 hr, after which there was little change. The means and ranges in the scorbutic guinea-pigs were not very different; at 45 min 11 (9–12)% was in ferritin; at 3 hr 29 (27–31)%, at 24 hr 27 (22–30)% and 48 hr 13 (12–15)%.

The major difference between the two groups was in the faction of ^{59}Fe present in the heat precipitate after subtracting haemoglobin iron ('insoluble iron'). In the control guinea-pigs the mean figure (range) rose from 2 (1–3)% at 45 min to 10 (9–11)% at 3 hr, and thereafter

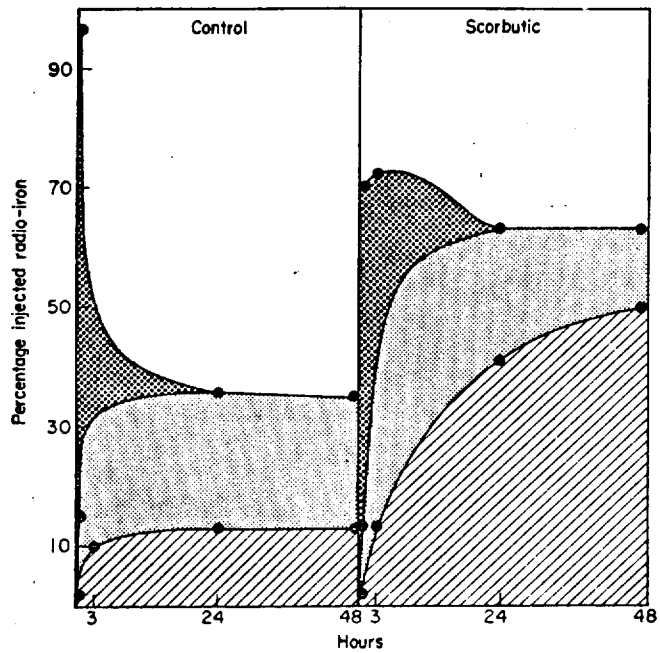

FIG 2. Distribution of ^{59}Fe in combined livers and spleens of control and scorbutic animals at various times after injecting denatured red cells containing ^{59}Fe-haemoglobin. Heavy stipple, haemoglobin; light stipple, ferritin; hatched, insoluble (haemosiderin).

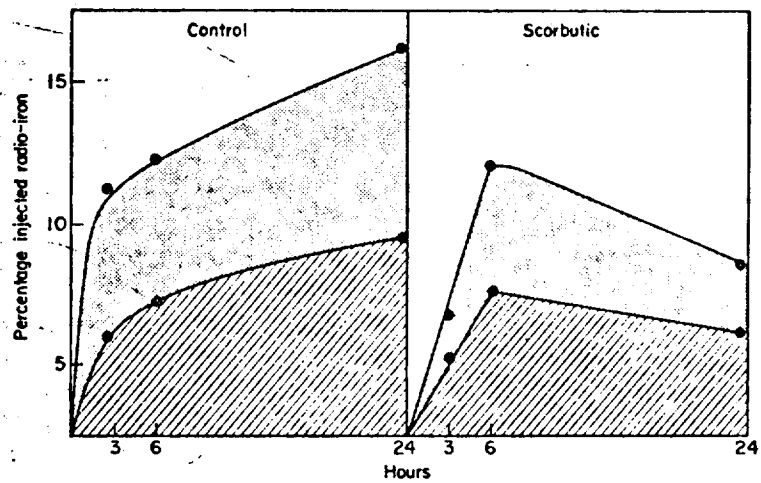

FIG 3. Distribution of ^{59}Fe in the livers of control and scorbutic animals at various times after injecting transferrin-bound ^{59}Fe. Stippled, ferritin; hatched, insoluble (haemosiderin).

was 13 (10–15)% at both 24 and 48 hr. In the scorbutic animals, on the other hand, the amou of radioactivity in this fraction continued to increase; at 24 hr a mean (range) of 41 (37–44) was in this form, and at 48 hr the figure had risen to 50 (47–53)%.

Uptake and Release of Transferrin-Bound ^{59}Fe

Nine scorbutic and nine control animals were given intravenous injections of 1 ml guine pig plasma labelled with ^{59}Fe. Three animals from each group were killed at 3, 6 and 24 respectively, and total hepatic radioactivity as well as the percentage present in ferritin a heat-insoluble fractions were determined.

The patterns were significantly different in the two groups (Fig 3). In the control anima the percentage of injected radioactivity present in the liver rose from a mean (range) of (10–15)% at 3 hr to 13 (11–15)% at 6 hr and 19 (16–22)% at 24 hr. In the scorbutic anim there was also an initial increase in hepatic radioactivity to a mean (range) of 13 (11–15)% 6 hr, but at 24 hr the figure had declined to 8 (7–10)%. The distribution of ^{59}Fe between t ferritin and heat-insoluble fractions was not very different in the two groups, the proporti which was insoluble being slightly more in the scorbutic animals.

This experiment was repeated using six control and six scorbutic guinea-pigs, all of whi had been hypertransfused with homologous packed red cells 7 and 6 days previously order to inhibit erythropoiesis. Haemoglobin values ranged between 24 and 26 g/100 n Three animals from each group were killed at 3 hr, and the remaining three at 6 hr. T mean percentage (range) of injected radioactivity in the livers of the control animals w 15 (13–16)% at 3 hr and 20 (18–23)% at 6 hr, while in the scorbutic group the correspondi figures were 20 (15–22)% and 30 (23–36)%. The mean percentages of injected radioactivi present in the form of ferritin at these times were similar in the two groups, but grea amounts were present as insoluble iron in the scorbutic animals. The mean figures (range) 6 hr were 7 (6–9)% and 15 (12–19)% in the control and scorbutic groups respectively.

Uptake and Release of $^{59}FeSO_4$

Twelve scorbutic and 12 control animals were given intravenous injections of 1000 iron as $^{59}FeSO_4$. Three animals from each group were killed at 12 hr, and at 2, 4 and 6 da thereafter, and the total hepatic radioactivity together with the fractions present as ferri and as insoluble iron determined.

The results resembled those obtained with plasma-bound ^{59}Fe. Initially similar p centages of injected radioactivity were present in the livers of each group, the mean figu (range) at 12 hr being 76 (74–77)% and 68 (65–71)% in scorbutic and control animals spectively. Thereafter there was a steady decline in radioactivity in the scorbutic gro whereas in the control animals the figures remained high. At 6 days the mean figures (ran were 39 (38–41)% and 72 (70–73)% respectively.

DISCUSSION

In the present study ascorbic acid deficiency was found to produce changes in the distributi of storage iron. The total non-haem iron concentration was lower in the livers and consid ably higher in the spleens of the scorbutic animals than it was in the controls (Fig 1). Not o

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was there a redistribution of storage iron between the two organs, but the proportion present in each of the two iron storage compounds was also different, much less being present as ferritin and much more as haemosiderin. These results confirm and extend those of Banerjee & Chakrabarty (1965), who reported a higher total iron concentration in the spleens of scorbutic guinea-pigs compared with pair-fed control animals; in the livers the total iron concentration was not diminished, but in the scorbutic group the soluble iron fraction was smaller and the insoluble fraction larger. In the present study it was possible to demonstrate the role of ascorbic acid deficiency in the production of these changes. After a single intraperitoneal injection of the vitamin 24 hr before death, there was a drop in mean total non-haem iron concentration in the spleens of the treated animals to less than half that in the untreated group. This indicated that a considerable amount of storage iron had been released. In addition, there was a very considerable decrease in haemosiderin and an absolute increase in the amount of ferritin, which now formed more than half the total as compared with less than 10% in the scorbutic animals. In the livers the relative proportions of the two storage compounds also returned to normal on injecting ascorbic acid, but the total non-haem iron concentration did not change significantly. These observations suggest that the balance between ferritin and haemosiderin is shifted towards the insoluble form in the presence of ascorbic acid deficiency, and that the balance is rapidly restored when the deficiency is corrected. In this connection, the observations *in vitro* of Matioli & Baker (1963) may be relevant. These workers have obtained evidence suggesting that haemosiderin is formed as a result of the oxidative denaturation of ferritin. It therefore seems possible that the proportion of storage iron present in each form might be influenced by the cellular concentration of a reducing agent such as ascorbic acid.

The increase in total storage-iron concentration in the spleens of the scorbutic animals together with the decrease in the livers suggested that ascorbic acid deficiency might have different effects on the release of iron from stores in different types of cell. This possibility was strengthened by the marked effect on splenic-iron release when ascorbic acid was administered to scorbutic guinea-pigs, and the absence of such an effect in the liver. Hepatocytes might conceivably be capable of different metabolic reactions from reticulo-endothelial cells. However, since hepatic storage-iron is located not only in hepatocytes but also in cells of the reticulo-endothelial system, further studies were necessary in order to test this hypothesis. Iron within each cell type was separately labelled with ^{59}Fe by injecting the isotope in different forms. It is generally agreed that damaged red cells are not engulfed by hepatocytes, so that ^{59}Fe in the form of haemoglobin within heat-denatured red cells was located in reticulo-endothelial cells, in the liver as well as in the spleen (Hosain *et al*, 1962). Iron bound to transferrin, on the other hand, does not enter reticulo-endothelial cells (Hosain *et al*, 1962). Most of it is taken up by the erythroid bone marrow and incorporated into developing red cells, but a fraction goes to the liver and enters hepatocytes. Furthermore, free iron injected intravenously is predominantly taken up by the parenchymal cells of the liver (Finch *et al*, 1950). The effect of ascorbic acid deficiency upon the uptake, storage and release of ^{59}Fe injected in these three forms was therefore studied.

Significant differences between scorbutic and control guinea-pigs were observed (Fig 2). In normal animals, iron was rapidly freed from haemoglobin and released into the plasma, whereas in scorbutic animals very little ^{59}Fe left the reticulo-endothelial cells. There was, in

addition, some suggestion that a smaller percentage of the damaged erythrocytes had been taken up by the scorbutic animals, and that the rate of liberation of iron from haemoglobin was slower. Much more striking, however, was the progressive build-up of radioactivity in haemosiderin (heat-insoluble fraction) in these animals, whereas in the control animals there was little change after the first 3 hr. These observations therefore provide support for the suggestion that the effect of ascorbic-acid deficiency is to inhibit the release of iron from reticulo-endothelial cells, and to promote the formation of haemosiderin at the expense of ferritin.

The behaviour of hepatocytes in terms of the handling of storage iron was found to be different to that of reticulo-endothelial cells (Fig 3). When ^{59}Fe-labelled plasma was injected intravenously there was a steady build-up in hepatic radioactivity between 6 and 24 hr in the control animals. However, in the scorbutic animals the radioiron levels diminished over the same period, thus indicating a release of ^{59}Fe into the plasma. These conclusions were confirmed by the results of the experiment with $^{59}FeSO_4$.

Mazur *et al* (1960) have suggested that ascorbic acid is necessary for the incorporation of plasma iron into hepatic ferritin. However, no evidence to support this contention was obtained in the present study. In the experiment in which erythropoiesis was inhibited by hypertransfusion, similar quantities of labelled ferritin were formed from plasma-bound ^{59}Fe in both control and scorbutic animals, while the *total* percentage incorporated into storage complexes (ferritin + haemosiderin) was actually greater in the scorbutic group.

The observations made in the present study may well have relevance to the cellular and organ distribution of storage iron in Bantu subjects with iron overload. The siderosis in these individuals is a consequence of the excessive amounts of available iron in the diet, and over the years there is a steady accumulation of iron in storage depots (Walker & Arvidsson, 1953; Bothwell & Bradlow, 1960). In the majority of affected subjects the most striking deposits are found in reticulo-endothelial cells throughout the body. This distribution differs from that found in idiopathic haemochromatosis, in which reticulo-endothelial deposits are much less prominent than those in parenchymal cells. The differing hepatic localization of iron in the two conditions has, in fact, been shown to be distinctive enough to allow for differentiation on histological appearances alone (Bothwell *et al*, 1965). Several possible reasons for the difference in iron distribution have been advanced, and it has even been suspected that it may hold a clue to the aetiology of the idiopathic condition. The results of the present study however, suggest that the answer may lie in the low ascorbic acid content of the Bantu diet (Lynch *et al*, 1967) compared with that of most subjects with idiopathic haemochromatosis. Significant deficiency of ascorbic acid in the Bantu might inhibit the release of iron from reticulo-endothelial sites sufficiently to allow for the slow accumulation of storage iron in these cells. This notion is supported by the low serum-iron concentrations in such subjects when frank scurvy is present, and the sharp increase in serum-iron concentration on administering ascorbic acid (Bothwell *et al*, 1964). Although this suggests that the release of iron from stores is inhibited, it should be noted that the plasma-iron turnover in scorbutic Bantu is usually between two and three times normal, and that it does not change when ascorbic acid is first administered (Bothwell *et al*, 1964). It is obvious, therefore, that a considerable amount of iron does enter the plasma in scorbutic individuals; the present study suggests that most of it comes from hepatic parenchymal cells rather than reticulo-endothelial cells

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Unanswered questions relate to the factors responsible for the increased release of iron from hepatocytes under these circumstances, and the relationship (if any) between the inhibition of iron release from reticulo-endothelial cells and the enhanced conversion of ferritin to haemosiderin.

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STUDIES IN ASCORBIC ACID TASTE THRESHOLD CIRCADIAN RHYTHM IN RELATION TO PLASMA ASCORBIC ACID LEVELS

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Taste thresholds for ascorbic acid have been measured in twenty-three female and twenty-eight male students. Aqueous dilutions of ascorbic acid ranging from 10.16 mg litre to 1200 mg litre were used according to the method for measuring alcohol taste thresholds (McAirt. et. al., Irish J. Med. Sci., 1, 579, 1968). The sour taste threshold for ascorbic acid lies in the range 162.5 mg litre to 650 mg litre for these subjects. Circadian rhythms for ascorbic acid taste threshold were investivated in ten males and fourteen females during three twenty-four hour periods. In one experiment the circadian rhythm for plasma ascorbic acid was also determined.

In males the lowest taste threshold occurred at 16.00 hours (338.77 mg litre) and the highest at 24.00 hours (293.25 mg litre). In females a similar rhythm occurred, the highest value being at 24.00 hours (430.10 mg litre) and the lowest at 12.00 hours (260.89 mg litre). In the plasma circadian rhythm, the highest level occurred at 12.00 hours (0.9 mg 100 ml for males; 1.13 mg 100 ml for females) and the lowest at 24.00 hours (0.75 mg 100 ml for males, 1.10 mg 100 ml for females).

The differences between the highest and lowest mean levels in the ciradian rhythms are all statistically significant except for the plasma ascorbic acid levels in females.

The ascorbic acid taste threshold circadian rhythm is inversely proportional to the plasma ascorbic acid plasma.

Relationship Between Leucocyte and Plasma Ascorbic Acid Concentrations

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Summary

Leucocyte and plasma ascorbic acid values were measured in healthy students, adult factory employees, and old people not receiving supplementary vitamin C and in healthy old people receiving 500 mg of vitamin C daily. Significant positive correlations between leucocyte and plasma ascorbic acid were found in all the groups. The regression lines differed significantly between one another within the sexes, but the pooled lines for each sex did not differ significantly in the unsupplemented groups. The relationship between plasma and leucocyte ascorbic acid values in the supplemented group differed significantly from that in the pooled unsupplemented groups.

There was a limited range of variation in leucocyte ascorbic acid values compared with the range in plasma values in the supplemented group, whereas there was a wider range of variation in the leucocyte values in the unsupplemented groups. Leucocytes can therefore achieve a saturation level of ascorbic acid. Measurement of leucocyte ascorbic acid concentrations alone does not 'de a reliable guide for the estimation of tissue ; of ascorbic acid in normal individuals. Leucocyte concentrations provide a measure of the availability of ascorbic acid for storage, and plasma levels give an indication of its metabolic turnover rate. When these values are related the regression lines provide information about the storage and metabolism of ascorbic acid in normal individuals.

Introduction

Leucocyte vitamin C concentrations are measured to provide information about the tissue ascorbic acid status of an individual so as to maintain his health (Andrews, 1968). The leucocyte ascorbic acid concentration is stated to be the most reliable guide to use for measuring tissue status (Griffiths, Brocklehurst, Scott, Marks, and Blackley, 1967; Andrews and Brook, 1968) This statement is based on the original observations of Bartley, Krebs, and O'Brien (1953), who measured leucocyte ascorbic acid concentrations to find out whether deprivation of vitamin C was associated with a reduction in blood ascorbic acid levels and the appearance of clinical signs of scurvy. They reported that ascorbic acid levels fell more quickly in the plasma than in the leucocytes after vitamin C deprivation.

Andrews and Brook (1966) and Griffiths *et al.* (1967) drew attention to the wide variation in leucocyte ascorbic acid concentrations under normal conditions, though Loh and Wilson (1971a) pointed out that this may be partly attributable ' -rtefacts produced by the method of estimation. Loh and n (1970a) showed that ascorbic acid can move rapidly f the leucocytes into the plasma and back again. On this basis, ascorbic acid can be justifiably described as a labile component of the blood (Andrews and Brook, 1966, 1968; Griffiths *et al.*, 1967). If that is the case it is clearly desirable that the relationship between levels of ascorbic acid in the leucocytes and plasma should be investigated. Plasma and leucocyte ascorbic acid levels have therefore been measured in different population groups, some of whom also received vitamin C supplements. A relatively extended range of ascorbic acid values was obtained for both plasma and leucocytes, and the correlation between them has been investigated.

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Experimental Method

Leucocyte ascorbic acid concentrations and plasma ascorbic acid levels were measured by the methods described previously (Loh and Wilson, 1971b). Blood samples were removed from all the subjects between 9.30 a.m. and 12 midday. Two populations were investigated. In one the subjects received no vitamin C supplements and in the other the subjects were given daily supplements of vitamin C 500 mg over a period of 14 weeks (Loh and Wilson, 1971c). The unsupplemented population consisted of a group (B) of adult males and females, a group (M1) of geriatric subjects living in an old people's home, a group (K) of subjects in a geriatric hospital, and groups (US) of healthy male and female university students (Table I). Blood samples were withdrawn from the students on six different occasions. The subjects supplemented with vitamin C comprised groups of geriatric males and females (M2). Blood samples were taken from the geriatric subjects on seven different occasions.

Results

The value for the leucocyte ascorbic acid concentrations and plasma ascorbic acid levels in the subjects who had not received any supplementary vitamin C are shown in Table I, together with their ages. The correlations between leucocyte and plasma ascorbic acid values and the regression coefficients for the lines are also indicated. These correlations extended over the range +0·39 to +0·93. Positive regression coefficients indicated direct relationships between the two variables, so that an increase in the plasma ascorbic acid was associated with an increase in the leucocyte ascorbic acid concentration.

The range of variation in the regression angle was wider in the female than in the male subjects. Comparison of the regression lines within the sexes showed that the individual lines differed significantly. However, the two pooled lines, obtained by combining all the values for each sex separately, did not differ significantly from each other. The values for the two sexes were therefore combined and the overall correlation for the unsupplemented subjects was found to be +0·51 (Table II).

Supplementary vitamin C raised the concentrations of ascorbic acid significantly in the leucocytes and plasma, though the amount of the rise in the plasma and leucocyte values did not differ significantly between the sexes. In consequence all the values from the supplemented subjects have been combined (Table II). The combined correlation coefficient in the supplemented groups was +0·26.

When the values from treated and untreated groups were combined, amounting to a total of 246 observations, a correlation

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TABLE I—*Ages, Leucocyte Concentrations (μg/10⁸ cells), and Plasma Levels (mg/100 ml) of Ascorbic Acid in Subjects of Different Age Groups who did not receive Vitamin C Supplements. Correlation and Regression Coefficients for the Leucocyte and Plasma Ascorbic Acid Values, Leucocyte being the Dependent Variable*

Group		No.	Age		Ascorbic Acid				Correlation	Regression Coefficient	t	P
					Leucocyte		Plasma					
			Mean	Range	Mean	S.D.	Mean	S.D.				
					Males							
University	US	39	22	18-27	25·20	12·11	0·83	0·43	0·4814	13·43	3·34	<0·002
Adult	B	14	39	33-49	30·43	8·65	0·87	0·31	0·4896	13·78	1·95	0·05 – 0·1
Geriatric	M1	16	71	56-88	24·42	13·59	0·41	0·39	0·4284	15·07	1·77	0·05 – 0·1
Geriatric	K	9	80	70-92	28·23	15·26	0·49	0·37	0·9299	38·87	6·69	<0·001
					Females							
University	US	24	20	18-23	23·27	5·58	0·97	0·24	0·3904	9·06	1·99	0·05 – 0·1
Adult	B	17	34	22-45	33·99	10·85	0·97	0·41	0·7314	19·15	4·15	<0·001
Geriatric	M1	29	74	51-87	23·43	11·34	0·37	0·22	0·7580	38·57	6·04	<0·001
Geriatric	K	18	75	65-87	22·83	6·93	0·35	0·33	0·4841	10·07	2·21	<0·05

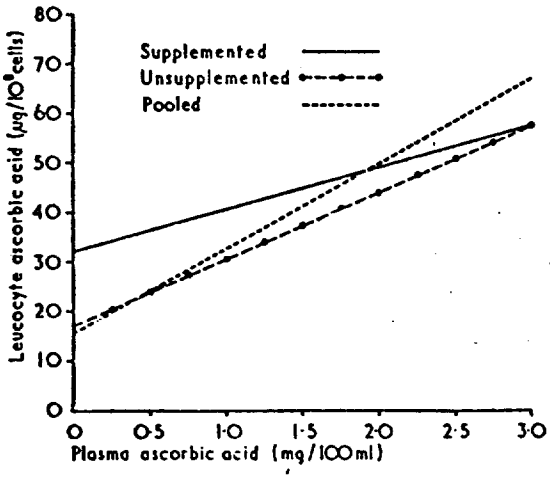
Analysis of variance of regression lines for leucocytes and plasma absorbic acid values within the sexes, and between the pooled values for the sexes: males $P<0·001$; females $P<0·001$; pooled males versus pooled females $P>0·05$.

TABLE II—*Leucocyte Concentrations (μg/10⁸ cells) and Plasma Levels (mg/100 ml) of Ascorbic Acid in Unsupplemented and Supplemented Subjects and both together (Pooled). Correlation and Regression Coefficients are shown together with Significance of Individual Lines, Leucocyte being Dependent Variable. Tangent of Angle Corresponding to Regression Coefficient is indicated*

Group	Ascorbic Acid				Correlation	Regression Coefficient	Angle	P
	Leucocyte		Plasma					
	Mean	S.D.	Mean	S.D.				
Unsupplemented	25·79	11·07	0·68	0·43	0·51127	13·2787	53	<0·001
Supplemented (M2)	43·94	12·34	1·47	0·38	0·25798	8·2889	39	<0·05
Pooled	31·69	14·29	0·93	0·56	0·65397	16·8322	59	<0·001

coefficient of +0·654 was obtained between the leucocyte and plasma ascorbic acid values. The leucocyte ascorbic acid values varied from 5·6 to 72·4 μg/10⁸ cells, encompassing a 13-fold range of variation. The corresponding range in the plasma values extended from 0·1 to 2·5 mg/100 ml, covering a 25-fold range of variation.

The regression lines for the unsupplemented, supplemented, and pooled groups differed significantly from each other ($P<0·001$). All had a positive correlation, indicating that a rise in the plasma level of ascorbic acid was directly associated with an increase in the leucocyte concentration (see Chart). The rise in the plasma level was associated with a greater range of



Calculated regression lines relating leucocyte with plasma ascorbic acid values in the group receiving no supplementary vitamin C, in the group receiving supplementary vitamin C, and in both groups pooled together.

increase in the leucocyte concentration in the unsupplemented than in the supplemented group, as shown by the significantly larger regression angle among the unsupplemented subjects. In the supplemented subjects a rise in the plasma level was associated with a more limited change in leucocyte concentration. The angle of the pooled regression line for the unsupplemented and supplemented subjects together fell between the angles of the lines for these subjects separately.

Discussion

The metabolism of ascorbic acid differs between males and females (Loh and Wilson, 1971b), and it has also been shown that females have higher ascorbic acid plasma levels. Furthermore, plasma levels alter with age (Dodds, 1959, 1969). In our investigations there was no significant difference between the leucocyte concentrations or the plasma levels individually in the two sexes. In both sexes there was a significant positive correlation between these variables.

Within the sexes the correlations changed significantly with age. Relatively higher leucocyte values tended to be correlated with lower plasma values, as shown by the increase in the regression coefficient of the groups as their ages increased. It seems that transfer of ascorbic acid from the leucocytes into the plasma diminishes and storage of ascorbic acid in the leucocytes tends relatively to increase as people get older. Metabolic transfer of ascorbic acid into the plasma from the more highly loaded leucocytes tends to be greater in adolescence and early adulthood. During the ageing process overall storage of ascorbic acid in the leucocytes falls (Allen, Andrews, and Brook, 1967; Brook and Grimshaw, 1968; Loh and Wilson, 1971b). As age increases metabolic demands for ascorbic acid become less and plasma levels diminish, particularly at the end of the reproductive period in women. Concurrently concentrations of ascorbic acid in the leucocytes diminish. Measured independently these variables provide little information; measured together they evaluate the dynamic relationship between storage and metabolism of ascorbic acid in normal subjects.

The existence of a linear correlation of +0·59 between leucocytes and plasma ascorbic acid values has been demonstrated in unsupplemented elderly subjects (Andrews and Brook, 1966, 1968; Griffiths *et al.*, 1967). Depending on the sex and ages, in the present investigation the correlation coefficient varied from +0·39 to +0·93 in the unsupplemented subjects. The coefficient of the whole group was +0·51, which corresponded closely to that reported elsewhere for unsupplemented subjects.

Ascorbic acid can be transferred rapidly from the plasma to the leucocytes and back again during the 24-hour circadian rhythm in normal young adults (Wilson and Loh, 1969a; Loh and Wilson, 1970b). The rapid rate of change of ascorbic acid

concentrations in the plasma and leucocytes during these circadian periods indicates the extreme lability of leucocyte ascorbic acid. This metabolic lability differs in character from the physiological variation in the different population groups within each sex illustrated in the present investigations. The physiological variation is related to the demand for and utilization of ascorbic acid in the different groups. Groups having relatively high storage of ascorbic acid and low metabolic turnover have a high regression coefficient, as in the female geriatric M1 group, and groups having high metabolic turnover and low storage, as in the female students US, have low regression coefficients.

Administration of various doses of vitamin C causes an increase in mean leucocyte ascorbic acid concentrations (Lowry, Bessey, and Birch, 1952; Mašek and Hrubá, 1964; Brocklehurst *et al.*, 1968; Andrews, Letcher, and Brook, 1969). Brocklehurst *et al.* (1968) reported a concentration of 59 μg/10⁸ cells after 12 months' administration of 200 mg daily to elderly subjects, and Mašek and Hrubá (1964) reported the highest concentrations of 60 μg/10⁸ cells after giving 1,500 mg to adults for 38 days. Concentrations of 61 μg/10⁸ cells were recorded by Loh and Wilson (1971b) in schoolboys after they had received 500 mg of vitamin C daily for 14 weeks.

The mean leucocyte concentration in geriatric supplemented subjects was 47 μg/10⁸ cells in males and 50 μg/10⁸ cells in females (Loh and Wilson, 1971c). At this concentration it seems that the capacity of the leucocytes to absorb further ascorbic acid is becoming limited (Loh and Wilson, 1970a) because saturation is being reached. The highest concentrations for ascorbic acid reported in the leucocytes are in the region of 60 μg/10⁸ cells, though the doses administered have ranged from 200 to 1,500 mg daily. No reports indicate that males and females differ significantly in their maximum leucocyte ascorbic acid concentrations after they have received adequate supplementation.

Clearly, measurement of leucocyte ascorbic acid concentrations alone does not provide a reliable guide for the estimation of tissue status of ascorbic acid in normal individuals. Concentrations of ascorbic acid in the leucocytes provide a measure of the availability of ascorbic acid for storage in the body. Plasma ascorbic acid levels give an indication of the transfer of ascorbic acid between tissues at a specific time and in particular metabolic circumstances. When leucocyte concentrations are related to plasma levels the significance of the correlation and angle of the regression line together provide information about the rate of use of vitamin C for metabolic purposes and the availability of excess ascorbic acid for storage.

The relationship between vitamin C and specific physiological and pathological processes such as haemopoiesis (Andrews, Fairley, and Barker, 1967; Loh and Wilson, 1971b, 1971c, 1971d), menstruation (Loh and Wilson, 1971e), disease of the alimentary canal (Cohen and Duncan, 1967), joint disease (Sahud and Cohen, 1971), and upper respiratory inflammation (Wilson and Loh, 1969b) has been investigated under clinical conditions. In these studies vitamin C status was evaluated by measurement of ascorbic acid in the plasma, leucocytes, or urine. Andrews (1968) drew attention to the variation found in leucocyte and plasma estimations, and Booth and Todd (1970) demonstrated the variation in measurements of urinary ascorbic acid excretion.

The continuous and varying flow of ascorbic acid between the tissues (Wilson, 1971) explains the discrepancies in the reported results. Only the fraction of ascorbic acid being transferred through the plasma, or the labile pool temporarily being stored in the leucocytes, or the fraction of the plasma ascorbic acid transferred into the renal tubules is being measured at a specific time in these circumstances. These fractions individually give little indication of the state of ascorbic acid metabolism for clinical assessment of the patient. The ascorbic acid status of the patient can be evaluated by correlating two of these criteria simultaneously or by correlating one of them with a separate but physiologically related criterion which provides an adequate comparison for the ascorbic acid.

We wish to acknowledge the helpful advice, suggestions, and collaboration received during these investigations from colleagues in universities and hospitals and in the pharmaceutical industry. We would also like particularly to thank Mr. Paul Dempsey for technical help, and Mr. P. J. Byrne and Mr. Kevin Foran for their help in many ways. We are grateful to Miss Collander and Miss Roberts for secretarial assistance. We wish to thank Roche Products Ltd. for supplies of Redoxon and Abbott Laboratories for supplies of Ferrogradumet and Ferrogradumet C.

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Preliminary Communication

RELATIONSHIP OF HUMAN ASCORBIC-ACID METABOLISM TO OVULATION

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Summary Ascorbic-acid excretion has been measured daily in the first morning specimen of urine in five ovulating and five non-ovulating females who were receiving 500 mg. ascorbic acid each morning at breakfast throughout the menstrual cycle. Basal body-temperatures were taken daily before the subjects got out of bed. The ascorbic-acid excretion reached a peak and then diminished three days before ovulation. It increased at the time of ovulation. Two smaller post-ovulatory peaks also occurred. Control subjects did not show comparable fluctuations in ascorbic-acid excretion. The pattern of ascorbic-acid excretion closely resembles the excretion of luteinising hormone at the time of human ovulation.

INTRODUCTION

DEFICIENCY of vitamin C has been shown to be associated with clinical signs of scurvy [1,2]; and a daily intake of 30 mg. has proved necessary for the adult population, including non-pregnant women, in order to prevent signs of deficiency of ascorbic acid. This has been recommended as the desirable intake for the population of the United Kingdom.[3] The report [3] did not state whether this daily requirement could be met from a normal diet, but such surveys as have been carried out suggest that in old people the normal dietary intake of vitamin C is insufficient to supply this amount.[4,5] In addition to the possibility that dietary intake of vitamin C may be deficient in sections of the population, there is evidence that an increased intake of vitamin C is desirable in some circumstances. The plasma-ascorbic-acid falls significantly in pregnant women after the twentieth week [6] and there is an increased demand for ascorbic acid to maintain alimentary mucosal integrity [7] and to combat upper respiratory infection.[8] Since larger quantities of ascorbic acid appear to be needed when cells are dividing rapidly or being replaced, it seemed possible that the female reproductive organs might require more ascorbic acid during their phases of maximal activity in the menstrual cycle. We describe here the results of tests to investigate the relationship between ascorbic-acid metabolism and the menstrual cycle.

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SUBJECTS AND METHODS

The subjects comprised five unmarried female university students and secretarial staff, aged 18–22 years. They all had normal menstrual cycles of 3–4 weeks and menstruation lasted 3–5 days. The subjects recorded their morning temperatures before getting out of bed and collected their first morning urine daily from the first day of menstruation until the last day of the succeeding menstrual period. Temperatures were taken by all the subjects in the same way, the thermometer being kept under the tongue for 5 minutes immediately on waking. The subjects took 500 mg. of vitamin C daily at breakfast for a week before the investigation and also during the period of the investigation. The periods of menstruation and the daily ingestion of the vitamin-C tablet were noted on the temperature-record cards. Symptoms and signs of upper respiratory infection were recorded on special cards.[8] The subjects were requested to note any other symptoms on their temperature-record cards. The five control females consisted of four menstruating and one prepubertal subject. Of the four menstruating subjects three were aged 18–22, and one was aged 44 years; none of these subjects ovulated, as shown by absence of any rise in basal body-temperature. The prepubertal subject was aged 12 years and had not attained her menarche.

The bottles were delivered daily to the laboratory for estimation of ascorbic-acid content. During the weekends the samples were kept in the refrigerator, and were delivered to the laboratory on Monday morning. Urinary ascorbic acid was measured by the method of Denson and Bowers.[9]

RESULTS

The mean temperatures and excretion of ascorbic acid in the ovulating subjects are shown in fig. 1a. The

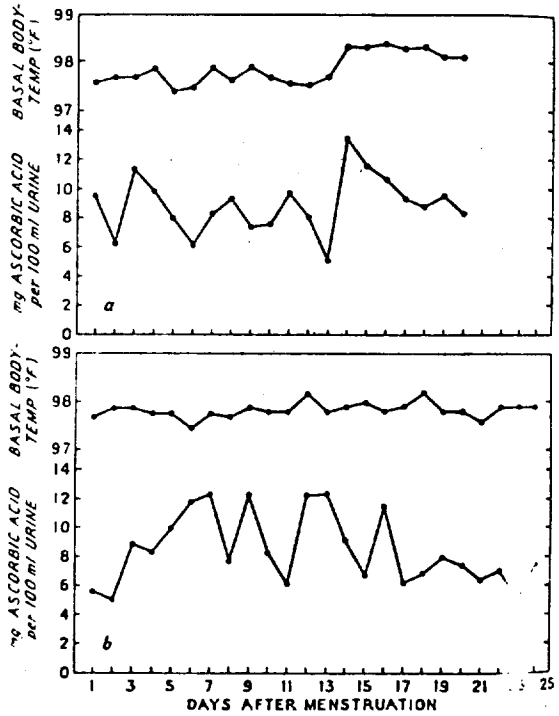

Fig. 1—**Basal body-temperatures and concentration of ascorbic acid in morning specimens of urine in (a) five ovulating and (b) five non-ovulating females during menstrual cycle.**

The rise in basal body-temperature has been adjusted to occur on day 14 in all the subjects.

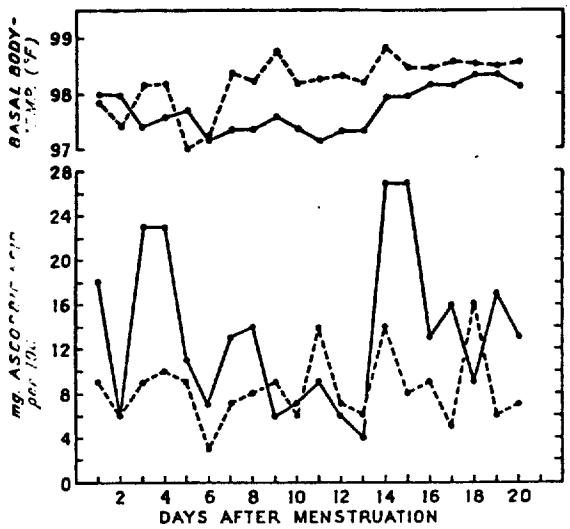

Fig. 2—Basal body-temperatures and concentration of ascorbic acid in morning specimens of urine in two subjects during menstrual cycle.

records have been adjusted so that the day on which the intermenstrual rise of temperature took place corresponded in all the subjects. In consequence the mean values are shown only for 7 post-ovulatory days. During the investigation none of the subjects noted any symptoms or signs suggestive of upper respiratory infection, and there was no indication of other circumstances which might have interfered with their ascorbic-acid metabolism. Mean temperatures and urinary excretion of ascorbic acid in the control subjects are also shown in fig. 1.

In the ovulating females the excretion of ascorbic

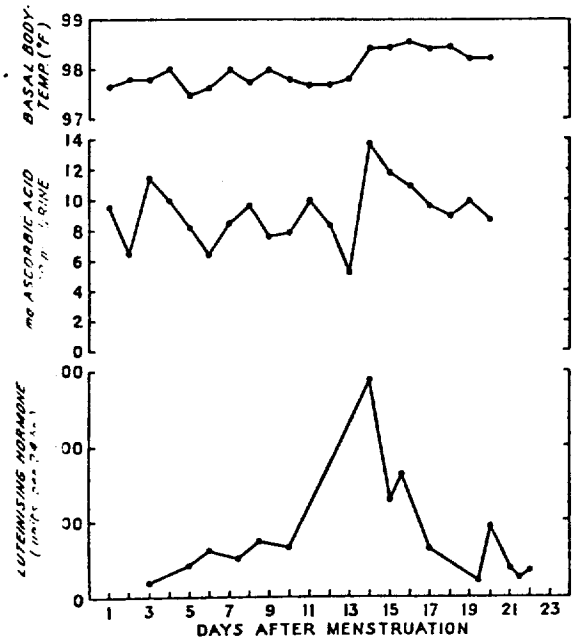

Fig. 3—Mean daily values for basal body-temperature and excretion of ascorbic acid in morning specimen of urine from five female subjects superimposed on mean values for excretion of luteinising hormone in morning specimen of urine from five other comparable ovulating subjects (● ●).

acid showed four variations associated with the alteration in basal body-temperature. 3 days before the rise in temperature, ascorbic-acid excretion rose to a peak. It then diminished, reaching its minimum value on the day before the temperature rise. The excretion rose dramatically at the same time as the elevation in basal body-temperature on the 14th day. There was a slight fluctuation during the subsequent fall in ascorbic-acid excretion on day 16, and the excretion diminished further until the 18th–19th day, when a small elevation again appeared. The rise and fall in excretion of ascorbic acid before ovulation and the two post-ovulatory peaks are illustrated in fig. 2.

DISCUSSION

Changes in the ascorbic-acid concentration of the ovaries at the time of ovulation have been described in guineapigs,[10] and measurement of the depletion of ascorbic acid in the ovaries of rats at the time of ovulation has been used as the basis for biological assay of luteinising hormone (L.H.).[11,12] Experimental evidence therefore suggests that there might be comparable changes in human ovarian metabolism of ascorbic acid at the time of ovulation. On account of the considerable variation in the excretion of ascorbic acid in human urine from day to day, it is difficult to detect significant biological alterations in the ascorbic-acid-excretion rhythm with the accuracy needed to relate it to changes in hormonal activity.[13] If, however, the tissues are saturated by daily administration of 500 mg. of ascorbic acid for a week, ascorbic-acid excretion attains a relatively high stable level and alterations in it reflect changes in the metabolic use and availability of ascorbic acid in the tissues.

The ascorbic-acid concentration in the morning specimen of urine can be measured accurately. It is then possible to relate changes in the ascorbic-acid concentration to alterations in the concentration of other urinary constituents on a temporal basis. Taubert et al.[14] have measured the excretion of L.H. daily in the first morning specimens of urine from ovulating women throughout the menstrual cycle. Their values for the excretion of L.H. are superimposed on the values for the excretion of ascorbic acid in relation to basal body-temperature in fig. 3. The only major difference in the excretory patterns of the compounds occurs during the 2 days preceding ovulation. A secondary rise in the excretion of L.H. was found in five subjects within 30 hours of the ovulatory peak.[15] A similar biphasic peak is illustrated in two of the subjects in whom the ascorbic-acid excretion was measured. The second peak can just be seen in fig. 3. A more definite and completely separate increase in L.H. excretion has been described 3–4 days after the appearance of raised L.H. values in the plasma and urine at the time of ovulation[16] and is evident in fig. 3. This is reflected by the rise in ascorbic-acid excretion on days 18–19.

Comparison of the excretory patterns of L.H. and ascorbic acid at the time of ovulation suggests that in the human menstrual cycle these two compounds are metabolically related. The rise followed by the fall in ascorbic-acid excretion, when compared with the progressive rise in L.H. excretion before ovulation, suggests that their metabolic functions may not be completely interdependent. The similarity in their

excretory patterns following the rise in body temperature suggests that their metabolic paths are closely interlinked after ovulation.

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THE EFFECT OF ORGANIC COMPOUNDS UPON VITAMIN C SYNTHESIS IN THE RAT*

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A continued investigation of pure substances which accelerate vitamin C synthesis and excretion in rats (1, 2) has shown that several new series of compounds are as active as the terpene-like substances reported previously. Individual compounds in the new series, many of which are widely used clinically and in experimental work, are much more active than any of the compounds referred to in earlier reports. Although the very active substances differ greatly in chemical structure, they have an interesting common characteristic *in vivo*, functioning as nerve depressants. Among the most active compounds are four series of anesthetics (barbituric acid derivatives, polymeric aldehydes, sulfonemethanes, and halogenated aliphatic compounds) and two antipyretics, aminopyrine and antipyrine. Many of the bactericidal agents such as sulfapyridine, salicylates, and phenols are active to a much less degree. The more active compounds cause the animals to excrete from 10 to several hundred times more vitamin C than would otherwise be excreted on a given diet. In view of the fact that without any intake the quantities excreted each day are far in excess of the total body stores, the phenomenon obviously involves an increased rate of synthesis unless one postulates a normal very rapid turnover (synthesis and disposal) that might be blocked at the second step with resultant accumulation of the ascorbic acid.

* Contribution No. 405 from the Department of Chemistry, University of Pittsburgh.

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A summary of the data included in the present paper was presented at the meeting of the American Institute of Nutrition at New Orleans, March 13, 1940.

There is evidence in the literature of a disturbance in vitamin C metabolism due to anesthesia. Zilva (3) found that when the vitamin was injected intravenously guinea pigs excreted more ascorbic acid during ether anesthesia than they did after similar injections when no anesthetic was used. The anesthetic did not prevent the fixation of the vitamin in the tissues, since higher amounts were found with anesthesia than without it. Zilva was not inclined, however, to regard his results as indicative of abnormal metabolism of the vitamin. Bowman and Muntwyler (4) observed a 10- to 15-fold increase in the urinary excretion of ascorbic acid following ether anesthesia in the dog; smaller increases were noted in both the rat and the guinea pig. The ascorbic acid content of the liver and kidney was found to be increased in the etherized rat (5); decreases were noted in rat adrenals (5, 6) and in several tissues of the guinea pig (5), and the serum ascorbic acid in the guinea pig was increased (7). In profound amytal narcosis, Lombroso and Cera (8) observed a decrease in the total ascorbic acid content of rat liver and a slight change in the ratio of dehydroascorbic acid to ascorbic acid.

The influence of narcosis on vitamin C economy has been studied by Bersin, Lauber, and Nafziger (9–12). These authors (9) measured the urinary excretion of ascorbic acid by rabbits after narcosis or local anesthesia and after various operations. They interpreted the decreased excretion values obtained by all procedures as indicative of an increased utilization (10). Bartlett, Jones, and Ryan (13) have also pointed out the increased need for vitamin C in surgical cases, but without specific reference to the possible effect of anesthetics. Wildbolz (14), however, could find no statistical justification for the opinion that ascorbic acid prevented or lessened the postoperative complications in which he was interested. In another study, Lauber *et al.* (15) found that narcotics generally produced a decrease in the vitamin C content of the adrenals and other "storage" organs.

The vitamin C content of the cerebrospinal fluid and brain was not affected by prolonged narcosis with opium derivatives or amylene hydrate in either humans or rabbits (16). Neither chloroform nor ether had a significant effect on the vitamin C content of the cerebrospinal fluid of apes or rabbits (17). A study of alcoholic patients has shown that a low vitamin C content in

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the blood and cerebrospinal fluid is frequently observed without evidence of peripheral neuritis or psychosis, but, with the onset of the latter, there is a definitely subnormal content. Wortis *et al.* (18) believe that ascorbic acid may play a rôle in the metabolism of nerve tissue, and the work of Michetti *et al.* (19) contributes to this point of view. Melka (20) has shown that the ascorbic acid content of brain varies markedly in different sections, from 0.13 to 0.26 mg. per gm., compared to only 0.03 mg. per gm. in peripheral nerves. Dainow (21) believes that vitamin C exerts a definite control over the autonomic nervous system.

Evidence of the importance of vitamin C in detoxication processes has accumulated steadily in the literature. Papers from this and other laboratories have shown repeatedly that the tissue concentration of vitamin C is markedly lowered by some of the bacterial toxins and other toxic materials, and that there is a reduced tolerance for such substances by animals whose vitamin C reserves are depleted. Much of this literature has been referred to in recent reviews (22, 23), and additional investigations have pointed in the same direction. Thus the fatty degeneration of various organs after acute phosphorus poisoning was lessened in rabbits by treatment with vitamin C (24, 25); rabbits were also partially protected from phenol poisoning when vitamin C was administered (26); guinea pigs treated with 50 mg. of ascorbic acid for 8 to 10 days were made more resistant to lethal doses of phenylquinolinecarboxylic acid (27); chronic benzene poisoning was frequently accompanied by diminished urinary excretion of the vitamin (28) and a lowered blood concentration (29), and both man and experimental animals (30) appeared to be benefited by treatment with ascorbic acid (29, 31); in human lead poisoning the administration of ascorbic acid was followed by more rapid recoveries (32); in experimental animals (rats, guinea pigs, and rabbits) lead poisoning was alleviated by feeding apples (33), implicitly on the basis of providing vitamin C; and several investigators (34, 35) have reported a protective function of vitamin C against certain arsenicals.

In 1936, Daniels and Everson (36) reported a definite increase in ascorbic acid excretion when children (three) were given aspirin. Youmans and his colleagues (37) and van Eekelen (38) did not confirm this finding in healthy adults but Keith and Hickmans

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(39) were able to produce a significant increase in vitamin C excretion in a large group of children with rheumatic fever by the administration of 50 grains of sodium salicylate and 100 grains of sodium bicarbonate. In similar patients Richeri and Litter (40) found no influence on the blood level of ascorbic acid. Samuels, Ritz, and Poyet (41) confirmed our observation (2) of the increased excretion of ascorbic acid by rats when fed acetyl salicylate and extended their studies to include guinea pigs. The diuretics, salyrgan and neocinchophen, did not produce such an effect, though caffeine was slightly active. The effect upon guinea pigs was only demonstrable when large dosages of ascorbic acid were given (33 mg. per 100 gm. of body weight). Svirbely (42) reported tissue concentration changes with some of the above compounds, Einhauser (43) reported vitamin C to be effective in increasing the resistance of rats against lethal doses of barbiturates, and Haas (44) observed that the vitamin prevented the pyretic effect of 2,4-dinitrophenol. Vedder and Rosenberg also observed a protective rôle for the vitamin in rats which were fed a peculiarly toxic jewfish oil (45).

The rôle of ascorbic acid as a detoxicating agent *in vivo* has been established from the types of evidence cited above, without a clear picture of the chemical basis for its action. Other substances upon which the organism is known to rely for detoxication have been isolated frequently as conjugates of the toxic or foreign material. References to conjugation with glucuronic acid, sulfates, glycine, cysteine, glutamine, ornithine, and methyl groups have been cited in reviews by Quick (46) and Young (47). It is generally accepted that such conjugation is for the purpose of either blocking active groups in or facilitating the removal of undesirable substances. If ascorbic acid could be isolated from the urine of animals as a conjugated product with substances known to be toxic to the animal body following their administration, then direct and more satisfactory evidence would be available that ascorbic acid acts as a detoxifying agent. Such evidence of conjugation has not yet been presented and experiments reported in this paper indicate that stable conjugation products do not occur in rat urine. In our previous studies, however, it was shown that substances like the ionones, menthol, borneol, carvone, isophorone, piperitone, thujone, pulegone, and camphor, all of

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which are known to be conjugated with glucuronic acid and often with sulfates, produce a simultaneous rise in the urinary excretion of ascorbic acid. The results provided by the earlier study and by the present data show a striking correlation between the metabolism of *d*-glucuronic acid and *l*-ascorbic acid. The relationship is all the more interesting because of the close analogy in their molecular structures.

EXPERIMENTAL AND DISCUSSION

A standardized technique, described in earlier publications (1, 2), was used in making the assays. Briefly, it consisted of adding definite amounts of pure substances each day to a basal diet of evaporated milk and feeding this mixture to albino rats maintained in individual metabolism cages. 24 hour samples of urine were collected in sufficient 12 per cent freshly prepared metaphosphoric acid, saturated with 8-hydroxyquinoline, to maintain a final concentration of not less than 3 per cent acid. In practice, 8 to 10 ml. of acid were found adequate for this purpose. The vitamin C content of these 24 hour urine specimens was determined by direct titration with 2,6-dichlorophenol indophenol and verified by a photoelectric colorimeter so that a correction could be made for other possible interfering agents (48, 49). Not less than nine 250 to 300 gm. standardized animals and usually ten or more were used to test each substance. The rats were raised from weaning on a stock ration (Dog Chow) until ready for the assays. On this ration they excrete about 2 mg. of vitamin C per day. A 2 day inanition period followed by 2 days on the milk diet alone was usually sufficient to reduce their excretion level to 0.2 mg. per day, at which value it remained constant on the milk diet alone. Contrary to a recent suggestion (50), we have recognized from the first that the composition of the diet *per se* is very important in relation to ascorbic acid excretion, but nominal variations in general composition do not exert changes that are comparable with those induced by specific stimulants. Furthermore, the response to specific stimulants is markedly dependent upon the basal diet used; hence our use of a standard assay diet of evaporated milk.

It will be noted that the substances listed in Table I are extremely active in causing an increased excretion of ascorbic acid.

TABLE I

Vitamin C Synthesis Induced in Rats

Daily ration, 35 ml. of evaporated milk + 20 mg. of test substance; nine or more animals for each test.

Test substance (barbiturates, hypnotics, and antipyretics)	Vitamin C excreted per 24 hr. period						
	1 day	2 days	3 days	4 days	5 days	6 days	7 days
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Evaporated milk only	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Barbital	0.4	0.9	1.4	4.0	6.7	7.9	8.6
Sodium phenobarbital	0.4	1.0	3.7	5.3	6.1	9.6	8.6
Phanodorn	0.6	1.2	2.7	4.1	3.4	4.2	
Evipal	0.5	1.2	1.4	1.6	1.8	1.4	1.8
Sodium amytal	0.3	0.2	0.4	0.6	0.8	1.0	1.3
Seconal	0.3	0.2	0.5	1.1	1.5	1.3	1.7
Nembutal	0.7	1.2	2.1	3.2	3.8	4.2	
Pentothal	1.4	2.6	3.0	3.9	4.2	3.8	4.2
Ipral	1.1	3.4	7.7	8.8	10.2	9.9	10.6
Alurate	0.7	1.2	1.8	2.1	2.0	1.7	2.4
Dial	0.3	0.4	0.3	1.0	2.0		
Chloral hydrate	0.6	0.7	0.7	0.8	0.7	0.8	
Paraldehyde	2.9	5.8	3.9	7.9	11.4	10.7	10.9
Chloretone	1.1	8.5	11.3	13.4	15.9	16.2	18.3
tert-Butanol (then changed to chloretone)	0.4	0.5	0.4	0.4	0.5	(To chloretone)	
Chloretone	3.4	8.0	11.5	14.6	16.8		
Bromural	0.5	1.2	2.2	2.6	2.1	1.3	
Tribromoethanol	0.3	0.2	0.3	0.5	0.5	0.6	
Trional	0.5	1.1	2.3	3.7	5.3	4.6	3.5
Urethane	1.0	0.4	0.4	0.8	0.7	0.4	0.7
Aminopyrine	0.9	1.8	3.0	4.2	5.9	7.4	
Antipyrine	0.4	2.3	5.4	6.1	6.5	4.7	
Dipyrone	0.5	0.8	1.6	2.6	1.6	1.7	
Acetanilide	0.4	0.4	1.0	1.1	1.2	1.2	
Phenacetin	0.3	0.4	0.4	0.4	0.4		
Aniline	0.3	0.3	0.4	0.5	0.6		
p-Aminophenol	0.2	0.4	0.7	1.0	0.8	0.7	0.8

The effect is interpreted as primarily the result of an increased rate of synthesis. All of the barbituric acid derivatives brought about this phenomenon and similar results were obtained with a

number of hypnotics and antipyretics. The most active compounds assayed were chloretone, paraldehyde, sodium phenobarbital, and calcium ipral. With these compounds the ascorbic acid excretion was regularly raised from the 0.2 mg. per day level on evaporated milk alone to 10 to 20 mg. The pyrazolone derivatives, aminopyrine and antipyrine, also exerted a marked stimulating action on ascorbic acid synthesis and excretion. From a structural point of view it was of interest to note that *tert*-butanol had very little effect compared with its trichloro substitution product.

The vitamin C content of urine from animals receiving 20 mg. of sodium phenobarbital daily was assayed biologically,[1] as described in the earlier paper. The biological data were in good agreement with the chemical data for determining the amount of vitamin C present. When an amount of urine equivalent to 0.5 mg. of ascorbic acid, determined by titration, was given as a daily supplement to the Sherman assay diet, seven guinea pigs, previously depleted for 12 days, gained an average of 10 gm. during a 21 day period. Their average scurvy score (on the basis of 0 to 24) was 5.3, and the average score for a control group of seven animals receiving a standard vitamin solution (0.5 mg. per day) was 4.7.

For the biological assay, urine with a high vitamin C content (20 to 25 mg. per day) was collected from the same animals over a period of 1 month. There was no evidence of injury to the animals during the test period. During a 2nd month the excretion level dropped to 12 to 20 mg. per day. A similar long continued excretion had been observed in earlier experiments with carvone and isophorone. When rats were continued for a long period on evaporated milk to which 20 mg. of chloretone were added daily, the urinary ascorbic acid levels continued to rise steadily to a maximum of 20 to 30 mg. per day, which was reached on the 12th to 14th day, and remained at that level for a 3 month test period. The high continued excretion level has been observed

[1] We are indebted to Mr. C. S. Spiegl and Mr. C. E. Neubeck for their assistance in conducting the biological assays reported in this paper. The assay diet consisted of rolled oats, wheat bran, heated skim milk powder, butter fat, and cod liver oil. A 12 day depletion period was followed by 21 days of test feeding, as outlined previously (1).

for over 100 animals given chloretone for other test purposes. In several individual cases when urine was collected directly on a watch-glass and titrated immediately, values of 3.5 to 4.0 mg. of ascorbic acid per ml. were obtained, which, when calculated on the basis of average daily urine output, indicated a total 24 hour excretion of 50 to 60 mg. of the vitamin. Since the amount of ascorbic acid excreted per day through a long period was far in excess of the total quantity normally present in the animal, this finding considerably strengthens the probability of an increased rate of synthesis. It is also of interest that this concentration of ascorbic acid was much greater than had been found previously in any natural product.

The effects of a number of organic compounds of clinical and research interest are given in Table II. Several of these compounds are known to be detoxified by glucuronic acid (46, 47, 52). Sulfapyridine was only slightly active, but elicited a greater excretion than hydroxyethylapocupreine or sulfanilamide. The salicylates and phenols exerted little stimulation. Nicotinic acid and narcotine were fairly active. A biological assay of the vitamin C content of urine from animals receiving narcotine showed again that the chemical method gave essentially reliable results and that the values obtained were not due to other reducing substances. An amount of urine equivalent to 0.5 mg. of ascorbic acid produced an average weight gain of 50 gm. in six guinea pigs during a 21 day test period. The average scurvy score was 4.0. Alkaloids which were tested and found inactive or only slightly active (0.4 to 0.6 mg. of ascorbic acid per day) were papaverine, codeine, atropine, pilocarpine, cocaine, procaine, pantocaine, and intracaine. Caffeine, theobromine, and quinine were also inactive at a test level of 20 mg. per day. Among the alkaloid type of substances there was no apparent relation between nerve depressant effect and ascorbic acid excretion as noted for many of the other types of compounds.

In an earlier series of experiments the recovery of definite amounts of ingested ascorbic acid in the urine of rats was determined. The per cent of recoveries decreased rapidly as larger amounts of ascorbic acid were fed; with 100 mg. of ascorbic acid, very little more was recovered than when 50 mg. were fed. However, in more recent tests, intraperitoneal injections of 10

and 50 mg. of ascorbic acid dissolved in physiological salt solution permitted 60 per cent (range, 55 to 70 per cent) to be recovered during a 7 day test period with five animals. These values are similar to results obtained by Reiser (53). It might be argued from such data that not more than 60 per cent of the ascorbic

TABLE II

Vitamin C Synthesis in Rats Induced by Organic Compounds of Pharmacological Interest

Basal ration, 35 ml. of evaporated milk; nine or ten animals for each test.

Test substance	Vitamin C excreted per 24 hr. period						
	1 day	2 days	3 days	4 days	5 days	6 days	7 days
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Sulfanilamide, 50 mg.	0.2	0.3	0.5	0.6	0.4	0.5	0.5
Sulfapyridine, 50 "	0.3	0.4	0.7	0.9	1.2	1.1	1.3
Hydroxyethylapocupreine,* 50 mg.	0.2	0.2	0.3	0.2	0.4	0.5	0.4
Narcotine,† 20 mg.	1.4	3.6	4.1	4.7	5.5	5.3	5.2
Nicotinic acid, 50 mg.	0.9	1.1	1.3	0.6	2.2	3.6	3.6
Phenol, 25 mg.	0.7	0.6	0.5	1.1	1.3	1.0	1.0
Guiacol, 20 "	0.3	0.4	0.5	0.8	0.7		
Sodium diethyldithiocarbamate (20 mg.)	0.4	0.6	0.7	1.4	1.6		
Sodium salicylate, 20 mg.	0.7	1.0	0.9	1.3	1.2		
Phenyl " 20 "	0.3	0.3	0.4	0.8	1.0	1.1	1.4
Methyl " 20 "	0.3	0.3	0.4	0.4	0.4	0.5	

* Kindly supplied by Dr. L. H. Cretcher, Head of the Department of Pure Research, Mellon Institute.

† Alkaloids found to be essentially inactive, *i.e.* causing excretions less than 0.6 mg. per day, were codeine, atropine, papaverine, pilocarpine, cocaine, procaine, quinine, caffeine, and theobromine. The reported function of narcotine as an antiscorbutic agent *per se*, or as a supplement to *d*-glucuronic acid, has repeatedly been shown to be untrue (*cf.* Neuwiler (51)).

acid synthesized as a result of the ingestion of such compounds as reported here would be excreted in the urine, and hence that the excretion values represent minimum figures for the ascorbic acid actually synthesized rather than definite totals.

Attempts were made to isolate from the urines conjugation products of ascorbic acid with a number of the various compounds

listed above. These were all unsuccessful when procedures were followed of the type that lead to concentration or isolation of glucuronic acid conjugates. Neither was it possible to obtain an additional amount of reducing substance by acid hydrolysis of the urines. In further experiments to test for the presence of conjugated ascorbic acid, urine was collected directly from the animals onto a watch-glass and titrated immediately without acidification, with faint acidification with acetic acid (pH 6.5), and after hydrolysis for 10 minutes at 60° with acetic acid or hydrochloric acid at pH 3.5. In no case could additional ascorbic acid be obtained by acidification or hydrolysis. Neither could reducing material be extracted from cold acidified urine with ether, as should be true if the product were conjugated through other groups than those involved in the reducing action. Consequently, unless direct evidence becomes available to indicate conjugation, the ascorbic acid synthesized and excreted in rat urine can be regarded as unconjugated.

The properties of the vitamin contained in the rat urines with respect to precipitation by lead salts, solubility in organic solvents, and reaction with dinitrophenylhydrazine were identical with those of the free vitamin in solution. A discussion of the problem of separating the dinitrophenylhydrazine derivative from urine has been published by Roe and associates (54).

The present data considerably extend the number and types of molecular structures which produce accelerated ascorbic acid synthesis and they serve to emphasize further the improbability that these substances are direct precursors of the vitamin. Detailed studies of the synthesis by tissue slices will be recorded in a later paper (55). It is believed that the ascorbic acid is of endogenous origin, formed from tissue metabolites in much the same way as Lipschitz and Bueding (56) have demonstrated in the case of glucuronic acid.

The functional relationships of the active compounds cited in Table I point toward a close connection between vitamin C and the metabolism of nerve tissue; the increased synthesis of ascorbic acid constitutes a fairly rapid and continued response of the rat to at least an extensive number of nerve depressants. It is possible that the accelerated ascorbic acid synthesis is a protective mechanism available to the animal against foreign toxic substances

but there is no direct evidence that this is so. The phenomenon may also represent a disturbance of normal intermediate metabolism in which high ascorbic acid production is only one of a number of disturbed reactions.

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SUMMARY

1. Ascorbic acid excretion by albino rats was greatly accelerated by a series of compounds that are widely used as nerve depressants in laboratory research and clinical practice.

2. All barbituric acid derivatives assayed were effective in stimulating ascorbic acid excretion. Sodium phenobarbital and calcium ipral were the most active; 20 mg. of each per day raised the average ascorbic acid excretion values from 0.2 to 10 mg. per day within a few days.

3. A group of chemically unrelated hypnotics had a similar effect; paraldehyde and chloretone in doses of 20 mg. per day caused average daily excretions of ascorbic acid of 11 and 18 mg. respectively.

4. Among the antipyretics which caused rats to excrete vitamin C, the pyrazolone derivatives, aminopyrine and antipyrine, were most effective; 20 mg. per day caused excretions of approximately 7 mg. of ascorbic acid within 5 to 6 days.

5. Phenols, salicylates, sulfanilamide, and sulfapyridine were only slightly active; 20 mg. of narcotine and nicotinic acid induced excretion of 4 and 5 mg. of ascorbic acid per day, respectively. Hydroxyethylapocupreine and a number of alkaloids caused practically no increased excretion. There was no evidence of correlation between nerve depressant action and causation of ascorbic acid excretion in this group of compounds.

6. Guinea pig assays for vitamin C in rat urines from animals receiving narcotine and sodium phenobarbital were conducted to verify the nature and amount of the reducing substance titrated with 2,6-dichlorophenol indophenol.

7. Rats continued to synthesize vitamin C for 3 months in

response to being fed 20 mg. daily of either sodium phenobarbital or chloretone, during which period they excreted 20 to 30 mg. of ascorbic acid per day. Individual animals excreted as high as 50 to 60 mg. per day (3.5 to 4.0 mg. of ascorbic acid per ml. of urine).

8. A much greater recovery of ascorbic acid was obtained in the urine of rats after intraperitoneal injection than resulted when the acid was fed.

9. No evidence was obtained to indicate that the urinary ascorbic acid was conjugated with any of the toxic substances fed, but its endogenous production appeared to be related to the animal's detoxication processes.

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Influence of Time and Temperature on Ascorbic Acid Stability[1]

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CITRUS JUICES AND FRUITS have traditionally been the principal sources of ascorbic acid in the American diet. Their importance was demonstrated by Bessey and White (1), who found that the amount of ascorbic acid in the vegetables and fruits available to city children in 1942 was not enough to affect the blood plasma values unless citrus fruits or tomatoes were among the foods eaten daily. Recent advances in processing and marketing of many other fruits and vegetables, such as green peppers, broccoli, and melons, have resulted in significant increases in sources of ascorbic acid. Obviously, the importance of the citrus juice or any food as a source of ascorbic acid depends on the amount of the vitamin in the juice or food consumed.

It is well known that ascorbic acid is a substance which is readily oxidized and that destruction of its biologic activity is facilitated by such factors as oxygen, alkaline *pH*, metallic ions, particularly copper and iron, and that all of these reactions are accelerated by increases of temperature. However, the lability of ascorbic acid to oxidative reactions is frequently exaggerated; the fact is that, when properly handled, it is a rather stable chemical. This is not well appreciated, and thus the concept that ascorbic acid is extraordinarily labile can be misleading.

To provide objective information regarding the stability of ascorbic acid under conditions of controlled time and temperature, several orange juice preparations, including fresh, frozen, and canned, and a number of chemical mixtures containing ascorbic acid were subjected to room temperature and refrigerator temperatures for periods of 24, 48, 72 hr., and eight days; in addition, similar samples were boiled for 10 min.

Two other extremely important nutrients in orange juice were also studied: sodium and potassium. Citrus juice, and particularly orange juice, enjoys a favorable position as an excellent low-sodium food and a rich source of potassium. Therefore, fresh, canned, and frozen orange juices were compared with two chemical synthetics with respect to sodium and potassium content. The details of this investigation are presented below.

Method

The following products were purchased at the local supermarket: fresh oranges from which freshly squeezed orange juice was made, frozen orange juice which was reconstituted for use, canned orange juice, and two synthetic orange juices. An aqueous solution of ascorbic acid containing 50 mg. per 100 cc. was prepared in the laboratory. All of the products were prepared as for table use with distilled water following the directions on the labels.

Ascorbic acid was determined by a technique based on the one of Roe and Oesterling (2). Appropriate aliquots of the above samples were taken for analysis, and ascorbic acid determinations were carried out on each sample in duplicate at two levels of concentration, thereby giving four values for each determination. The ascorbic acid analyses were conducted immediately after the samples were prepared and following storage at room temperature (24° to 28°C.) or in the refrigerator (4°C.) for periods of 24, 48, and 72 hr., and eight days. The results thus represent the average of the four values

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TABLE 1 **Effect of different storage times and temperatures on ascorbic acid in orange juices**

SAMPLE	ASCORBIC ACID								
	Before storage	24-hr. storage		48-hr. storage		72-hr. storage		8-day storage	
		Room temperature	Refrigerator	Room temperature	Refrigerator	Room temperature	Refrigerator	Room temperature	Refrigerator
	mg. 100 cc.								
Fresh orange juice	55	53.5	53.5	54.5	54.5	54.5	55.0	69.0*	55.5
Canned orange juice	35	32.7	33.3	33.0	33.0	34.0	33.0	34.0	34.0
Synthetic orange juice									
Brand A†‡	63	63.0	63.0	61.0	61.0	60.0	59.5	61.0	61.0
Brand B†‡	63	66.0	65.5	63.5	63.0	64.0	63.2	58.0	64.7
Frozen orange juice†‡	44	45.0	46.0	44.0	44.5	44.5	42.5	45.0	42.8
Ascorbic acid solution†‡	49	48.5	50.0	50.0	49.0	48.0	48.2	48.0	50.0

*Mold contamination.
†Prepared using distilled water.
‡Strength: 50 mg./100 cc.

for each sample. These checked within the limits established for the method.

It should be emphasized that these samples were all prepared with distilled water and that storage was in tightly covered glass containers. Therefore, the oxidative effects of oxygen (air) and of metallic ions, such as copper and iron, would be held to a minimum.

In an attempt to imitate the conditions of use of these juices, the ascorbic acid content was also determined in (duplicate) samples prepared with either distilled or tap water.

To further demonstrate that ascorbic acid is actually a remarkably stable chemical, aliquots of the previously described samples were placed in glass containers and boiled at 100°C. in open air for 10 min. After boiling, the samples were cooled, readjusted to their original volume to compensate for the evaporative losses of water, and then assayed for ascorbic acid by the same method.

Samples of fresh orange juice, canned orange juice, the two synthetic orange juices, and frozen orange juice, prepared as for consumption according to label directions, and the ascorbic acid solution were also analyzed for sodium and potassium, utilizing the flame photometer.

Results and Discussion

An examination of the data in Table 1 reveals a remarkable stability of ascorbic acid in all of the samples, even when stored at room temperature for as long as eight days. Actually, the sample of fresh orange juice stored for eight days had a higher-than-original ascorbic acid content, which may be explained by the fact that significant mold contamination developed. These data provide sound evidence that under appropriate storage conditions, i.e., either in covered glass or plastic containers, and in the absence of undue exposure to air or added metallic ions, ascorbic acid is remarkably stable.

Under the conditions of our experiments, no

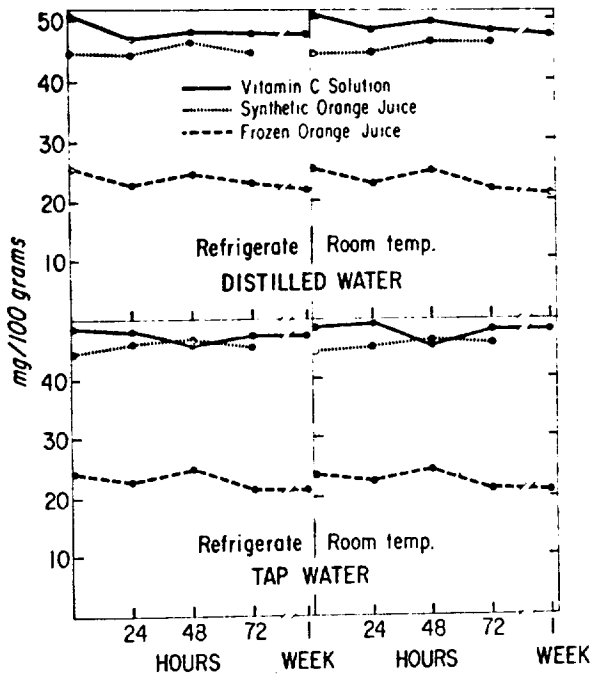

FIG. 1. *Effect of distilled vs. tap water on reconstituted synthetic and frozen orange juice and on an ascorbic acid solution.*

difference was observed in the stability of the naturally occurring ascorbic acid or the artificially added ascorbic acid in some of the products assayed. These data should provide reassurance to homemakers and professional nutritional advisors that, with proper storage, there is little need to fear loss of ascorbic acid in appreciable amounts from citrus products. Citrus juices, whether squeezed, canned, or reconstituted, can be kept in the refrigerator for several days before any measurable ascorbic acid is lost. These results are in agreement with those of Miller and Marsteller (3), Noel and Robberstad (4), and Pelletier and Morrison (5). Figure 1 also illustrates

TABLE 2 Effect of 10-min. boiling on ascorbic acid in orange juices

SAMPLE	ASCORBIC ACID		
	Before boiling	After 10-min. boiling	Loss
	mg./100 cc.	*mg./100 cc.*	*%*
Fresh orange juice	48.2	42.1	12.7
Canned orange juice	41.0	37.0	9.8
Synthetic orange juice			
Brand A*	65.5	54.0	21.3
Brand B*	63.5	54.5	14.2
Frozen orange juice*	52.5	44.5	15.3
Ascorbic acid solution*	50.3	41.5	17.5

*Prepared with distilled water.

findings when samples made up with tap water were compared with those in which distilled water had been used. Stability was also unaffected by the type of water used.

Obviously, losses of ascorbic acid should be expected with improper storage. This may in part explain the fact that the ascorbic acid values of the fresh juice (Table 1) are higher than those of the canned orange juice, and this is about what would be expected as a result of storage (6, 7). Even drastic treatment, such as boiling for 10 min. (Table 2), revealed a surprisingly high degree of stability of the ascorbic acid in these preparations, as measured by the method of this study. In no case was more than 25 per cent of the vitamin lost. This assumes, of course, that the method measures biologically active ascorbic acid. The possibility exists that during storage, other non-active ascorbic acid substances can be produced which are also measured by the 2-4 dinitrophenylhydrazine method. We are now studying this possibility. One should not interpret the variations in percentage loss presented in Table 2 as reflecting any significance in favor of one preparation over another. (It is conceivable that canned orange juice which has previously undergone a fair amount of processing may be a product in which ascorbic acid is even more stable.)

Of interest are the results presented in Table 3 on the sodium and potassium content of fresh, canned, and frozen orange juice vs. the two synthetic orange juices (chemical mixtures simulating orange juice). It is evident that both chemical mixtures contain appreciable amounts of sodium, whereas, as is well known, orange juice does not. Conversely, all of the orange juice preparations provide significant amounts of potassium while the two synthetic mixtures provide relatively small amounts of this important element. Here, again, it is important to emphasize that the consumer should be

TABLE 3 Sodium and potassium content of orange juices

SAMPLE	SODIUM		POTASSIUM	
	mEq/liter	mg./100 cc.	mEq/liter	mg./100 cc.
Fresh orange juice	0	0	48	187.0
Canned orange juice	0	0	44	182.0
Synthetic orange juice				
Brand A*	15	34.5	2	7.8
Brand B*	15	36.8	1	3.9
Frozen orange juice*	1	23.0	51	199.0
Ascorbic acid solution*	0	0	0	0

*Prepared with distilled water.

aware of what he is buying and that all that is orange is not orange juice and may not have the same nutritional value as orange juice. For example, if a product with a low-sodium, high-potassium content is desired, the natural product, i.e., orange juice, would be the choice.

Summary

This study demonstrates that ascorbic acid in citrus juices and in certain chemical solutions containing ascorbic acid is remarkably stable, even for long periods of storage at room temperature, provided storage and dilution conditions are satisfactory. It is emphasized that storage in covered glass or plastic containers permits little loss of ascorbic acid due to oxidation. Even vigorous boiling for 10 min. in glass and with the use of distilled water as diluent revealed that ascorbic acid is surprisingly stable. The synthetic products made to simulate orange juice did not do so with respect to sodium and potassium content.

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ASCORBIC ACID, GLUCOSE-1-PHOSPHATE, AND LYSERGIC-ACID DIETHYLAMIDE IN RHEUMATOID ARTHRITIS

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Long and Miles (1950) found that cortisone and adrenocorticotrophic hormone (A.C.T.H.) diminished experimental tuberculin hypersensitivity in the guineapig. Postulating a possible analogy between this form of allergy and the tissue reaction in rheumatoid arthritis, Long et al. (1951b) suggested that other factors influencing experimental tuberculin hypersensitivity might also influence arthritis in man. They described several such factors (Long et al. 1951a and b, Fisher et al. 1951, Cornforth and Long 1952). In man we found that cortisone and A.C.T.H. diminished the tuberculin reaction more conspicuously than other inflammations studied, and we considered the possibility that this reaction might reflect the nature of the disease process in rheumatoid arthritis (Lovell et al. 1953). We therefore tried to discover if some of the factors shown by Long and his colleagues to effect experimental tuberculin hypersensitivity influenced rheumatoid arthritis; we also studied their effect on the tuberculin reaction in man. We report these studies here.

Long et al (1951a) suggested that, in guineapigs, the desensitising action of cortisone and A.C.T.H. was mediated by an indirect effect on the metabolism of ascorbic acid. In laboratory animals deficient in ascorbic acid but not clinically scorbutic, fed on a synthetic pelleted diet, sensitivity to tuberculin was high and could be diminished by giving ascorbic acid but not by giving the hormones. The hormones did not enhance the desensitising action of ascorbic acid. If tuberculin sensitivity in the guineapig and rheumatoid arthritis in man were analogous, and the mode of action of cortisone and A.C.T.H. was comparable in the two conditions, a deficiency of ascorbic acid might be expected to enhance the inflammation in rheumatoid arthritis and at the same time to diminish the responsiveness of the disease to the hormones. In a state of depletion of ascorbic acid, treatment with ascorbic acid might diminish the activity of the arthritis. In our first study we examined these possibilities.

Our second study was of the effects on rheumatoid arthritis and tuberculin reactions of two substances which modified experimental tuberculin hypersensitivity in the guineapig—namely, glucose-1-phosphate and lysergic-acid diethylamide (L.S.D.). Cornforth and Long (1952) found that tuberculin sensitivity in guineapigs was reduced by a subcutaneous injection of glucose-1-phosphate 0·01 mg. per kg. body-weight and by L.S.D. 0·00018 mg. per kg. body-weight. We are not aware of any reports of the effects of glucose-1-phosphate in man. The effects of L.S.D. both in normal and in psychopathic patients have been reported by several workers—e.g., Rinkel et al. (1952). It causes transient mental changes and disturbances in the autonomic nervous system. The effects have sometimes been observed with doses as small as 0·02 mg., and 0·04–0·1 mg. is reported to be active in most cases, though individual variation in responsiveness seems to be great.

Methods

Ten patients studied had active rheumatoid arthritis, and one had polyarteritis nodosa with predominant involvement of the joints. All were treated by rest in bed, controlled exercises, and, when necessary, splinting of acutely inflamed joints.

All the patients received acetylsalicylic acid ('Disprin'), gr. 30–60 daily, and sometimes tab. codeine co. *B.P.* We have found in man that tuberculin reactivity is unaltered by salicylates, and Long (1952) has found salicylates to be without effect on tuberculin sensitivity in guineapigs. Salicylates do not modify the beneficial therapeutic effects of cortisone and A.C.T.H. We did not therefore feel justified in withholding these analgesics. Their use, however, implies a more severe test of factors possibly giving mild relief of symptoms in arthritis than would have been imposed if they had been withheld;

TABLE I—SUMMARY OF RESULTS OF ASCORBIC-ACID STUDIES

Patient	Study	Régime	Duration in weeks	Blood ascorbic acid (mg. per 100 ml.)	Course of arthritis
1	A	(a) Ordinary diet	..	0·3	..
		(b) Scorbutic diet	11	0·3→<0·1	Gradual improvement
		(c) Scorbutic diet + 1 g. ascorbic acid daily	5	2·1–2·5*	Improvement continued
		(d) Scorbutic diet + 1 g. ascorbic acid daily + cortisone	1½	1·9	Conspicuous improvement
		(e) Ordinary diet + ascorbic acid 1 g. daily	2	2·1	Relapse lasting 2 weeks
	B	(a) Scorbutic diet	10	2·1→<0·1	Fluctuating
		(b) Scorbutic diet + cortisone	3	<0·1	Conspicuous improvement
		Scorbutic diet + A.C.T.H.	3½		
		(c) Scorbutic diet	4	<0·1	Relapse after 3 weeks
		(d) Scorbutic diet + 0·5 g. ascorbic acid daily	1	1·9*	Relapse continued
		(e) Ordinary diet	8	..	Relapse continued 3 weeks, then improvement
2	A	(a) Ordinary diet	..	0·4	..
		(b) Ordinary diet + 100 mg. ascorbic acid daily	3	1·0–1·3	Slight improvement
		(c) Ordinary diet + 100 mg. ascorbic acid daily + cortisone	2	0·9–1·2	Conspicuous improvement
		(d) Ordinary diet	6	..	Relapse after 4 weeks
	B	(a) Scorbutic diet	9	0·13→<0·1	Fluctuating
		(b) Scorbutic diet + cortisone	3	<0·1	Conspicuous improvement
		Scorbutic diet + A.C.T.H.	3		
		(c) Scorbutic diet	4	<0·1	Moderate relapse
		(d) Scorbutic diet + 0·5 g. ascorbic acid daily	1	1·4†	Unchanged
		(e) Ordinary diet	8		Unchanged
3		(a) Ordinary diet	..	0·3	..
		(b) Scorbutic diet	9	0·3→<0·1	Unchanged
		(c) Scorbutic diet + 1 g. ascorbic acid daily	2	1·9	Unchanged
		(d) Scorbutic diet + 1 g. ascorbic acid daily + A.C.T.H.	3½	1·6	Conspicuous improvement
		(e) Ordinary diet	..	..	Relapse after 3 weeks

* Attained with 3 g. ascorbic acid. † Attained with 2·5 g. ascorbic acid.

for salicylates afford some such relief. However, it was against the more conspicuous effects of cortisone and A.C.T.H. treatment that we wished to measure an effect, if any.

The arthritis was assessed at least twice a week in the long studies, and daily in the shorter ones, by measurements of heat, swelling, tenderness, and functional capacity of the joints. To the patients treated with glucose-1-phosphate and L.S.D., dummy preparations were usually administered for up to 14 days before treatment.

Cortisone (Merck) 200 mg. daily was given orally in case 1 and by intramuscular injection in case 2. A.C.T.H. (Armour) 100 mg. daily was given by intramuscular injection. Ascorbic acid (Roche) was given orally.

When a scorbutic diet was given, all the cooking was done in copper vessels. Fruit, fruit juices, and vegetables were not allowed. All the milk was boiled. Potatoes were boiled, mashed, and kept hot for at least an hour before being served. Ascorbic acid in whole blood was measured at least once a week by the method of Roe and Kuether (1943).

The α-D-glucose-1-phosphate was prepared by Roche Products Ltd. as a solution containing 50 mg. of the dipotassium salt per ml. The preparation was confirmed by Dr. D. A. Long to be active in reducing tuberculin hypersensitivity in guineapigs. Intramuscular and subcutaneous injections caused intense pain: this could be mitigated by mixing the solution with procaine hydrochloride, but such a mixture was found by Dr. Long to be inactive in the guineapig. The glucose-1-phosphate was therefore given intravenously, by intermittent injections, or by continuous intraveonus drip in physiological saline solution. The doses ranged from 4 to 500 mg. daily.

Lysergic-acid diethylamide was supplied by Sandoz Products Ltd. both as a powder and as a liquid. Because of its instability, solutions made from the powder were freshly prepared every 5 days; fresh ampoules of liquid were opened for each dose. The drug was given by mouth in about 30 ml. of water before breakfast. Doses ranged from 0·005 to 0·1 mg. daily.

Tuberculin reactivity was tested by intradermal injections of serial dilutions of purified protein derivative (P.P.D.), as described by Lovell et al. (1953). In patient no. 3, the tests were made at intervals of 1–2 weeks. In the other cases comparisons were based on between one and four tests done before and during treatment.

Eosinophils in capillary blood were usually counted twice weekly and erythrocyte-sedimentation rates (E.S.R.) were measured once a week.

Results

ASCORBIC ACID

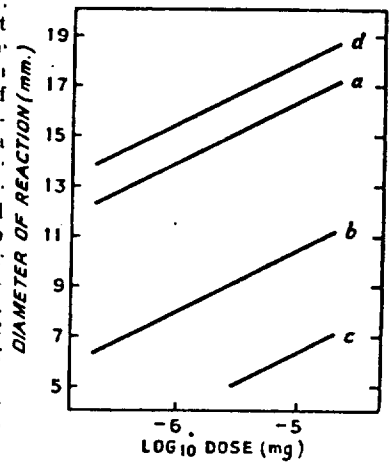
The results are summarised in table I.

Patient 1.—Female, aged 34. Active rheumatoid arthritis for 8 months had involved almost all joints. During 11 weeks on scorbutic diet the arthritis improved. When the patient was saturated with ascorbic acid, the trend of improvement (already established) continued, and later there was a conspicuous clinical response to cortisone. During a subsequent 10 weeks on scorbutic diet the arthritis fluctuated in severity, but there was no definite trend in its course. While the patient was still on scorbutic diet the response to cortisone was again conspicuous and the improvement was sustained during a course of A.C.T.H.

Patient 2.—Female, aged 17. Active rheumatoid arthritis for 2 years affecting fingers, hands, wrists, elbows, and knees. During 9 weeks on scorbutic diet the rheumatoid arthritis fluctuated in severity, but no definite trend was discernible. The therapeutic response to cortisone when the patient was depleted of ascorbic acid appeared as full as an earlier response induced when there was no depletion. The disease responded to A.C.T.H. during the 13th to 15th weeks of scorbutic diet. Saturation with ascorbic acid after 19 weeks on scorbutic diet, and subsequent return to full diet caused no appreciable change in the course of the disease.

Patient 3.—Female, aged 50. Rheumatoid arthritis for 20 years. Active inflammation in hands, wrists, right hip, and both knees. During 9 weeks on scorbutic diet, there was no change in the arthritis which could be attributed to this régime, nor was there one when the patient was saturated with ascorbic acid. When the patient was saturated with ascorbic acid the symptoms were improved while A.C.T.H. was given.

Tuberculin reactions were studied in patient no. 3. At the beginning of the scorbutic diet, P.P.D. solutions weaker than 0·2 mg. per ml. did not evoke a response and this solution caused a lesion of only 10 mm. diameter. After a month's scorbutic diet, when the blood-ascorbic-acid level had fallen to about 0·1 mg. per 100 ml., the reactivity was increased, a solution of 0·002 mg. per ml. giving a response just short of necrosis. Tests with serial dilutions were made thereafter with 0·0002, 0·00002, and 0·000002 mg. per ml. (see figure). The reactivity was high when the blood-ascorbic-acid level was less than 0·1 mg. per 100 ml.; it fell when the patient was saturated with ascorbic acid and it fell further when A.C.T.H. 100 mg. daily was given. After A.C.T.H. was stopped reactivity rose to approximately the same level that was attained in the scorbutic state, though now the patient was still saturated with ascorbic acid.



Patient 3.—Calculated mean parallel regressions of diameters of reactions on log. dose of P.P.D. injected during (a) ascorbic-acid depletion, (b) ascorbic-acid saturation, (c) A.C.T.H. treatment, (d) after A.C.T.H. For differences between (a) and (b), $0·02 < P < 0·05$; (b) and (c), $0·05 < P < 0·1$; and (c) and (d), $0·01 < P < 0·02$.

Conclusion.—Rheumatoid arthritis was uninfluenced by ascorbic-acid depletion or saturation, nor did these factors appear to influence the therapeutic effects of cortisone and A.C.T.H. In one patient reactivity to tuberculin increased during depletion of ascorbic acid, decreased after saturation, and decreased still further when A.C.T.H. was given. When A.C.T.H. was withheld the reactivity to tuberculin rose to a level comparable to that attained during the period of ascorbic-acid depletion.

GLUCOSE-1-PHOSPHATE

Patient 4.—Male, aged 47. Active rheumatoid arthritis for 3 years. Main disability now due to inflammation in the knee-joints. Glucose-1-phosphate 500 mg. in 1 litre of physiological saline solution was given daily for 8 days by intravenous drip. Each infusion lasted 12–16 hours. There was no modification of the arthritis, which had improved conspicuously in the first 8 days of cortisone administration a year earlier. Tuberculin reactivity was not changed significantly (table II), though it had been reduced about 100-fold during previous cortisone treatment (case 6 in paper by Lovell et al. 1953).

Patient 5.—Male, aged 56. Active rheumatoid arthritis for 6 months involving mainly fingers, hands, wrists, and knees. Received six intravenous injections of glucose-1-phosphate in doses of 10, 20, 50, 100, 200, and 200 mg. There was no subjective or objective change in the arthritis. Injections of 100 and 200 mg. caused rigors owing to a pyrogen in the solution injected.

Patient 6.—Male, aged 46. Rheumatoid arthritis for 7 years; and a severe relapse for past 3 months with active inflammation in fingers, hands, shoulders, hips, and knees. Glucose-1-phosphate 100 mg. was given intravenously twice daily for 4 days and 200 mg. twice daily for 3 days. There was no subjective or objective change in the arthritis and no significant change in tuberculin reactions (table II).

Patient 7.—Male, aged 52. Polyarteritis nodosa for 5 years, involving mainly the joints and skin. A maintenance dose of cortisone 50 mg. daily was incompletely suppressing the arthritis, which was most conspicuously active in wrists and knees. Intravenous glucose-1-phosphate 1 mg. was given four times daily for 1 day, 100 mg. for 3 days, and 200 mg. for 3 days. There was no subjective or objective change in the arthritis, though, when the treatment was stopped, there was relief of pain with objective improvement 4 days after the dose of cortisone had been increased to 100 mg. daily.

Conclusion.—In the doses used glucose-1-phosphate did not influence the course of the arthritis in four patients or the size of the tuberculin response in the two patients tested. No effects were discerned on eosinophil-counts or E.S.R.

LYSERGIC-ACID DIETHYLAMIDE

Patient 8.—Female, aged 39. Active rheumatoid arthritis for 18 months, with inflammation mainly in hands, wrists, shoulders, and right knee. Subjective improvement in the arthritis took place during a fortnight's observation on dummy medicine. L.S.D. was given for 9 days, starting with 0·005 mg. and increasing to 0·025 mg. daily. The patient was troubled by flushing of the face during treatment. Subjective improvement continued, but there was no objective change in the joints.

Patient 9.—Male, aged 33. Rheumatoid arthritis for 4 years, and a relapse of 2 months' duration. Wrists, finger, elbow, shoulder, and knee joints were inflamed. L.S.D. was given for 11 days, starting with 0·025 mg. and increasing to 0·1 mg. daily. There were no side-effects, no objective change in the arthritis, and no significant change in tuberculin reactions (table II). This patient had shown a conspicuous therapeutic response to A.C.T.H. during an episode of active arthritis a year previously.

Patient 10.—Female, aged 52, had 10 years' history of active rheumatoid arthritis with advanced structural changes in many joints. Active inflammation in the wrists and knees. L.S.D. was given for ten days, starting with 0·025 mg. and increasing to 0·1 mg. daily. No side-effects and no subjective or objective changes in the arthritis were observed.

Patient 11.—Male, aged 23. Acute polyarthritis for 10 months and bronchiectasis. Acute inflammation in hands, wrists, elbows, shoulders, hips, knees, and feet. L.S.D. was given for 11 days, starting with 0·025 mg. and increasing to 0·1 mg. daily. Conspicuous dilatation of the pupils developed during treatment. There were no subjective or objective changes in the arthritis. The size of the tuberculin reaction was not significantly changed (table II).

TABLE II—CHANGES IN DIAMETERS OF TUBERCULIN REACTIONS DURING TREATMENT

Patient	Treatment	Mean lesion diameter before treatment (mm.)	Change in diameter during treatment ± standard error	Significance
4	Glucose-1-phosphate	11·5	+1·5 ± 0·9	0·3 < P < 0·4
6	Glucose-1-phosphate	16·7	−1·7 ± 2·5	0·5 < P < 0·6
9	Lysergic acid ..	9·4	−1·3 ± 1·3	0·3 < P < 0·4
11	Lysergic acid ..	12·7	−1·0 ± 2·3	0·6 < P < 0·7

Conclusion.—In the doses used L.S.D. did not influence the course of rheumatoid arthritis in four patients or the size of the tuberculin reaction in the two patients tested. No effect was evident on eosinophil-counts or the E.S.R.

Discussion

Although cortisone and A.C.T.H. diminish both experimental tuberculin hypersensitivity and the inflammation in rheumatoid arthritis, the disease has proved to be unaltered by other factors affecting experimental hypersensitivity. The degree of ascorbic-acid saturation appears to be without influence on the disease, or on its responsiveness to cortisone and A.C.T.H., and glucose-1-phosphate and L.S.D. have no discernible cortisone-like effect, even in doses proportionally greater than those which diminish experimental hypersensitivity. These studies therefore provide no evidence to support an analogy between experimental tuberculin hypersensitivity and the tissue reaction in rheumatoid arthritis. On the other hand, because of the difficulties inherent in duplicating experimental conditions in man and the guineapig, such an analogy is not refuted. In the ascorbic-acid studies especially, there were the inevitable differences in the basal diets.

In patient no. 3 the trends of change in the tuberculin reactions during ascorbic-acid depletion and saturation were comparable to those described in guineapigs (Long et al. 1951a). We should hesitate, however, to conclude, from this single observation, that the changes observed were not fortuitous, for we have not previously studied tuberculin reactivity so long in untreated persons. The possibility of an association between ascorbic-acid saturation and tuberculin reactivity in man merits further study.

Though cortisone and A.C.T.H. diminish the tuberculin reaction in both the guineapig and man, the reaction in man appears to be uninfluenced by glucose-1-phosphate and L.S.D., which have cortisone-like effects in the guineapig. We have therefore been unable to test the possibility that substances other than cortisone and A.C.T.H., which diminish the tuberculin reaction in man, may also affect the tissue reaction in rheumatoid arthritis. The study in patient no. 3 has confirmed our earlier impression that there is ordinarily no correlation between the level of tuberculin reactivity in a given person and the activity of the arthritis.

It is possible that the means by which cortisone diminishes the tuberculin reaction are different in the guineapig and in man. Though the dose used by Long and Miles (1950) in guineapigs was, on the basis of weight, comparable to that which we found necessary to reduce the tuberculin reaction in man (Lovell et al. 1953), the resulting reduction in reactivity in the guineapig was about 4-fold, whereas in man the reduction ranged between 15-fold and 170-fold. However, the variability in species responses to the hormones, and the fact that Long and his colleagues usually confined their observations to effects attained within a few hours of single injections of hormones, make comparison in the two species difficult.

Long and his colleagues have interpreted the cortisone effect in the guineapig as reflecting a reduction in its hypersensitivity. Some support for this thesis has been provided by Leahy and Morgan (1952), who found that cortisone inhibited the cytotoxic action of tuberculin on sensitised guineapig cells in vitro. The means whereby cortisone diminishes the tuberculin reaction in man has not been identified. In the doses we used we found that the inflammation evoked by a direct irritant, manganese butyrate, was also reduced by cortisone, and a similar effect has been described on the inflammation evoked in human skin by ultraviolet light (Järvinen 1951). Possibly therefore, the action of cortisone on the tuberculin reaction in man reflects, at least in part, a non-specific diminution of an inflammatory response, rather than an interruption of the immunological mechanism which initiates a hypersensitivity reaction.

Summary

The effects of some factors which modify experimental tuberculin hypersensitivity have been studied in patients with rheumatoid arthritis.

Ascorbic-acid depletion and saturation did not influence the course of rheumatoid arthritis or the therapeutic effects of cortisone and A.C.T.H.

α-D-glucose-1-phosphate and lysergic-acid diethylamide had no effect on rheumatoid arthritis or on the tuberculin reaction in man.

The possibility is discussed that the means by which cortisone diminishes the tuberculin reaction in the guineapig and in man may differ.

We gratefully acknowledge the coöperation of the patients taking part in this study. We are indebted to the Nuffield Foundation for their generosity in making the grant which enabled these studies to be undertaken; to Merck & Co. for their gift of cortisone to the joint committee of the Nuffield Foundation and the Medical Research Council, part of which was used in these studies, and to this same committee for A.C.T.H.; to Dr. H. H. Green, of the Ministry of Agriculture veterinary research laboratories, for the gift of P.P.D.; and to Dr. H. M. Sinclair for his advice on ascorbic-acid estimations. We also thank Roche Products Ltd. for giving us glucose-1-phosphate, and Sandoz Products Ltd. for giving us lysergic acid. Finally we are indebted to Prof. G. W. Pickering for his encouragement and to Dr. D. A. Long for much helpful discussion and for suggesting the trial of glucose-1-phosphate and lysergic acid.

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THE INTERRELATIONSHIP OF DIETARY, SERUM, WHITE BLOOD CELL, AND TOTAL BODY ASCORBIC ACID

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The white blood cells are living, actively metabolizing cells which are accessible for analysis. If the ascorbic acid concentration of these cells could be shown to be a valid index of the ascorbic acid level of the body as a whole, white cell assays should be a valuable means of determining the status of the individual in regard to this vitamin. The analysis of blood or urine yields information which is of value chiefly if it may be interpreted in terms of function of the organism, or in terms of the tissue concentrations on which functional activity surely depends. The establishment of the interrelationships among serum, urine, white cell, and tissue concentrations should enhance the value of existing ascorbic acid data on serum and urine.

Stephens and Hawley (1) and Butler and Cushman (2) found that white blood cells and blood platelets are rich in ascorbic acid in normal persons. The latter investigators found the level very low in scorbutic patients. (White blood cells and platelets contain essentially the same concentration of ascorbic acid and are usually measured together. They will be referred to collectively, hereinafter, as white cells.) Crandon *et al.* (3) observed that on an ascorbic acid-free diet the serum level of the vitamin fell almost to zero, after which the white cell level slowly fell and approached zero at the time frank signs of scurvy appeared. These data have created the impression that the white cells, and hence presumably the other tissues, contain undiminished amounts of the vitamin as long as ascorbic acid is demonstrable in the serum. Pijoan and Lozner (4) have presented data in support of this conclusion. Such data do not, however, concur with the repeated observation that considerable amounts of ascorbic acid may be retained (in a load test) by persons with low but definite serum ascorbic acid concentrations (5).

Clearly more information on the relationship between intake and serum and white cell levels is desirable.

An exceptional opportunity for making the desired comparison was offered by an experiment conducted by the Royal Canadian Air Force. Over 100 vigorous RCAF personnel were maintained for 8 months on

controlled ascorbic acid intakes. Monthly serum ascorbic acid determinations were made by the primary investigators. Group Captain Frederick F. Tisdall very kindly invited this laboratory to obtain blood samples from these subjects for determination of white cells and serum ascorbic acid. The development of a rapid procedure for measuring white cell ascorbic acid concentrations on finger blood (0.1 ml.) made such determinations feasible.[1]

In addition to observing the effect of different ascorbic acid intakes on the serum and white cell concentrations, the extent of the tissue depletion was measured by determining the amount of ascorbic acid necessary to restore the concentration in the white cell and in the body as a whole to maximal values. These measurements permitted an evaluation of the white cell as an index of the total ascorbic acid in the body.

Materials and Methods

The details of the RCAF experiment have been described by Linghorne *et al.* (6). In brief, the personnel were divided into four groups. Group I received a palatable basal diet which was repeatedly shown by analysis to contain an average of 8 mg. of ascorbic acid per day. Group II received the same diet plus a raw cabbage supplement containing (by analysis) 15 mg. of ascorbic acid (average total 23 mg.). Group III received the basal diet plus 70 mg. per day of synthetic ascorbic acid (total 78 mg.). Group IV received the regular station ration which furnished an average of 78 mg. of ascorbic acid per day.

Ascorbic acid was measured in duplicate on (*a*) 10 c.mm. serum samples (7), (*b*) the white blood cells plus platelets obtained from 0.1 ml. of blood,[1] and (*c*) 0.025 to 0.1 ml. aliquots of 24 hour urine specimens. The procedure for urine was essentially the same as that used for serum except that larger volumes were employed. The white cell method has since been improved and is reported elsewhere.[1]

Results

Groups I and II receiving 8 and 23 mg. of ascorbic acid per day had average white blood cell values only about half as great as did Groups III and IV, receiving 78 mg. per day (Fig. 1). The average serum value for both Groups I and II was the same (0.2 mg. per cent). No serum values of zero were encountered and all but two samples were 0.1 mg. per cent or over. As was found for the serum values, there was no statistically significant difference between the white cell averages for Groups I and II. There was also no significant difference between the serum and white cell averages for Group III receiving synthetic ascorbic acid and Group IV receiving the vitamin in natural form (total intakes 78 mg. daily).

[1] Bessey, O. A., Brock, M. J., and Lowry, O. H., in preparation.

On inspection of the graph in Fig. 1 it will be seen that a definite relationship exists between ascorbic acid concentration in serum and white cells. Of the thirty-nine subjects with serum values greater than 0.4 mg. per cent, only one white cell value was less than 20 mg. per cent, whereas out of the forty-two with serum ascorbic acid less than 0.3 mg. per cent, only in one subject did the white cell level exceed 20 mg. per cent. In the intermediate zone of serum values (0.3 to 0.4 mg. per cent) both

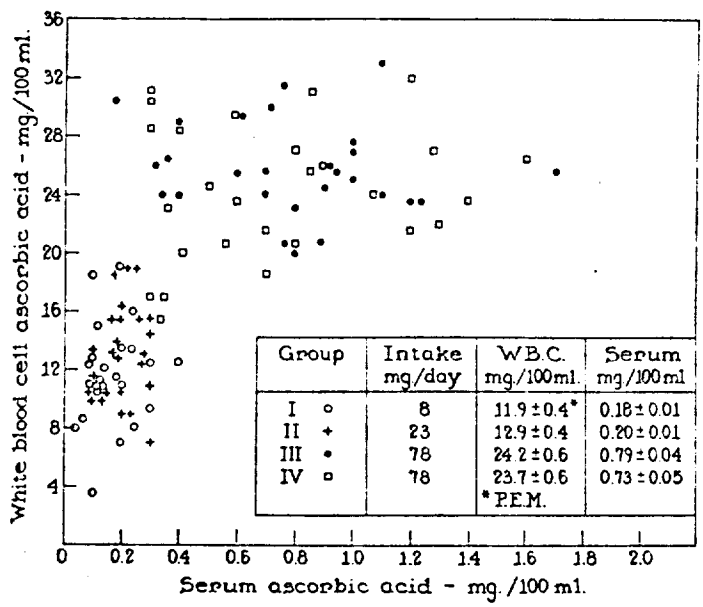

FIG. 1. Comparison of the ascorbic acid concentration in serum and white blood cells of a group of 103 subjects maintained for 8 months on 8, 23, or 78 mg. of ascorbic acid daily.

high and low white cell levels were encountered. The relation between intake and serum and white cell levels is evident.

At the end of 8 months on the rigidly controlled ascorbic acid intakes, thirty subjects, chiefly from Group I (8 mg. per day), were given large amounts of ascorbic acid and the course of realimentation was studied. Each day for 4 days 500, 1000, or 2000 mg. of ascorbic acid were given by mouth in 8 or 10 hourly doses. 24 hour urine samples were collected and each morning, before any ascorbic acid was received, white cell and serum samples were collected.

The data summarized in Figs. 2 and 3 show that for the subjects who had been receiving 8 mg. of ascorbic acid per day recovery of both serum

and white cell levels was nearly complete in 1 day with 2000 mg. of ascorbic acid per day (four subjects), in 2 days with 1000 mg. per day (six subjects), and in 3 or 4 days with 500 mg. per day (four subjects). Thus both serum and white cell values can respond with great rapidity to a sufficiently large dose of the vitamin. The changes in ascorbic acid levels in white cells *versus* serum follow essentially the same pathway (but at different rates) whether the ascorbic acid dose is 500, 1000, or 2000 mg. per day (Fig. 3).

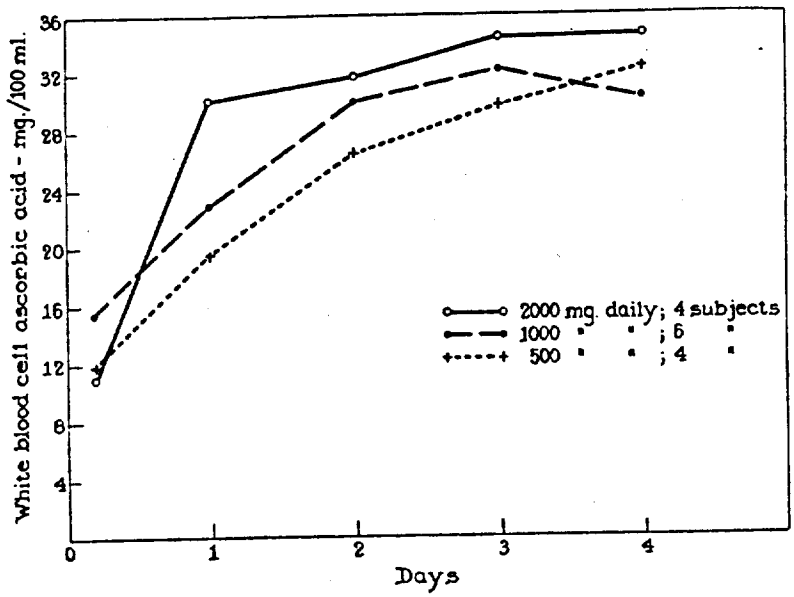

FIG. 2. Summary of changes in ascorbic acid content of white blood cells upon realimentation of subjects previously maintained on low ascorbic acid intakes.

Furthermore, this pathway nearly coincides with the curve obtainable from Fig. 1 for static values of white cell and serum ascorbic acid attained by the ingestion of different amounts of ascorbic acid. This suggests that the white cells and serum are in equilibrium with each other under the conditions of the experiment.

The serum and white cell response of six subjects (data not shown) who had been receiving 23 mg. of ascorbic acid per day was essentially the same as for those who had been receiving 8 mg. Although the average serum levels of a control group of four subjects (from Group III, which had been regularly receiving 78 mg. of ascorbic acid daily) were raised by the large test doses, the white cell levels were not further raised; on the contrary, they decreased slightly (about 15 per cent) during the 4 days of the experiment. This suggests that factors other than ascorbic acid intake may at

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least to a small degree affect the ascorbic acid level of white blood cells. Similar unexplained changes have been encountered by others (Wilson and Lubschez (8)).

The urinary excretion (Table I) was uniformly low during the first 24 hours except in the control group (No. III). It is surprising that, even when 2000 mg. of ascorbic acid were ingested, only 0.5 per cent of the dose was lost in the urine. On subsequent days the excretion rose to 75 to 90 per cent of the intake. An estimate of the ascorbic acid retention was

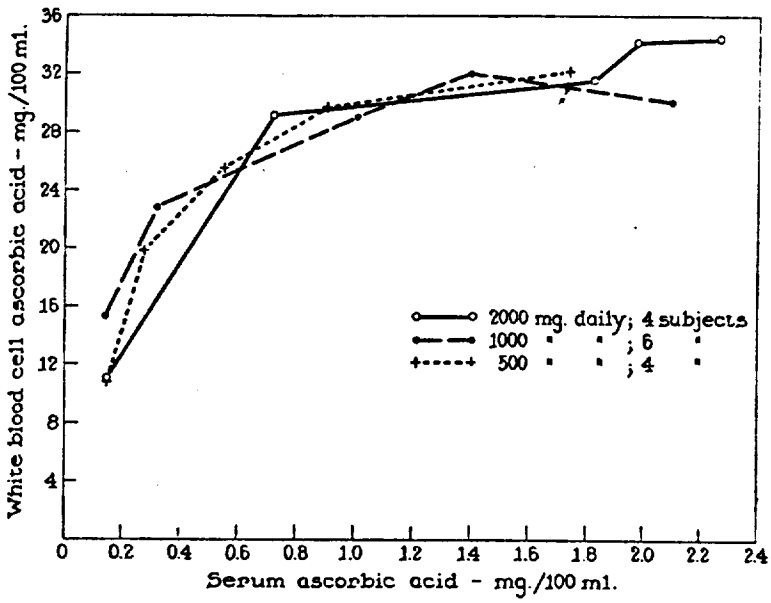

FIG. 3. Comparison of the ascorbic acid content of white blood cells and serum during realimentation of subjects previously maintained on low ascorbic acid intakes.

made with the following assumptions: (*a*) that the ingested vitamin was in part retained, in part destroyed, and in part excreted in the urine; (*b*) that during the last 24 hour period the control group (No. III), receiving 500 mg., and the groups receiving 1000 and 2000 mg. of ascorbic acid were no longer retaining ascorbic acid, in which case the destruction in the body would be equal to the difference between intake and output; (*c*) that this destruction was the same on all 4 days, and was equal in the three groups receiving 500 mg. per day.

If these assumptions are valid, the retention each day may be calculated by subtracting the ascorbic acid excreted that day from the amount excreted at a time when no retention was taking place. For example, the

group receiving 1000 mg. excreted 1, 125, 495, and 767 mg. on the 4 successive days; the calculated retention was, therefore, 766, 642, 272, and 0 mg. respectively, or a total of 1680 mg. This is presumably a lower limit, since there is quite possibly less destruction of ascorbic acid during the 1st day or 2. Considering this uncertainty, the agreement in retention with

TABLE I

Average Daily Urinary Excretion and Total Retention of Ascorbic Acid during Realimentation

Group No.	Previous intake	Test dose	Excretion				Retention
			1 day	2 days	3 days	4 days	
	mg. per day	*mg. per day*	*mg. per day*	*mg. per day*	*mg. per day*	*mg. per day*	*mg.*
I	8	500	2	22	72	167	1625
II	23	500	1	13	77	260	1537
III	78	500	154	371	470	474	419
I	8	1000	1	125	495	767	1680
I	8	2000	11	980	1403	1539	2223

TABLE II

Comparison of Retention of Ascorbic Acid to Increase in White Blood Cell Ascorbic Acid

Group No.	Previous intake	Test dose		Change as per cent of maximal change	
				After 18 hrs.	After 42 hrs.
	mg. per day	*mg. per day*			
I	8	500	Total body*	29	57
			White blood cells†	40	68
I	8	1000	Total body	46	87
			White blood cells	44	81
I	8	2000	Total body	69	
			White blood cells	78	
II	23	500	Total body	31	60
			White blood cells	33	83

* Retention as per cent of total retention.

† Increase in concentration as per cent of total increase in concentration.

different dosages is satisfactory. The average retention for the fourteen subjects in Group I was 1844 mg. Group II, which had been initially receiving 23 mg. of ascorbic acid per day, retained nearly as much ascorbic acid as Group I (initially receiving 8 mg. per day), whereas the control group retained only about one-fourth as much.

In reference to the question whether the white blood cell ascorbic acid level can serve as an index of general tissue levels of ascorbic acid, it is

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useful to correlate the total daily retention of ascorbic acid by the body as a whole with the rate at which the white cells return to normal (Table II). Total retention is recorded as per cent of the ultimate total retention, and the white blood cell changes are recorded as per cent of the total change during the 4 days; *i.e.*, if the initial, 1st day, and 4th day white cell ascorbic acid values were 12, 21, and 30 mg. per cent, the change after 1 day would be recorded as 100 × 9/18 or 50 per cent. It will be seen (Table II) that the white cell recovery parallels the ascorbic acid retention within the limits of experimental variations expected for this type of study. This suggests that the white blood cells are actually a valid measure of the level of the body content of ascorbic acid. If this is true, the total body ascorbic acid may be calculated: Since the white cell ascorbic acid concentration approximately doubled during the retention of 1800 mg. of ascorbic acid, the final body content of ascorbic acid is presumably twice 1800 mg. or nearly 4 gm.

DISCUSSION

It seems permissible to conclude (1) that with the ingestion of 8 to 23 mg. of ascorbic acid daily by adults the white blood cell ascorbic acid is maintained at only about half the concentration attained with intakes of 78 mg. per day; (2) that with 8 to 23 mg. of ascorbic acid per day a tissue deficit from the maximal attainable concentration amounting to nearly 2 gm. is encountered; (3) that with white cell ascorbic acid values half of normal, serum ascorbic acid levels are not necessarily zero but lie rather in the concentration range of 0.1 to 0.4 mg. per cent; (4) that the white cell level appears to be an index of the total body level of ascorbic acid; and (5) that given an opportunity, through a sufficiently large intake of ascorbic acid, the white cells and serum levels will return to normal within 24 hours, with a concomitant retention of very large amounts of ascorbic acid by the body.

An important question to public health is what tissue concentration is necessary for optimal ascorbic acid nutrition. In this study the control group receiving 78 mg. per day was approximately 90 per cent saturated (400 mg. retention with a calculated 4 gm. of ascorbic acid content at saturation). Groups I and II with 8 and 23 mg. of ascorbic acid per day were 50 or 60 per cent saturated. Careful studies of the gums of these subjects by Linghorne *et al.* (6) revealed a clearly defined handicap to the gingival tissue in Groups I and II. This tissue is continually exposed to mechanical trauma and bacterial invasion. It is precisely such conditions which are likely to expose handicaps attributable to suboptimal nutritional states. These findings suggest, therefore, that with somewhere between 60 and 90 per cent tissue saturation handicaps attributable to ascorbic acid deficiency become demonstrable.

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The degree of tissue undersaturation appears to be measured in a general way by the ascorbic acid concentration in the white blood cells. It will be frequently desirable, however, to have a more direct and sensitive measure of the tissue deficit, particularly when the depletion is not great. The usual saturation tests are not as sensitive a measure of this deficit as they could be. Common practice is to give a single fairly large dose, perhaps 200 to 500 mg., and to measure the amount of ascorbic acid which appears in the urine. In any case, but particularly if this dose is given by vein, the vascular system is flooded with ascorbic acid and the amount excreted depends on how well the kidney competes with the rest of the body for the vitamin. When given by mouth, the serum level and, hence the urinary excretion, will depend in part on the rate of absorption. Thus the information obtained gives relative but not absolute information concerning the tissue deficit. An alternative procedure is suggested by the experiments reported here: A total dose should be given which is somewhat larger than the expected deficit; *e.g.*, 750 to 1000 mg. for persons receiving over 50 mg. per day, and for persons thought to be seriously depleted, 2 or 3 gm. The ascorbic acid should be given in many divided doses spread out during the day, and all the urine excreted during a 24 hour period, commencing with the first dose, should be collected. With allowance for an estimated 25 per cent destruction, the ascorbic acid retention can be estimated. This procedure is more involved than the ordinary tolerance test, but it has the virtue of giving a measure of the actual tissue depletion. Hathaway and Meyer (9) have used this type of test in studies with children, but they used much smaller doses administered over a much longer period of time. The relatively small amount of destruction of ascorbic acid by the body in comparison to that of thiamine, riboflavin, and niacin creates an especially favorable opportunity for the measurement of tissue depletion by this method (10).

SUMMARY

1. White blood cell and serum ascorbic acid concentrations were measured in subjects who had received standardized diets for 8 months containing 8, 23, and 78 mg. of ascorbic acid per day. With 8 and 23 mg. of the vitamin per day the white cells averaged about 12 mg. per cent of ascorbic acid compared to 25 mg. per cent of ascorbic acid for subjects receiving 78 mg. per day. The serum levels were not zero even with the low intakes and low white cell levels, averaging 0.2 mg. per cent for those receiving 8 and 23 mg. of ascorbic acid per day.

2. After 8 months on a diet containing 8 mg. of ascorbic acid per day an average of about 1800 mg. of ascorbic acid was retained following the ingestion of large amounts of ascorbic acid.

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3. Since the increase in white cell ascorbic acid concentration paralleled the retention of ascorbic acid, the white blood cells appear to be a valid index of the total body concentration of ascorbic acid. This leads to the conclusion that the normal adult contains nearly 4 gm. of ascorbic acid.

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Effects of Prolonged High Dosage with Ascorbic Acid.* (19623)

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In recent years relatively large doses of ascorbic acid have been prescribed in the treatment of a variety of diseases including hypertension and allergic conditions. It has been generally assumed that daily doses of one gram are innocuous although there have been reports that vagotonic symptoms occur in children and that animals receiving large quantities of ascorbic acid may lose weight(1).

Wilson and Lubschez(2) have reported preliminary data suggesting that possibly the ingestion of 500 to 1000 mg ascorbic acid per day for a long period might not be innocuous. These investigators found a paradoxical decrease in the ascorbic acid of the white blood cells after a time on high dosage of the vitamin and suggest that their results might arise from the organism becoming too efficient in the disposal of ascorbic acid.

Accordingly, it was felt desirable to investigate further the effect of prolonged high dosage with ascorbic acid. One woman and 3 men were each given 1000 mg/day for 3 months. Measurements were made at appropriate intervals of the ascorbic acid concentration in the serum, the concentration in the white blood cells plus platelets, the ascorbic acid tolerance curve, and the urinary output of the vitamin.

Methods and procedure. All analyses were performed at least in duplicate. The white blood cells plus platelets from 0.1 ml of finger blood were analyzed as previously described (3). The serum ascorbic acid was measured using 0.01 ml samples. The original procedure(4) was altered as follows: (a) charcoal was omitted from the precipitating acid; (b) the dinitrophenyl hydrazine reagent was prepared as follows: to a 2.2% dinitrophenyl hydrazine solution in 10 N H_2SO_4 is added 5 volumes of 5% thiourea solution and 5 volume % of 0.6% $CuSO_4 \cdot 5H_2O$ solution. The reagent is satisfactory for use at least a week if held at 4°C. It is prepared from the more stable stock solutions; and (3) the incubation time was increased from 3 to 4 hours at 38°C. The copper, which is easier to use, substitutes for charcoal in converting ascorbic acid to dehydroascorbic acid. This change gives results which average 0.1 mg % higher than with charcoal, a difference of no consequence in the present study. The urine was preserved with 0.1 N acetic acid and analyzed in the same manner as serum except the use of charcoal was retained as it has been found necessary to remove the urinary pigments. A single urine sample analyzed with dichlorophenol indophenol gave a value of 7% less than with dinitrophenyl hydrazine. This may indicate some conversion to dehydroascorbic acid prior to analysis, and suggests an advantage in the use of dinitrophenyl hydrazine which measures both ascorbic and dehydroascorbic acids. The 1000 mg daily supplement of ascorbic acid was administered in 3 divided doses (with meals). The 100 mg pills used were analyzed and found to be within 2% of the stated value.

Results and conclusion. The data for the 4 subjects were so concordant, they have been averaged together and treated as replicates. There is clearly no progressive change in either the serum or white cell level, tolerance curve, or urinary excretion (Table I). The averages obscure the fact that the individual tolerance curves differed a little in shape. However, the individual character of the curves remained essentially unchanged throughout. There seems, therefore, to be no reason to believe that the prolonged high dosage of ascorbic acid had any qualitative or quantitative effect on the manner of disposal of excess acid by the body. Furthermore, in these four

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TABLE I. The Ascorbic Acid of Urine, White Cells, and Serum of 4 Subjects Receiving 1000 mg Ascorbic Acid per Day.

Day of exp.	Urine, mg/day	White cells * mg %	Serum after 400 mg test dose (mg %) 0	1 hr	3 hr	5 hr	7 hr
0		27 ± 2*	1.22 ± .09	1.78 ± .13	1.97 ± .11	1.80 ± .06	1.63 ± .04
5	[illegible] ± 35*	2[illegible] ± 3	1.81 ± .09	2.45 ± .09	2.39 ± .08	2.14 ± .11	1.94 ± .04
21	[illegible] ± [illegible]	3[illegible] ± 1	1.7[illegible] ± .06	2.28 ± .11	2.35 ± .05	2.19 ± .11	2.03 ± .04
39	714 ± 37	2[illegible] ± 1	1.82 ± .13				
55		2[illegible] ± [illegible]	1.54 ± .08				
67		31 ± [illegible]	1.9[illegible] ± .15	2.45 ± .17	2.48 ± .20	2.19 ± .24	2.09 ± .26
98	822 ± 47	2[illegible] ± 1	1.64 ± .1[illegible]	2.24 ± .99	2.14 ± .08	1.83 ± .13	1.76 ± .06

* White blood cells = platelets. * ± Stand. error. ‡ 3rd day of exp. § 43rd day of exp.

persons, at least, no harmful effects whatever were observed during the 3 months with 1000 mg daily intake.

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SOME OBSERVATIONS ON THE EFFECT OF SULFA DRUGS ON BLOOD PLASMA ASCORBIC ACID LEVELS IN CALVES*

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Sulfa drug therapy for the treatment of calfhood diseases has been reported to be quite successful by Herriott (6), Thorp (10), Thorp and Shigley (11), Thorp, Shigley and Anderson (12), Ehlers (4), and Farquharson (5). The success was attributed to the control of bacteria responsible for the disease. Studies at this station (Lundquist and Phillips (7)) have demonstrated a relationship in calves between blood plasma ascorbic acid concentrations and certain active infections of the type reported to be relieved by sulfonamide therapy. This would suggest that subnormal blood plasma ascorbic acid levels would be increased by sulfa drug therapy in such cases. A preliminary observation has indicated this to be true. In fact, the blood plasma ascorbic acid concentration in affected calves returned to normal more quickly when the sulfa drug was given than when ascorbic acid was given alone. The purpose of this paper is to present further observations on the effect of certain sulfonamides on blood plasma ascorbic acid levels. The method of Mindlin and Butler (8) was used in determining the blood plasma ascorbic acid values reported herein.

THE EFFECT OF SUCCINYL SULFATHIAZOLE IN THE NEWBORN

In the present experiment succinyl sulfathiazole was given in capsules to the newborn calf at the rate of two grams per day (50 to 70 mg. per kg. body weight) for a three-day period. It was also given for seven days to a group of calves raised from birth on a vitamin supplemented skim milk ration. This was much below the recommended therapeutic dose of 3.5 to 5.0 grains per pound of body weight. It is apparent from table 1 that the administration of the succinyl sulfathiazole prevented the normal disappearance of blood plasma ascorbic acid and maintained higher concentrations during the first week of life. The higher concentration of ascorbic acid in the blood stream in those calves fed the skim milk ration was due to the administration of one-fourth gram of ascorbic acid daily. The difference in blood plasma ascorbic acid concentration between the skim milk and the whole milk fed calves under the influence of the drug was distinctly in favor of the former.

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THE EFFECT OF CERTAIN SULFA DRUGS IN HEIFERS

The effect of succinyl sulfathiazole, sulfapyridine, and sulfathiazole on blood plasma ascorbic acid was studied in Holstein heifers. These heifers were eight to nine months of age and had an average weight of 445 pounds. Succinyl sulfathiazole and sulfathiazole was given at the rate of 2, 10, and 15 grams (approximately 10, 50 and 75 mg. per kg. respectively) daily. Sulfapyridine was administered at the rate of 10 and 15 grams (approximately 50 and 75 mg. per kg. respectively) daily. Eight heifers were used for the 2-gram dosages, and two heifers were used for each of the other levels fed, with the exception of the 15-gram dose of sulfapyridine, when it became necessary to use only one heifer. Two heifers were kept as controls and given no treatment. They were used to observe daily blood plasma

TABLE 1

The effect of succinyl sulfathiazole on blood plasma ascorbic acid of the newborn calf (mg./100 ml.)

Age in days	Skim milk* diet from birth				Colostrum—whole milk diet			
	Control (no drug)		2 gm./day for 7 days		Control (no drug)		2 gm./day for 3 days	
	No. cases	Mg. % C	No. cases	Mg. % C	No. cases	Mg. % C	No. cases	Mg. % C
Birth	6	0.99	10	0.81	3	0.86	5	0.96
1	5	0.67	10	0.79	5	0.53	5	0.61
2	6	0.55	9	0.75	5	0.46	5	0.53
3	.....	.....	.....	.....	5	0.41	5	0.44
4	6	0.44	9	0.47	5	0.42	5	0.47
5	.....	.....	.....	.....	5	0.43	5	0.55
6	5	0.37	9	0.43	5	0.45	5	0.52
7	.....	.....	.....	.....	4	0.39	4	0.51
8	3	0.43	6	0.38	.....	.....	.....	.....
14	2	0.27	6	0.24	5	0.34	5	0.31

* Supplemented with 250 mg. ascorbic acid daily for 10 days.

ascorbic acid variations. The 2-gram dose was given for a period of three days, and the higher doses were given for seven days.

Succinyl sulfathiazole did not appear to influence blood plasma ascorbic acid concentrations at the dosages fed (table 2). Variations which occurred do not indicate an effect which can be attributed to the sulfonamide, since their range was only slightly greater than that of the control animals. These variations are considered to represent normal daily fluctuations and present a much narrower range than that reported by Bortree, Huffman and Duncan (1).

Sulfapyridine, when given at the rate of 10 and 15 grams daily, did not increase the blood plasma ascorbic acid above that usually found in heifers of this age. The blood plasma ascorbic acid in the two heifers used in this part of the experiment averaged 0.53–0.56 mg. per 100 ml. for the first eleven

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days. At this point it became necessary to continue with only one of the two heifers. When she was given 15 grams daily of sulfapyridine, there was an increase of from 0.46 mg. per 100 ml. on the first day to an average for the week of 0.53 mg. per 100 ml. in blood plasma ascorbic acid level. Following the administration of the drug, the blood plasma ascorbic acid concentration returned to an average of 0.45 mg. per 100 ml.

TABLE 2

The effect of certain sulfonamides on the blood plasma ascorbic acid in Holstein heifers (average values in mg./100 ml.)

Before and after treatment	Controls (no treatment)	Succinyl sulfathiazole			Sulfapyridine		Sulfathiazole		
		Daily dose			Daily dose		Daily dose		
		2 gm.	10 gm.	15 gm.	10 gm.	15 gm.	2 gm.	10 gm.	15 gm.
	Number of animals per lot								
	2	8	2	2	2	1	7	2	2
days†									
− 3	.....	.....	.....	0.60	.....	0.60	0.59	.....	0.57
− 2	0.57	.....	.....	0.64	.....	0.66	0.58	.....	0.60
− 1	0.61	.....	0.51	0.58	0.37	0.57	0.59	0.55	0.58
0	0.59	0.65	0.53	0.65	0.46	0.46	0.51	0.52	0.60
+ 1	0.52	0.62	0.57	0.67	0.55	0.46	0.61	0.57	0.56
+ 2	0.55	0.60	0.57	0.59	0.52	0.50	0.55	0.62	0.64
+ 3	0.56	0.56	0.62	0.61	0.55	0.51	0.61*	0.55	0.58
+ 4	0.59	0.52	0.66	0.57	0.61	.....	.....	0.66	0.66
+ 5	0.51	0.50	0.59	0.65	0.51	0.54	.....	0.68	0.72
+ 6	0.58	0.52	0.54	.....	0.47	0.62	.....	0.64	0.73
+ 7	0.57	.....	0.63*	0.65*	0.52*	0.55*	.....	0.64*	0.75*
+ 8	.....	.....	0.63	0.64	0.55	0.43	.....	0.59	0.72
+ 9	.....	.....	0.66	0.65	0.53	0.49	.....	0.66	0.63
+ 10	.....	.....	0.66	0.60	0.59	0.48	.....	0.51	0.64
+ 11	.....	.....	0.63	0.56	0.58	0.46	.....	0.56	0.63
+ 12	.....	.....	0.57	0.65	.....	0.42	.....	0.51	0.59
+ 13	.....	.....	0.68	0.55	.....	0.45	.....	0.60	0.50
+ 14	.....	.....	0.60	0.60	.....	0.43	.....	.....	0.53

† − before treatment, + after treatment.
* Drug discontinued.

Sulfathiazole had no effect when 2.0 grams daily was given, and only slightly increased blood plasma ascorbic acid levels during the last four days of the feeding period when 10 grams daily was fed. When 15 grams daily was given, the ascorbic acid in the blood plasma rose from an average of 0.59 mg. per 100 ml. to 0.75 mg. per 100 ml. in the course of one week. This is a significant increase and indicates a decided stimulatory effect. During this same seven-day period the blood plasma ascorbic acid concentrations in the two control calves remained quite constant, averaging 0.55 mg. per 100 ml. with a range of from 0.51 mg. per 100 ml. to 0.59 mg. per 100 ml. The blood level in those calves receiving the drug declined to normal levels within five days after it was discontinued, indicating little or no residual effect.

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THE EFFECT OF CHLOROBUTANOL FOLLOWING SULFA DRUG ADMINISTRATION

The results obtained with sulfathiazole suggested an action somewhat similar to that of chlorobutanol. This latter drug has been demonstrated to increase blood plasma ascorbic acid in the bovine by Bortree, Scheidenhelm and Huffman (3) and Bortree, Huffman and Duncan (2). For this reason four of the heifers were selected on the basis of previous solfonamide treatment and given 3 grams daily of chlorobutanol for two weeks. Calf No. 1 had no previous treatment, No. 2 had been given 15 grams succinyl sulfathiazole for one week just previous to the initiation of the chlorobutanol feeding, Nos. 3 and 4 had previously received 10 grams sulfathiazole daily for seven days. The chlorobutanol administration of these latter two heifers was initiated on the sixth day after the completion of the sulfathiazole feeding period. Heifers 1, 3 and 4 were given the chlorobutanol for a two-week period while No. 2 received it for only a one-week period.

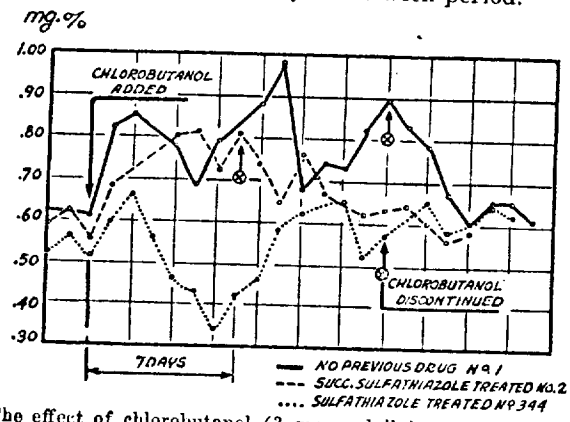

FIG. 1. The effect of chlorobutanol (3 grams daily) on the blood plasma ascorbic acid following sulfa drug administration.

Chlorobutanol materially increased the blood plasma ascorbic acid levels in the heifer which had not previously received any drug treatment (Fig. 1). This increase was maintained throughout the two-week chlorobutanol feeding period, after which it returned to normal levels. The average blood plasma ascorbic acid concentration before the start of chlorobutanol feeding was 0.62 mg. per 100 ml., while the average during the course of this feeding period was 0.79 mg. per 100 ml. with a variation of from 0.68 to 0.98 mg. per 100 ml. The heifer which had previously received succinyl sulfathiazole (No. 2) without showing an effect on blood plasma ascorbic acid levels averaged 0.59 mg. per 100 ml. before chlorobutanol feeding and during the feeding period 0.79 mg. per 100 ml., with a range of from 0.68 to 0.81 mg. per 100 ml.

The blood plasma levels of calves 3 and 4, previously given sulfathiazole, present a strikingly different picture. Results indicate a decidedly depress-

ing effect (Fig. 1). Instead of any significant increase the ascorbic acid levels decreased from an average of 0.53 mg. to 0.34 mg. per 100 ml. in six days. The decrease was accompanied by a temporary loss in appetite. This decrease was followed by an increase which reached normal on the ninth day. The maximum blood plasma ascorbic acid level attained was 0.65 mg. per 100 ml.

DISCUSSION

Although the 2-gram and 15-gram doses of succinyl sulfathiazole, given to the newborn calf and to older heifers respectively, represent only about 10 per cent of the therapeutic dose (Thorp (10)), the results seem to demonstrate an effect which differs as the animal matures. The maintenance of somewhat higher blood plasma ascorbic acid concentrations during the first few days of life in calves receiving the drug, as compared to the controls, may be due to the control of the coliform organisms of the gastro-intestinal tract; or it may exert its influence through the thyroid gland, since Schochner (9) has shown that certain of the sulfa drugs inhibit the production of thyroxine by this gland.

The results obtained when sulfapyridine was fed to the heifers are difficult to interpret. The calves involved in this part of the experiment showed some variation in their blood plasma ascorbic acid levels previous to the initiation of treatment. However, it does appear that the 10-gram daily dose of sulfapyridine produced an increase in blood plasma ascorbic acid when it was below normal but did not raise this level above normal. The maintenance of normal levels following the drug administration may have been due to a residual effect of the drug. However, normal levels were not maintained after treatment with 15 grams daily. Thorp *et al.* (12) and Thorp (10), in using sulfapyridine for treatment of calf pneumonia, have pointed out that toxic symptoms are apt to develop when blood concentrations of free drug exceed 10 mg. per 100 ml. They also point out that for most effective results the amount of drug should be decreased 0.3 grain per pound body weight every second day from the initial therapeutic dose of 0.7 to 1.0 grain per pound body weight. They also present some evidence to show that the drug may be cumulative in the body. In this experiment the dosage was maintained constant; and although it was less than recommended for a therapeutic dose to begin with, it was considerably above recommended dosage before its administration was discontinued. The possibility of toxicity may have been present, although no visible evidence of it was observed. Blood levels of the drug were not determined, nor was the animal sacrificed for autopsy.

Sulfathiazole definitely effected an increase in blood plasma ascorbic acid when given at the rate of 15 grams daily for seven days. This was not evident at the 2-gram daily dose, and only slightly apparent at the 10-gram daily dose. Since it has been recommended that the dosage of sulfathiazole

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be reduced after the third day, as in the case with sulfapyridine, the 15-gram daily dose which we fed does exceed the therapeutic dose only on the last two days of the seven-day feeding period. In this latter case the dosage may have approached a level that was toxic. However, it is clear that the rise in blood plasma ascorbic acid is gradual throughout the seven-day experimental period and indicates an effect when the dose could not have been considered toxic. This increase appeared to be somewhat similar to that found by other workers when chlorobutanol was fed.

It is interesting to note the relation which succinyl sulfathiazole and sulfathiazole have to the effects of chlorobutanol feeding on the blood plasma ascorbic acid level. The succinyl sulfathiazole had no measurable effect on the chlorobutanol-ascorbic acid response. The sulfathiazole apparently interfered with the chlorobutanol-ascorbic acid mechanism or vice versa, since either one used alone stimulated an increased blood plasma ascorbic acid level above normal, but caused a reduction when given one following the other, even though there was a lapse of six days between the end of sulfathiazole administration and the beginning of the chlorobutanol feeding.

SUMMARY

The results of these experiments indicate that succinyl sulfathiazole, when fed at the low level of 2.0 grams per day to the newborn calf, was responsible for higher blood plasma ascorbic acid levels during the first week of life than those in untreated calves. No effect was noted when this drug was given at levels of 2, 10, or 15 grams daily for seven days to 8–9-month-old heifers.

Sulfapyridine, at 10- or 15-gram daily dose, did not appear to effect an increase in blood plasma ascorbic acid above normal. However, administration of the drug at the rate of 10 grams daily for seven days apparently increased subnormal levels up to, but not above, normal levels.

Sulfathiazole, when fed at the rate of 15 grams daily, definitely stimulated an increase in blood plasma ascorbic acid similar to that of chlorobutanol, but different in that it required a longer period to reach peak levels.

Chlorobutanol, fed at the rate of 3 grams daily for seven days, increased blood plasma ascorbic acid levels. This chlorobutanol-ascorbic acid response was not interfered with by the previous use of succinyl sulfathiazole; but a marked reduction in ascorbic acid concentration occurred when the chlorobutanol feeding period was preceded by the feeding of sulfathiazole, even though there was an interval of six days between these two feeding periods.

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Effect of Ascorbic Acid in Aniline Poisoning

ASCORBIC acid has been utilized extensively in large doses in the therapy of industrial methæmoglobinæmia, although Halsted[1] commented that at developments greater than 60 per cent methæmoglobinæmia, it is ineffective because of its lack of ability to bring on rapidly a reversion mechanism. But the rapidity of reversion is only the first problem. The second problem to be settled is whether, in chemical cyanosis, the effect of ascorbic acid may not be restricted only to bring about the reduction. It is known that ascorbic acid promotes the rate of hydroxylation of aniline to *p*-aminophenol[2], which is a potent agent in the production of Heinz bodies not only *in vivo* but also *in vitro*[3], and ascorbic acid is able to produce Heinz bodies *in vitro* without any toxic agents[4]. We have found that in rats poisoned with aniline the formation of Heinz bodies is promoted by treatment with ascorbic acid.

Twenty male white rats each weighing 270–320 gm. were used. All animals received intraperitoneal injections of 1 per cent aniline (0·08 ml. aniline/kgm.), and 250 gm. ascorbic acid (in 10 per cent buffered solution) was administered to 10 of them by the tail vein immediately before injecting aniline. The methæmoglobin concentration was determined as a percentage of total hæmoglobin by means of Evelyn and Malloy's method[5] modified by Magos[6] at 1, 3, 5, 7, 24 and 48 hr. after the treatment. Proportions of Heinz body red cells were determined at 48 hr. in wet preparations stained with methyl violet. Not only was the percentage of Heinz body red cells determined, but also the intensity of Heinz body formation by means of an index. 250 red blood cells were carefully examined and the intensity of Heinz body formation in a single cell was signed with index numbers as follows: no Heinz body, 0; one small Heinz body, 1; two small Heinz bodies or of one moderate size, 2; two moderate Heinz bodies or one conglomerate, 4. In the following equation n is the number of cells in the group.

$$\text{Heinz body index} = \frac{(1 \times n_1) + (2 \times n_2) + (4 \times n_4)}{n_0 + n_1 + n_2 + n_4}$$

On the basis of the results given in Table 1, it seems that, although ascorbic acid decreases the degree of maximal methæmoglobinæmia (the period of the methæmoglobinæmic phase remained unchanged), its effect on intensifying Heinz body formation is much more significant.

Table 1. MAXIMAL METHÆMOGLOBINÆMIA, HEINZ BODY RED CELLS (H.B.R.C.) AND HEINZ BODY INDEX (H.B.I.) IN ANILINE POISONING WITH OR WITHOUT ASCORBIC ACID TREATMENT

	Treatment				
	Aniline	Aniline ascorbic acid	S_D	t	P
Maximal methæmoglobinæmia	26·5 ± 4·27	22·7 ± 3·06	1·68	2·262	$0·05 > P > 0·02$
H.B.R.C.	46·5 ± 10·4	65·0 ± 11·4	4·88	3·813	$0·01 > P > 0·001$
H.B.I.	1·34 ± 0·54	2·23 ± 0·37	0·210	4·333	$0·001 > P$

These findings indicate that, apart from the lack of ability to bring about rapidly the methæmoglobin reduction, it is advisable to avoid the therapeutic use of ascorbic acid in chemical cyanosis because of its irreversible effect.

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Plasma Ascorbic Acid as an index of Vitamin C Nutrition

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ABSTRACT:

The study of plasma ascorbic acid levels in 500 children (100 healthy and 400 ill) showed that vitamin C subnutrition (ascorbic acid level below 1.0 mg%), due to an inadequacy of the diet or in its absorption was present in 94% of the healthy normal and 98.5% of the children. The wide overlap for plasma ascorbic acid levels in these two groups has been one conspicuous feature. Only 6% of the healthy children had plasma ascorbic acid above 1 mg% and 18% between 0.7 to 1.0 mg%. Amongst the ill children, the results in malnourished children showed that 5.8% had values between 0.7 to 1.0 mg% as against 13.2 and 11.2% respectively in cases with infections and gastrointestinal disorders.

It is concluded that plasma ascorbic acid, though not a measure of tissue saturation with vitamin C is, however, an important nutritive index as its value depends upon dietary intake. This study stresses the high prevalence of inadequate vitamin C intake, either primary due to lack in the diet or conditioned because of poor absorption or greater demands.

INTRODUCTION

Plasma ascorbic acid estimation has been employed extensively for surveying the extent and also the degree of vitamin C malnutrition.[1,2,3] The literature on the subject has grown very extensive with many sophisticated tests like "leading" or "saturation"[4,5,6,7] tests as well as simpler tests, as for example, intradermal test of Rotter[8,9] and capillary fragility test.[10,11] However, the large number of tests for assessing the vitamin C nutrition by themselves are an indirect indictment of their inadequacy.

The present study of plasma vitamin C levels in the healthy and ill children is an attempt to know the extent and degree of vitamin C malnutrition and to assess this method as an index of vitamin C nutrition.

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MATERIAL & METHODS

Five hundred children, both healthy and those suffering from various illnesses, with ages varying from one month to fourteen years, were categorised as follows:

Group 1	Apparently healthy children	100
Group 2	Malnutrition	97
	Scurvy	11
	Marasmus	68
	Kwashiorkor	11
	Rickets	7
Group 3	Infections	205
	Acute	175
	Chronic	30
Group 4	Gastrointestinal disorders	98
	Total	500

The method employed for the determination of ascorbic acid was the titration of 2:6 dichlorophenol indophenol in acid solution.[12]

OBSERVATIONS

Plasma ascorbic acid values in 500 cases found in various group are given in table I.

TABLE I. *Plasma Ascorbic acid levels in 100 normal and 400 ill children.*

Clinical group	Ascorbic acid level mg%										Total
	Above 1		0.7—1.0		0.4—0.7		0.2—0.4		Less 0.2		
	No.	%	No.	%	No.	%	No.	%	No.	%	
1. Normal	6	6.0	18	18.0	69	69.0	7	7.0	—	—	100
2. Scurvy	—	—	1	9.1	2	18.2	4	36.4	4	36.3	11
3. Malnutrition	—	—	5	5.8	62	72.1	19	22.1	—	—	86
A. Marasmus	—	—	3	4.5	46	67.6	19	27.9	—	—	68
B. Kwashiorkor	—	—	2	18.2	9	81.8	—	—	—	—	11
C. Rickets	—	—	—	—	7	100.0	—	—	—	—	7
4. Infections	3	1.4	27	13.2	133	64.9	42	20.5	—	—	205
A. Acute	3	1.7	26	14.8	114	65.1	32	18.4	—	—	175
B. Chronic	—	—	1	3.3	19	63.3	10	33.4	—	—	30
5. G.I. Disorders	—	—	11	11.2	72	73.5	15	15.3	—	—	98
Total	9	1.8	62	12.4	338	67.6	87	17.4	4	0.8	500

TABLE II. *Plasma ascorbic acid levels in 100 normal, 86 malnourished and 11 scorbutic children according to age.*

Age in months	Clinical Group	Ascorbic acid level mg%										Total
		Above 1.0		0.7—1.0		0.4—0.7		0·2—0.4		Less 0.2		
		No.	%	No.	%	No.	%	No.	%	No.	%	
Less Six	Normal	—	—	2	50.0	2	50.0	—	—	—	—	4
	Malnut.	—	—	—	—	8	80.0	2	20.0	—	—	10
	Scurvy	—	—	—	—	—	—	—	—	—	—	—
	Total	—	—	2	14.3	10	71.4	2	14.3	—	—	14
6-12	Normal	1	12.5	2	25.0	5	62.5	—	—	—	—	8
	Malnut.	—	—	2	14.3	5	35.7	7	50.0	—	—	14
	Scurvy	—	—	—	—	1	50.0	1	50.0	—	—	2
	Total	1	4.2	4	16.6	11	45.8	8	33.4	—	—	24
12-24	Normal	—	—	1	12.5	6	75.0	1	12.5	—	—	8
	Malnut.	—	—	2	11.8	13	76.5	2	11.7	—	—	17
	Scurvy	—	—	—	—	1	50.0	1	50.0	—	—	2
	Total	—	—	3	11.1	20	74.1	4	14.8	—	—	27
24-36	Normal	3	7.3	7	17.1	28	68.3	3	7.3	—	—	41
	Malnut.	—	—	1	4.2	18	75.0	5	20.8	—	—	24
	Scurvy	—	—	1	14.3	—	—	2	28.6	4	57.1	7
	Total	3	4.2	9	12.5	46	63.9	10	13.9	4	5.5	72
Above 60	Normal	2	5.1	6	15.4	28	71.7	3	7.8	—	—	39
	Malnut	—	—	—	—	18	85.7	3	14.3	—	—	21
	Scurvy	—	—	—	—	—	—	—	—	—	—	—
	Total	2	3.3	6	10.0	46	76.7	6	10.0	—	—	60
	Total	6	3.0	24	12.2	133	67.5	30	15.2	4	2.1	197

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Amongst the 100 normal children, none had plasma ascorbic acid below 0.2 mg%, 7 had concentration ranging from 0.2 to 0.4 mg% and 69% had levels ranging from 0.4 to 0.7 mg%. In 6, the values were above 1 mg%. Of the 11 cases of scurvy, 8 (72.7%) had values below 0.4 mg%, including 4 (36.3%) with levels below 0.2%. Two (18.2%) had levels between 0.4 to 0.7 mg% and in 1, the value was as high as 0.8 mg% but the possibility of technical error or else recent vitamin C intake in this case could not be excluded.

Amongst the 86 with malnutrition, there was none with value above 1 mg% and only (5.8%) had values from 0.7 to 1.0 mg%. The majority—81. (94.3%), had values below 0.7 mg%, 62 (72.1%) from 0.4 to 0.7 mg% and 19 (22.1%) between 0.2 to 0.4 mg%.

The general pattern of results in 205 with infections was similar to that seen in malnutrition. Thus, 153 (85.4%) had plasma ascorbic acid values below 0.7 mg%, 42 (20.5%) below 0.4 mg%. Values were, in general, lower in chronic infections as compared to acute cases as well as maluntrition. Unlike the ascorbutic cases, in both malnutrition and the infection groups (291 cases), there was none with levels less than 0.2 mg%.

Discussion

The extent of prevalent vitamin C malnutrition remains to be chartered. In spite of plethora of laboratory tests,[13] none is however specific in detecting early deficiency[14] As stated by Eddy and Dalldorf[15] "We are surfeited with tests for ascorbic acid deficiency but still hungry to know what deductions can safely be drawn from them." For everyday clinical practice, the fasting plasma ascorbic acid level or ascorbic acid content of the buffy layer is generally used. Various arbitrary levels of plasma ascorbic acid are taken as indices of the status of vitamin C nutrition. Thus, according to some observers values of 0.7 to 1.0 mg% indicate mild deficiency and those between 0.5 to 0.7 mg% indicate state of vitamin C depletion.[17,18] In the well fed infants, the values usually fall above 1.0%.[19,20] Adopting these as measure of the degree of vitamin C malnutrition, the results of the present study in the normals (100) and the ill children (400) are compared below:

	Plasma ascorbic acid mg%			
	0.7 to 1.0	0.5 to 0.7	0.2 to 0.4	Less than 0.2
Normal (100)	18(18.0%)	69 (69.0%)	7 (7.0%)	—
Ill children (400)	44(11.1%)	269 (67.5%)	80 (20.0)	4 (1.0%)

Johnson et al reported that values less than 0.3 mg% were indicative of chemical deficiency.[21] In cases of scurvy,[23,24] values ranging from 0.1 to 0.3 mg% were reported. Vilter[25] et al reported values as low as 0.0 mg% in scurvy, whereas McMillan and Inglis showed the average as 0.05.[26]

There was a considerable overlap of plasma ascorbic levels in the normal and the ill children in this study, except that 21% of the latter had values below 0.4 mg% as compared to only 7% of the healthy children.

Values between 0 to 0.3 mg% have been reported in the absence of scurvy or apparent ill health by various workers.[27,28,29] Low plasma ascorbic acid levels are in no way characteristic of scurvy as these may often be obtained in healthy children. On the other hand, presence of detectable amount of plasma ascorbic acid has been stated to be against the diagnosis of scurvy.[30] If values below 0.3 mg% have been considered indicative of vitamin C chemical deficiency then 5.6% of population falls into this group. In the present study of 11 scorbutic children, only 4 showed values below 0.2 mg%. On the other hand, none amongst the 100 normal, 75 cases of malnutrition (excluding scurvy) and 205 cases of various infections showed such low levels. High fasting plasma values indicate satisfactory nutrition. Low levels were frequently seen in this study and have also been reported in the past. In the present study, out of 175 cases of acute infections 32 (18.2%) had values from 0.2 to 0.4 mg%, 114 (67.1%) from 0.4 to 0.7 and 26 (18.2%) form 0.7 to 1.0 mg%. In thirty cases of chronic infections, the values were even lower as compared to acute infections. A fall in plasma ascorbic acid level by about 50% of the normal has been reported by Faulkner and Taylor.[33] The low plasma ascorbic acid levels in infections, despite decrease in its urinary excretion[31,35,36] were previously attributed to enhanced requirements resulting due to increase metabolic rate from pyrexia of infections. More recently, they are ascribed to result from the concentration of ascorbic acid in the leucocytes as their response to infections.[37]

The low plasma ascorbic acid levels in cases with gastrointestinal disorders follow closely the pattern obtained in malnutrition and infection cases, due either to primary dietary inadequacy of vitamin C in the diet or diminished alimentary absorption during episodes of diarrhoea or else due to both.

Most of laboratory tests are an attempt to study the individual's nutritional status with respect to ascorbic acid. Data on blood or urine estimations do not reflect truly the condition in the tissues. Tissue saturation tests involve the study of ascorbic acid excretion in urine in response to test dose.

This study demonstrates that plasma vitamin C level was less than 1 mg% in a vast majority of apparently healthy children and almost all the ill children. Sixty to 80% of all the cases had levels from 0.5 to 0.7 mg%.

There is a general consensus that ascorbic acid levels reflect the immediate nutritive levels and are thus dependent upon dietary intake.[40,41] Whereas, the low plasma levels may prevail in the healthy children, the fasting plasma ascorbic acid levels of more than 1.0 mg% imply optimal vitamin C nutrition. The study indicates that the dietary intake of vitamin C even in apparently healthy children, is suboptimal, and subclinical deficiency is frequent in malnourished children or children suffering from infections or gastrointestinal disorders.

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EFFECT OF VITAMIN C ON WORKERS EXPOSED TO LEAD DUST

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THE present study deals with a group of 303 regular employees, 80 per cent of whom have been in their present place of occupation for five years, and the balance for a longer period. These men are engaged in various types of work in the assembly of passenger automobiles.

The lead exposure in this work of assembly arises from the practice of covering body seams with solder (60 per cent lead), and grinding off the excess metal to form a flush surface. There is thus thrown into the air a large quantity of lead dust which is of variable particle size, the majority of particles being too large to be inhaled.

In a large series of air samples it was determined by standard methods that there was a variable concentration along the assembly line of inhalable lead dust (10 microns or less in size). This concentration varied from 3 to 15 mg. per 10 cubic meters of air.

The concentrations of dust and the particle size findings coincide with the report of Humperdinck[1] on similar lead hazards in European automobile assembly plants.

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Provision for ventilation consists of overhead and floor level exhaust ducts; and grinders and metal finishers are furnished with daily inspected respirators. Floors are kept clean by wet sweeping.

Since early in 1935 all men who were even remotely in contact with lead dust have been examined physically, and a basophilic aggregation count was made (McCord)[2] once a month, or oftener if complaints arose.

During the period in 1935 in which a wave of hysteria concerning lead poisoning was at its height, there were 340 definite complaints that ranged from abdominal cramps, nausea, and vomiting, to alleged weakness of the extensor muscles of the wrists and forearms. The majority of these illnesses were patently of psychologic origin, but there were 13 men in this group who were hospitalized for further observation and possible treatment for lead intoxication.

Urinalysis for lead, both before and after deleading treatment, failed to show an abnormal excretion of lead in any one of these 13 men; and the basophilic aggregation count, with the exception of three of the group, did not bear out a diagnosis of lead intoxication.

The three men who had a high basophilic aggregation were variously found to have a severe pyorrhea alveolaris and chronic cryptic tonsillitis, chronic appendicitis, and peptic ulcer. When treatment appropriate to these conditions was instituted, the blood picture returned to normal, and the lead poisoning disappeared.

At this time a routine form of medication for those exposed to lead dust was initiated. This treatment consisted of the administration once a week—Monday— of 1 ounce of a saturated solution of magnesium sulfate, to which was added 15 grains of citric acid and sufficient tincture of cudbear to color the mixture; this was given to the men when they obtained their tools, thus insuring that practically all the men would receive medication before starting work. The results of this treatment, judged by the basophilic aggregation count and lack of serious complaints, were good, but were met with strenuous objection from the men.

Beginning in June, 1939, the foregoing medication was discontinued, and in its place each man was given two pieces of gum containing a total of 50 mg. of ascorbic acid. These were distributed about one-half hour after the noon lunch period. The men made no objection to taking the gum, and considered it a treat. Physical examinations and basophilic aggregation counts were continued as previously.

After a short period there was a noticeable decrease in muscular fatigue. This was evident by an increase in efficiency and a feeling of well-being at the end of the work period. The men seemed more cheerful and much less inclined to moroseness.

During the five months from June to December, 1939, the basophilic aggregation count showed a slow but progressive decline. At this time further laboratory studies were undertaken as follows: (a) urinalysis for lead, (b) basophilic aggregation count, and (c) a stipple cell count. These determinations were made each week for seven weeks, covering a period of thirty-five working days of eight hours each.

During this test period production was at its peak, humidity was high, and there was a slow circulation of air due to the low temperature of the outside air.

Urine was collected at different periods of the working day to obtain a fair average of lead excretion. A total of 1,996 analyses for lead were made, or an average of 6.5 analyses per man.

The results of the lead urinalysis are shown in Table I.

TABLE I

NO. OF MEN	PER CENT	AVERAGE OF URINARY LEAD EXCRETION PER LITER (γ)
99	32.6	39.5
96	31.6	61.5
48	15.8	86.2
24	7.9	110.4
14	4.6	134.5
10	3.3	160.9
5	1.6	189.6
3	0.9	219.3
1	0.3	265.0
2	0.6	405.0
1	0.3	541.0

Those below 100 γ per liter, 243 men, or 80.2 per cent, averaged 62.4 γ per liter.
Those above 100 γ per liter, 60 men, or 19.8 per cent, averaged 157 γ per liter.
The average over-all excretion of 303 men equaled 77 γ per liter.

In this series was one man who excreted an average of 541 γ per liter. Further questioning revealed that he had had acute lead intoxication several years previously. He had not worked with lead for eight years, and was at least two city blocks away from any lead; therefore, his exposure has been nil. Under the influence of vitamin C, we are evidently releasing the fixed lead from his bones and he is excreting it without showing any clinical symptoms of lead intoxication.

The workers who showed an average of more than 100 γ per liter of urine—60 men—were studied further. The urine and blood counts were continued at fortnightly intervals. At the end of April, 1940, the lead excreted in the urine has shown no decline, and the basophilic aggregation and stipple cell counts have remained within the normal range.

DISCUSSION

From the foregoing it is apparent that this study confirms the contention of Holmes[3] that vitamin C (ascorbic acid) has a detoxifying action on lead in the human body. However, we cannot agree that the urinary lead excretion is decreased by the administration of vitamin C—since our findings show a continued high level of excretion over an observed period of five months in those workers continuously exposed to a high concentration of lead dust.

It is difficult to reconcile our findings with those of Shiels,[4] and Blumberg and Scott[5]; i.e., that there are definite levels of concentrations for lead, both in urine and blood, above which levels, one finds lead poisoning. Judged by the criteria of blood cell counts, physical condition, and lack of symptoms usually associated with chronic lead absorption, the subjects of this study may be said to show no evidence of lead intoxication whatever. Fairhall[6] has stated that a high urinary excretion of lead is not of itself evidence of lead intoxication, but rather of lead absorption only.

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We have found no evidence that the storage of lead is promoted by vitamin C. Rather the reverse is true, as shown by the workman with the highest level of excretion in the group. As stated, this man has been previously exposed and has been treated for lead intoxication.

Further investigation in the deleading properties of vitamin C are being undertaken.

It seems possible that the effect of lead on the human economy may be only indirectly toxic, the absorption of lead producing an avitaminosis C. This is the condition of vague symptoms known as chronic lead poisoning, but is in reality chronic subclinical scurvy.

CONCLUSIONS

1. The routine administration of 50 mg. of ascorbic acid daily appears to protect workers exposed to lead dust against the usual effects of chronic lead absorption.

2. The rate of urinary excretion of lead in workers continually exposed to lead dust is not decreased by the routine daily administration of 50 mg. of ascorbic acid.

I wish to express my appreciation to Dr. Milton H. Kronenburg, Chief of Division, and K. M. Morse, Engineer, of the Illinois Industrial Hygiene Bureau, for the air-borne dust determinations; and to Doctors E. K. Nielsen and H. A. Cranston for the determinations of urinary lead.

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THE VANDERBILT COOPERATIVE STUDY OF MATERNAL AND INFANT NUTRITION [1]

X. ASCORBIC ACID

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INTRODUCTION

The basic findings of the Vanderbilt cooperative study of maternal and infant nutrition have been reported (Darby et al., '53a, b; McGanity et al., '54), but little of the detail of the interrelationships of various factors was included. Analyses of the interrelationships of biochemical and dietary data in large groups of persons are rare. Such analyses are necessary, however, to the soundest interpretation of nutritional data. Accordingly, we have studied each of several nutrient groups separately and explored numerous possible relations. This paper deals with the dietary intake and the serum levels of ascorbic acid, the variation in each of these with time of gestation, season of the year, and with age, parity, height, weight, and physical findings of the mother. The variation in serum vitamin C with intake levels and the

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relation of both intake and serum levels to the course and outcome of pregnancy and to lactation are also explored. The data are for 2,129 consecutively encountered pregnant women, the outcome of the pregnancy being observed during the course of the investigation. The methods employed have been described in the earlier reports.

OBSERVATIONS AND DISCUSSION

Intake levels. The distribution of recorded intakes of vitamin C is given in table 1. This is based on a one-week diet record obtained from each patient once during each trimester of pregnancy. A wide range in reported intake is apparent, and there occurs a slight overall decrease in intake level from the first to the third trimester.

Median consumption in the study group was lower than the allowances recommended by the Food and Nutrition Board ('53) of 70 mg per day for adult women and 100 mg per day during the third trimester of pregnancy. Of those who had diet records for both the second and third trimesters, 14% reported daily intakes of less than 40 mg of vitamin C on both occasions, and 2% reported intakes of less than 20 mg on both records. Only 15% had intakes of 80 mg or more on both records.

Serum levels. As previously reported (Darby et al., '53b), serum vitamin C levels declined during pregnancy and were lowest at the time of the postpartum examination. Distributions are shown in table 1. Postpartum values were clearly lower for lactating than for non-lactating women, but a corresponding difference between these groups was not observed during pregnancy. In other words, the low values of serum ascorbic acid which are associated with lactation appear to be a reflection of lactation rather than the result of a pre-existing difference between those who lactated and those who did not.

Serum vitamin C levels during pregnancy and lactation have been studied by many investigators (Ingalls et al., '38;

TABLE 1

Distribution of dietary intakes and serum levels of vitamin C by period of the reproductive cycle

INTAKE				SERUM LEVELS					
Intake of vitamin C	First trimester	Second trimester	Third trimester	Serum vitamin C	First trimester	Second trimester	Third trimester	Postpartum [1]	
								Lactating	Non-lactating
mg/day	*Number of women*			*mg/100 ml*	*Number of women*				
0– 19	12	76	174	Under 0.20	72	315	638	510	235
20– 39	49	205	374	0.20–0.39	80	289	458	158	179
40– 59	59	308	392	0.40–0.59	53	236	316	67	84
60– 79	61	244	308	0.60–0.79	56	217	235	51	58
80– 99	34	174	180	0.80–0.99	32	146	163	24	49
100–119	33	91	104	1.00–1.19	23	95	89	13	22
120–139	13	69	68	1.20–1.39	17	48	41	2	7
140–159	9	27	24	1.40–1.59	1	17	12	1	2
160–179	5	14	15	1.60–1.79	3	3	5	1	1
180–199	2	4	10	1.80–1.99	1	1	2		
200 and over	1	9	16	2.00 and over		2	1		
Total subjects	278	1221	1665	Total subjects	338	1369	1960	827	637
Median intake (mg/day)	66	62	54	Median (mg/100 ml)	0.46	0.47	0.35	0.16	0.29

[1] Postpartum data do not include women who had abortions or twins.

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Teel et al., '38; Snelling and Jackson, '39; Lund and Kimble, '43; Anderson et al., '46; Young et al., '46; Munks et al., '47; Hoch and Marrack, '48; and Moyer et al., '54). The present analysis considers the influence of both intake levels and period of gestation on serum levels and hence offers an opportunity to extend our interpretation of such data.

Seasonal pattern. Seasonal variation occurred in both the intake and the serum vitamin C content (figs. 1, 2). The months of lowest recorded intake during the second trimester were January, February, June, July and September, and in the third trimester June, July, August, September and October. Serum vitamin C levels were lowest in the period of February through June. In other words, the season (July-September) of highest blood levels was a period when recorded intakes were relatively low. This inconsistency will be considered again in a later section. We have no explanation as to why the variation in intake with season was not identical for the second and third trimesters.

Correlation of values obtained for the same patient in different trimesters. In nutrition surveys or in the clinical assessment of nutriture the use of a one-week diet record or of a single laboratory determination is based on the assumption that each of these gives a reasonably reliable measure of the intake or serum level for the period in question. If the values vary greatly from one time to another, then a single record is obviously inadequate. It is of interest, therefore, to compare the records of the same patient in different trimesters in order to observe the constancy of findings over a time interval.

There is a moderate degree of correlation between the recorded vitamin C intake of the same individuals in different trimesters, as well as between serum vitamin C levels taken at different times. Tables 2 and 3 typify the degree of these correlations. Although there were some individuals having high values at one time and low values at another, the majority of cases showed reasonably good agreement. However, because of the large differences observed in some cases, it seemed

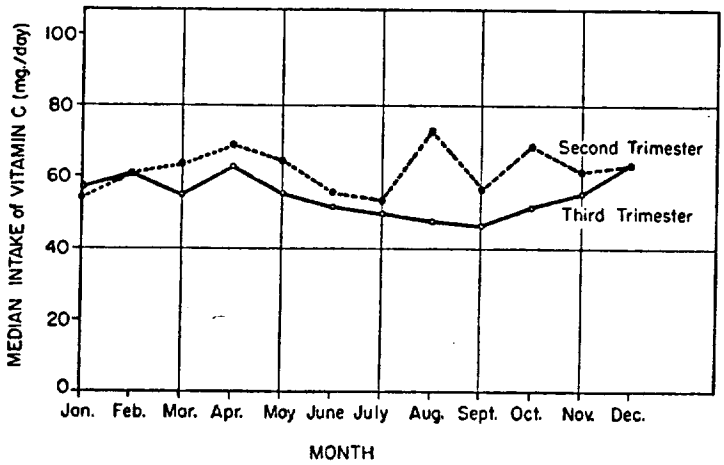

Fig. 1 Median dietary intake of vitamin C by month.

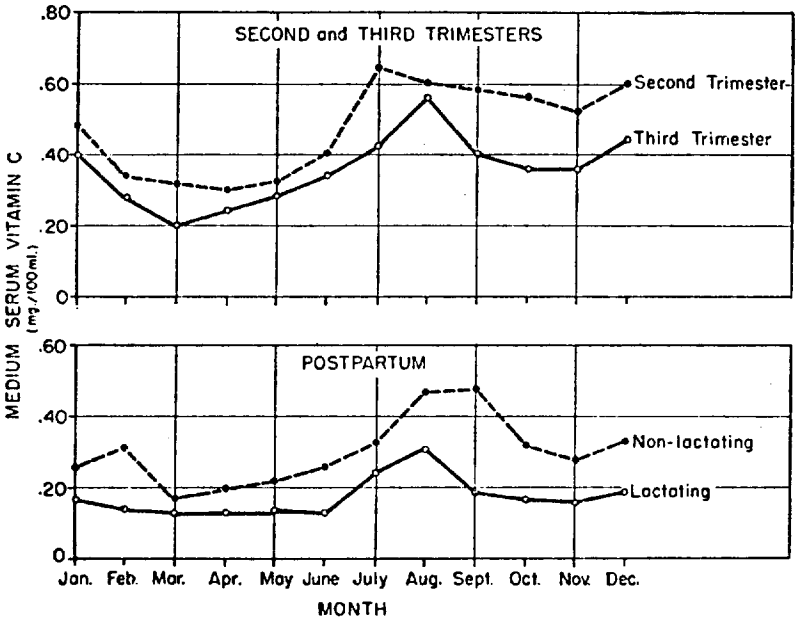

Fig. 2 Median serum levels of vitamin C by month.

likely that a comparison between those individuals who had either low or high values on each of two occasions might yield sharper contrasts than comparison made on the basis of a single determination. Consequently, in the latter part of

TABLE 2

Relation of dietary intakes of vitamin C in second and third trimesters

(Winter and spring seasons)

		SECOND TRIMESTER INTAKE (MG/DAY) (WINTER SEASON)							
		Under 20	20–39	40–59	60–79	80–99	100 and over	Total number patients	Median
		Number of cases							
THIRD TRIMESTER INTAKE (MG/DAY) (SPRING SEASON)	Under 20	3	6	8	3	2		22	45
	20–39	6	16	16	11	4	3	56	48
	40–59	2	8	12	11	12	6	51	66
	60–79	2	3	10	6	10	9	40	77
	80–99	2	2	6	3	6	6	25	77
	100 and over		1	6	7	6	16	36	93
	Total no. patients	15	36	58	41	40	40	230	63
	Median	35	35	48	52	64	87	55	

TABLE 3

Relation of serum levels of vitamin C in second and third trimesters

(Winter and spring seasons)

		SECOND TRIMESTER LEVELS OF SERUM VITAMIN C (MG/100 ML) (WINTER SEASON)						
		Under 0.20	0.20–0.39	0.40–0.59	0.60–0.79	0.80 and over	Total number patients	Median
		Number of cases						
THIRD TRIMESTER LEVELS OF SERUM VITAMIN C (MG/100 ML) (SPRING SEASON)	Under 0.20	51	27	12	8	8	106	0.21
	0.20–0.39	10	22	17	6	9	64	0.40
	0.40–0.59	10	9	12	8	8	47	0.48
	0.60–0.79	3	3	4	7	4	21	0.61
	0.80 and over	3	3	4	5	26	41	0.96
	Total no. patients	77	64	49	34	55	279	0.40
	Median	0.15	0.25	0.35	0.48	0.72	0.30	

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this paper groups having "consistenly" low, medium or high intakes or serum levels of vitamin C are reported.

Correlation of serum vitamin C with vitamin C intake. Serum levels of vitamin C vary with recorded intake, but the degree of correlation is not high (table 4). However, there were distinct differences in average values of serum vitamin C for groups at different intake levels. In each trimester daily intakes were grouped as follows: less than 40 mg, 40 to 79 mg, 80 to 119 mg, and 120 mg and over. Median serum vitamin C values were determined for each intake group, postpartum values being studied in relation to third trimester intakes. Cases were limited to those for whom the interval between the diet record and the serum determination was not more than three weeks (except for the postpartum group), and subdivision of the data by season (on the basis of laboratory values) was made as follows: winter (January through March), spring (April through June), summer (July through September), and fall (October through December). Table 5 gives median serum vitamin C values for the third trimester. Data for second trimester and postpartum show similar differences. Median serum levels increased with increasing intake. In addition, clear seasonal differences in the levels in the blood for similar recorded intakes were present, except possibly in the first trimester. For a given nutrient level, serum vitamin C values were highest in summer followed by fall, winter and spring, in that order. These seasonal differences were statistically significant except those between winter and spring. This same phenomenon was reflected in the difference in seasonal patterns between nutrient and serum vitamin C levels noted above. Among the influences which may contribute to these seasonal inconsistencies are seasonal variation in the vitamin C content of some foods (for which no allowance was attempted in calculations), and seasonal shifts in the composition of the intake groups. It is also possible that there is seasonal variation in vitamin C requirements, or in certain metabolic processes which affect serum levels of vitamin C.

Correlation of serum vitamin C with other laboratory determinations. Serum vitamin C levels were positively correlated with serum carotene levels and to a slight extent with serum vitamin A levels.[2] Intakes of vitamin C and carotene were also correlated. This is not unexpected, inasmuch as dietary

TABLE 4

Relation between nutrient intake and serum levels of vitamin C, winter season, third trimester

SERUM VITAMIN C	INTAKE OF VITAMIN C (MG/DAY)					
	Under 40	40–79	80–119	120 and over	Total	Median intake
mg/100 ml	*Number of cases*					
Under 0.39	107	91	45	14	257	47
0.40–0.79	26	51	22	14	113	66
0.80–1.19	8	19	18	10	55	81
1.20 and over	4	5	5	7	21	95
Total patients	145	166	90	45	446	57
Median serum level	0.21	0.36	0.40	0.62	0.33	

TABLE 5

Median levels of serum vitamin C in relation to nutrient intake, by season, third trimester

INTAKE	MEDIAN SERUM VITAMIN C				NUMBER OF CASES			
	Winter	Spring	Summer	Fall	Winter	Spring	Summer	Fall
mg/day	*mg/100 ml*							
Under 40	0.21	0.20	0.36	0.30	145	133	125	109
40–79	0.36	0.34	0.52	0.44	166	191	141	145
80–119	0.40	0.31	0.61	0.54	90	78	41	58
120 and over	0.62	0.57	0.80	0.52	45	31	14	35
All intake levels	0.33	0.29	0.46	0.40	446	433	321	347

sources of carotene and of ascorbic acid are often the same — especially greens of various sorts.

Serum vitamin C levels were also positively correlated with urinary excretion values, after a test dose, of niacin and, to perhaps a slight degree, of riboflavin and thiamine.

[2] Martin, Bridgforth, McGanity and Darby, unpublished data.

The number of women having edema increased from 2.4% in the first trimester to 10.8% in the third and returned postpartum to 2.1%. Only in the second trimester was there a significant relationship between serum level of ascorbic acid and edema. We were unable to detect a relationship between intake of vitamin C and the occurrence of edema in any trimester, even among the "consistent" groups. We, therefore, conclude that vitamin C is not an important factor in the occurrence of edema in our study group.

It was found (McGanity et al., '54) that women with anemia during pregnancy had lower intakes and serum levels

TABLE 7

Frequency of occurrence of single, live-born premature infants according to intake and serum levels of vitamin C in the third trimester of pregnancy

VITAMIN C INTAKE	TOTAL CASES	PREMATURES		SERUM VITAMIN C	TOTAL CASES	PREMATURES	
mg/day		*No.*	*%*	*mg/100 ml*		*No.*	*%*
Under 20	170	12	7.1	Under 0.20	622	34	5.5
20–39	371	10	2.7	0.20–0.39	449	19	4.2
40–59	385	8	2.1	0.40–0.79	537	17	3.2
60–99	475	14	2.9	0.80 and over	311	11	3.5
100 and over	233	6	2.6				
Total	1634	50	3.1	Total	1919	81	4.2

of vitamin C than the rest of the study group. Also, women with disease conditions complicating pregnancy [3] had somewhat lower intakes and serum levels during pregnancy. Relation of vitamin C to hematologic values will be considered again later in the present paper.

Vitamin C in relation to the course and outcome of pregnancy. As reported in a previous paper (McGanity et al., '54), mothers of premature [4] infants had lower serum vitamin C levels and lower intakes of vitamin C during pregnancy,

[3] Tuberculosis, venereal disease, endocrine disorders, heart disease, genitourinary diseases, and miscellaneous other conditions (McGanity et al., '54).

[4] Premature infants are those having a birth weight of 2500 gm or less.

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the lowering being significant in both the second and third trimesters. The increased frequency of occurrence of premature birth is limited to the group with intakes of vitamin C below 20 mg. Table 7 shows the results for single live-born infants for the third trimester. In the case of serum vitamin C levels, differences are not as large but it appears that the percentage of premature infants is higher for the group with the lowest serum levels. These differences are not necessarily indicative of a causal relation between vitamin C and premature birth since the group with low intakes and serum levels of vitamin C may have been different in other respects from the rest of the study group. The data do suggest an area for further study.

It is of interest to note that mothers of twins had significantly lower serum vitamin C levels in the second and third trimesters, while vitamin C intakes were not significantly different from those of the total study group. Cases of pre-eclampsia had generally lower intakes of all nutrients, including vitamin C, than the total study group. These patterns are believed to be reflections of the illness. There was no tendency for a difference in either vitamin C intakes or serum levels during pregnancy between mothers who later breast-fed their infants and those who did not. As previously noted there were differences in serum levels postpartum. Serum vitamin C levels postpartum tended to be high in those conditions, such as diabetes, where the mother did not nurse her infant.

Lund and Kimble ('43) found no relation of plasma vitamin C levels to the occurrence of toxemia, puerperal morbidity, or duration of labor in 197 cases. They concluded that low values found among cases of hyperemesis probably occurred as a result of the illness. We concur with this position.

It has been reported (Greenblatt, '53; and Javert, '54) that women who were habitual aborters responded favorably to a regime including supplements of vitamin C in the diet along with other measures. In the present study there was no indication of lowered vitamin C nutriture in the 40 women

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who aborted. Twenty-three of these had serum vitamin C determinations in the first trimester, and 18 in the second trimester. The distributions did not differ from those for the total study group. Although but a few had diet records, there was no indication of any unusual intake.

Consistent dietary intake groups. As noted earlier, individuals having high (or low) intakes on two or more diet records probably represent a group whose level of intake was generally high (or low). Study of such groups might be expected to show relationships not apparent when classification is based on a single diet record. Consequently, cases having two or more diet records were divided into subgroups as follows:

(1) "consistently low group" — 166 cases having intakes under 40 mg on at least two diet records;
(2) "consistently intermediate group" — 262 cases having intakes of 40 to 80 mg on at least two diet records;
(3) "consistently high group" — 193 cases having intakes of 80 mg or more on at least two diet records:
(4) cases not falling into any of the above groups.

Cases for whom three diet records were available were included in the above groups (1), (2), or (3) if two out of three records satisfied the necessary conditions.

Variation in serum levels of vitamin C for the consistent intake groups. For each of the "consistent" intake groups the median values of serum vitamin C were determined by weeks of gestation in each of two broad seasons, February through June, a period of low blood levels, and July through January, a period of high serum vitamin C levels. Postpartum values were further subdivided according to whether or not the mother was breast-feeding her infant. These are shown in table 8. The small size of the group of cases at 34 weeks gestation and over accounts for the irregularities in this group.

Differences by weeks gestation and weeks postpartum. The group with consistently high intakes maintained essentially

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the same average serum vitamin C levels throughout pregnancy but exhibited lowering of the concentrations during the postpartum period.

The group with consistently intermediate intakes showed a decrease in serum concentration between early and late pregnancy. It appears that their intake was sufficient to maintain their average level at about the same value as that of the high intake groups during the first trimester, but was not adequate to maintain these levels throughout pregnancy.

TABLE 8

Median levels of serum vitamin C by weeks gestation and postpartum within consistent intake groups

SEASON	LEVEL OF VITAMIN C INTAKE	WEEKS GESTATION					POSTPARTUM	
		13 and under	14–19	20–26	27–33	34 and over	Not nursing	Nursing
		Median serum vitamin C (*mg/100 ml*)						
Feb.–June	Low	0.18	0.20	0.28	0.14	0.12	0.14	0.12
	Medium	0.54	0.38	0.30	0.34	0.16	0.24	0.12
	High	0.46	0.68	0.58	0.54	0.80	0.36	0.22
July–Jan.	Low	0.40	0.38	0.58	0.30	0.54	0.24	0.16
	Medium	0.52	0.60	0.56	0.40	0.44	0.36	0.18
	High	0.52	0.76	0.56	0.64	0.44	0.48	0.28
		Number of cases						
Feb.–June	Low	24	41	39	71	7	19	20
	Medium	43	52	58	98	20	33	55
	High	28	29	40	85	8	36	43
July–Jan.	Low	32	42	44	77	10	31	55
	Medium	66	75	75	129	11	58	63
	High	41	58	63	85	13	34	42

Values in the postpartum period showed a further decline, significant only for the lactating group.

The group with consistently low intakes had low serum levels early in pregnancy, and a further decrease as pregnancy advanced. Postpartum values were still lower, although in the February-June season 70% of the cases already fell into the lowest grouping interval during the third trimester, so

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that there was not much opportunity for observing a further decline.[5]

While differences between third trimester and postpartum values of non-lactating women were not statistically significant within all the individual subgroups, the differences were in the direction of lower values in the postpartum period, and the combined effect of all intake groups in both seasons is significant.

Lactating women had lower serum C levels than non-lactating women in the same intake group, except for those with the lowest intakes where serum C levels of both lactating and non-lactating women were low.

In the consistently high intake group, there were 80 cases who had intakes of 100 mg or over on two or more diet records. During pregnancy their levels were not different from the remainder of the consistently high intake group, but 32 cases who were not nursing their infants at the time of the postpartum laboratory determination had median serum vitamin C levels of 0.70 mg/100 ml. This was significantly higher than the postpartum level of non-lactating women in the remainder of the consistently high intake group. The women who had had intakes of 100 mg or more and who were lactating at the time of the postpartum check-up had median levels of 0.30 mg/100 ml, which is not significantly different from the levels at somewhat lower intakes. Moreover, a group of 18 lactating women whose intakes had been

[5] Differences in median serum C levels between consistent intake groups were not statistically significant in the first trimester except that women with low intakes had lower serum C levels than those with medium and high intakes during the period February through June. In the second trimester, differences were definitely significant in the period February through June, and in the period July through January, those on low intakes had significantly lower serum C levels than those with medium and high intakes. Differences between all intake groups were clearly significant in the third trimester. During the postpartum period differences between intake groups were significant for both lactating and non-lactating women, except that there was little difference between low and medium intake groups among lactating women, both groups having very low serum C levels.

120 mg or more on at least two diet records during pregnancy had median postpartum levels of 0.27 mg/100 ml.

Within each intake group there was considerable individual variation in serum level about the median values, which may be due in part to individual differences in requirements. It is therefore difficult to interpret the results in terms of individual needs, but the figures indicate that on the average a dietary intake level of less than 40 mg was inadequate to support what might be regarded as acceptable concentrations of serum ascorbic acid during pregnancy or lactation. The intermediate daily intake range of 40 to 80 mg did support high levels of serum ascorbic acid early in pregnancy, but failed to do so during the latter part of gestation or during lactation. High serum concentration of vitamin C was supported throughout pregnancy by the consumption of 80 to 100 mg, but during the immediate postpartum period these levels were not attained unless the intake of vitamin C exceeded 100 mg per day. Finally, it appears that intakes in excess of even 120 mg per day did not maintain the average serum level of lactating women above about 0.3 mg.

With the knowledge available at present, it is not possible to say what levels of vitamin C in the serum are optimal, or whether any impairment of health results from lower levels. There is apparently a considerable margin of safety. In deprivation studies (Medical Research Council, '53) about 100 days elapsed between the virtual disappearance of vitamin C from the plasma and the appearance of the first clinical signs of scurvy.

Course and outcome of pregnancy in the consistent low, intermediate and high intake groups. Differences between the three intake groups for each of the following special conditions were within the limits of chance variation: hyperemesis, eclampsia and pre-eclampsia, puerperal fever, stillbirth, neonatal death, birth of single premature [6] infants, and congenital malformation (table 9). There were more cases of

[6] See footnote 4, page 211.

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disease conditions [7] in the low intake group, somewhat fewer in the medium intake group, and the smallest number in the high intake group. Low, medium and high groups were also compared for weeks gestation at delivery, labor complications, length of labor, various types of operative deliveries, lacerations occurring at delivery, placenta weight, birth weight of the infant, and type of infant feeding, whether breast or artificial. No significant difference was found. These findings for the "consistent" groups are confirmatory of the analysis

TABLE 9

Occurrence of special conditions in consistent vitamin C intake groups

SPECIAL CONDITIONS	VITAMIN C INTAKE GROUP					
	Low		Medium		High	
	No.	*%*	*No.*	*%*	*No.*	*%*
Disease complicating pregnancy	23	13.9	25	9.5	11	5.7
Hyperemesis	2	1.2	5	1.9	1	0.5
Eclampsia	1	0.6	3	1.1	1	0.5
Pre-eclampsia	12	7.2	14	5.3	9	4.7
Puerperal fever	8	4.8	21	8.0	8	4.1
Stillbirth	1	0.6	3	1.1	0	0
Neonatal death	3	1.8	4	1.5	3	1.6
Live-born premature, excluding twins	8	4.8	5	1.9	8	4.1
Congenital malformations	5	3.0	9	3.4	4	2.1
Total number in group	166		262		193	

of the whole study group (McGanity et al., '54), except for findings relating to premature birth.

Failure to find differences in the number of premature births was surprising in view of the increased incidence of this condition among mothers with intakes under 20 mg, reported above. However, in the present broader classification most of the low group had intakes above the 20 mg level, where no relationship was found. There were 23 women who had intakes of under 20 mg in both the second and third trimesters, and three of these delivered premature infants.

Relation to hematologic values. In view of the lower vitamin C intakes and serum levels among women with anemia

[7] See footnote 3, page 211.

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during pregnancy, it is of interest to compare hematologic values for the consistent intake groups, and at the same time to consider associated differences in iron intake.

In the low vitamin C intake group the average hemoglobin, packed cell volume, mean cell volume, mean cell hemoglobin, and mean cell hemoglobin concentration, but not the red cell count, were lower than in the other two groups. There was also a difference of about 4 mg in the daily intake of iron between the low and high vitamin C intake groups. In order to judge whether or not differences in hematologic values were attributable to associated differences in iron intake, those women with iron intakes of 10 mg or more in both second and third trimesters were selected within each of the vitamin C intake groups. Mean hematologic values in the third trimester for each of these groups are shown in table 10. Comparison of low and high groups showed significant differences in the second trimester for hemoglobin levels ($P = 0.03$), mean cell hemoglobin ($P = 0.02$) and for mean cell hemoglobin concentration ($P = 0.01$). The only difference which was significant in the third trimester was packed cell volume ($P = 0.05$). No significant differences existed postpartum. We are not able to say whether or not the small observed differences in hematological values are directly associated with vitamin C nutriture. They are of interest in view of the recent reports of Steinkamp, Dubach and Moore ('55) and of Moore ('55) on the effect of ascorbic acid on the absorption of radioiron. Interpretation of our observations is complicated by the possibility that the general abundance of the dietary of women in the high intake category is greater.

Consistently low, intermediate and high serum vitamin C groups. Cases were divided into low, medium and high serum vitamin C groups on the basis of second and third trimester determinations:

(1) "consistently low group" — 442 cases having serum vitamin C levels under 0.40 mg/100 ml in both the second and third trimesters or between 0.40 and 0.60 mg/100 ml in the second trimester and under

TABLE 10

Hematologic values in consistent vitamin C intake groups and in consistent serum vitamin C groups, third trimester

(Cases with iron intakes of 10 mg/day or more second and third trimesters)

CONSISTENT GROUP	NO. OF CASES	HEMOGLOBIN	RED BLOOD COUNT	PACKED CELL VOLUME	MEAN CELL VOLUME	MEAN CELL HEMOGLOBIN	MEAN CELL HEMOGLOBIN CONCENTRATION
		gm/100 ml	*million/mm³*	*%*	*μ³*	*μμg*	*%*
Vitamin C intake							
Low	67	11.44 ± 0.16	3.89 ± 0.05	34.6 ± 0.4	88.6 ± 1.0	29.24 ± 0.41	32.94 ± 0.25
Medium	199	11.55 ± 0.10	3.92 ± 0.03	35.0 ± 0.2	90.2 ± 0.6	29.76 ± 0.24	33.06 ± 0.14
High	164	11.72 ± 0.10	3.93 ± 0.03	35.5 ± 0.3	90.5 ± 0.7	30.02 ± 0.26	33.04 ± 0.16
Serum vitamin C							
Low	242	11.44 ± 0.09	3.92 ± 0.03	34.6 ± 0.2	88.8 ± 0.5	29.32 ± 0.22	32.98 ± 0.13
Medium	139	11.61 ± 0.11	3.96 ± 0.04	35.0 ± 0.3	89.3 ± 0.7	29.54 ± 0.29	33.08 ± 0.17
High	128	11.69 ± 0.12	3.87 ± 0.04	35.3 ± 0.3	91.4 ± 0.7	30.40 ± 0.30	33.12 ± 0.18

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0.20 mg/100 ml in the third trimester; those having serum levels under 0.20 mg/100 ml in both the second and third trimesters were later separated out from this "low" group and designated as the "very low" group (162 cases);

(2) "consistently intermediate group" — 249 cases having serum levels of vitamin C between 0.40 and 0.80 mg/100 ml in the second trimester and between 0.20 and 0.80 mg/100 ml in the third trimester;

(3) "consistently high group" — 198 cases with serum vitamin C levels of 0.80 mg/100 ml or more in either second or third trimester and 0.60 mg/100 ml or more in the other trimester.

The occurrence of various special conditions within the three groups is shown in table 11. The following conditions did not differ between the groups: hyperemesis, eclampsia and pre-eclampsia, stillbirth, neonatal death or congenital

TABLE 11

Occurrence of special conditions in consistent serum vitamin C groups

SPECIAL CONDITIONS	CONSISTENT SERUM C GROUPS									
	Very low		Medium low		Total low		Medium		High	
	No.	*%*	*No.*	*%*	*No.*	*%*	*No.*	*%*	*No.*	*%*
Disease complicating pregnancy	23	14.2	29	10.4	52	11.8	30	12.0	12	6.1
Hyperemesis	3	1.9	2	0.7	5	1.1	2	0.8	3	1.5
Eclampsia	2	1.2	1	0.4	3	0.7	3	1.2	1	0.5
Pre-eclampsia	10	6.2	17	6.1	27	6.1	12	4.8	6	3.0
Premature separation of placenta	2	1.2	7	2.5	9	2.0	1	0.4	0	0
Puerperal fever	6	3.7	23	8.2	29	6.6	15	6.0	5	2.5
Stillbirth	2	1.2	3	1.1	5	1.1	5	2.0	1	0.5
Neonatal death	3	1.9	7	2.5	10	2.3	2	0.8	4	2.0
Live-born premature, excluding twins	13	8.0	9	3.2	22	5.0	10	4.0	4	2.0
Congenital malformations	1	0.6	14	5.0	15	3.4	2	0.8	7	3.5
Total number in group	162		280		442		249		198	

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malformation. Puerperal fever was significantly lower in the group with highest serum levels, allowance being made for differences by season and parity in both serum vitamin C and in the occurrence of puerperal fever. No relation of puerperal fever to intake of vitamin C was observed, nor was any relation to serum levels apparent when data for single trimesters were studied. We conclude that ascorbic acid nutriture is not a major factor in the occurrence of puerperal fever.

Differences in per cent of premature [8] single births and disease conditions complicating pregnancy [9] were not statistically significant on the basis of the initial division of cases; however, when the low group was further subdivided so as to separate out 162 cases having values of less than 0.20 mg/100 ml in both trimesters, both the percentage of cases of premature single live births and percentage of cases having complicating diseases were significantly higher in the low group. The disease conditions showing higher than average frequency in this group were (a) venereal disease or a history of syphilis (9 cases), and (b) pyelitis, cystitis, pyelonephritis, and pyonephrosis (5 cases).

Among labor complications there was a significant difference for premature separation of the placenta (table 11). Serum vitamin C levels were not related to the gestation time at delivery. However, within the group of women who gave birth to premature infants (on the basis of birth weight) there was a tendency for the cases in the group with low serum vitamin C to have shorter gestations. Sixteen of 22 single live-born prematures in the low group had gestations of less than 38 weeks. For the medium group the corresponding numbers were 5 of 10, and for the high group none of 4. (Premature single live-birth was associated with premature separation of the placenta in only two cases.) Consistent serum vitamin C groups were also compared for length of labor, various types of operative deliveries, lacerations oc-

[8] See footnote 4, page 211.
[9] See footnote 3, page 211.

curring at delivery, placenta weight, birth weight of the infant, and type of infant feeding. No significant difference was found.

Hematologic values varied with serum vitamin C levels in a manner similar to that discussed above for intake values. Comparison of low and high groups (with iron intake of 10 mg or more in both second and third trimesters) showed significant differences for packed cell volume in all trimesters, and for hemoglobin, mean cell volume and mean cell hemoglobin in the third trimester (table 10 shows data for the third trimester).

In evaluating these results it should be noted that a causal relationship cannot be established from data of this type. Groups of cases selected on the basis of one characteristic, e.g. serum levels of vitamin C, will be expected to differ in other characteristics. In interpreting the data, we have allowed for differences recognized to exist, such as those for parity or season of the year, but it is obvious that other differences may be present. Such possibilities cannot be ruled out in an observational study.

SUMMARY

Data on intakes and serum levels of vitamin C in 2,129 pregnant women are studied in relation to many factors, including the course and outcome of pregnancy.

In general, serum levels decreased during pregnancy except in the group at a high level of intake. Values were further decreased postpartum, and were lower for lactating than for non-lactating women. Evidence is presented that on the average intakes of 80 to 100 mg daily supported high levels of ascorbic acid in the serum during pregnancy. The serum levels of non-lactating mothers averaged 0.7 mg per 100 ml during the puerperium on intakes (during pregnancy) of 100 mg or over per day; the serum concentration of lactating mothers did not average greater than 0.3 mg even on intakes exceeding 120 mg daily.

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Analysis of findings relative to the health of the mother and baby revealed only 5 categories which may possibly be associated with ascorbic acid nutriture: hematologic findings, gingivitis, premature separation of the placenta, premature birth, and puerperal fever. Increased frequency of premature birth was limited to the lowest intake levels and lowest serum concentrations. In none of the conditions was there a strong relation to both intakes and serum levels. Hence we believe that ascorbic acid nutriture is at most a contributary factor in any of these.

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ANTIRHEUMATIC ACTIVITY OF ASCORBIC ACID IN LARGE DOSES*

Preliminary Observations on Seven Patients with Rheumatic Fever

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THE purpose of this paper is to present observations suggesting that ascorbic acid possesses antirheumatic activity when administered in large doses. Because of the small number of patients treated so far, the relatively short period of follow-up study and the well known variability of rheumatic fever even when it is uninfluenced by therapy, the present communication must be considered preliminary in the strictest sense of the word. Nevertheless, the apparent responses of a group of 7 patients treated within the past three months have been sufficiently impressive to warrant this report.

OBSERVATIONS

Since January 2, 1950, ascorbic acid‖ has been given by mouth in doses of 1 gm. four times daily (total of 4 gm. per day) for periods varying from eight to twenty-six days to a total of 7 patients with rheumatic fever. There has been a follow-up period of twelve days to two months in 3 patients in whom therapy has been discontinued; at the time of this writing, March 20, 1950, 4 patients are still receiving treatment.

The results of treatment with ascorbic acid can best be presented by a brief description of the clinical course of each of the 7 patients:

CASE 1. P. L. is a 15-year-old boy whose pertinent findings prior to treatment included migratory polyarthritis, a temperature of 101° to 102° F. by rectum and elevation of the sedimentation rate. His heart seemed normal except for a slight systolic murmur. Ascorbic acid was given for a total of 8 days (January 2 through January 9, 1950). Within 24 hours of the beginning of treatment the joint manifestations had disappeared, and the fever had lessened. The temperature remained normal after the 2nd day of therapy, but the sedimentation rate continued to be elevated.

CASE 2. R. M. is a 14-year-old boy who has suffered for many months from persistent rheumatic fever, severe cardiac involvement and chronic hepatic congestion. Ascorbic acid therapy was begun on February 4, 1950, during a period of increasing rheumatic activity (possibly just a rheumatic-fever cycle), manifested by temperature elevation to 102°F. by rectum, rise in the sedimentation rate and increased congestion of the liver. The fever began to lessen on February 5, and the temperature has remained normal since February 6. The size of the liver has not changed appreciably, and the sedimentation rate is still elevated. Ascorbic acid was given for a total of only 8 days.

CASE 3. E. T. is a 15-year-old boy who for 6 weeks prior to therapy had been ill with migratory objective polyarthritis, fever, sinus tachycardia and a rapid sedimentation rate. Except for a slight systolic murmur, his heart seemed normal. With ascorbic acid therapy, which was given for a total of 15 days (February 21 to March 7, 1950), there was prompt improvement in symptoms and reduction in fever. Since the 2nd day of treatment the temperature has been entirely normal, and there have been no symptoms or signs referable to the joints. By March 20 the sedimentation rate had fallen to within normal limits.

CASE 4. W. D. is a 14-year-old boy with rheumatic heart disease whose symptoms of rheumatic fever had been controlled by acetyl salicylic acid. However, after the omission of this drug and prior to treatment with ascorbic acid he had elevation of the temperature to 102°F. by rectum, several nosebleeds and a swollen painful knee. Ascorbic acid therapy was begun on February 22, 1950, and continued to the present time. Improvement was definite but gradual over a 4-day period. He has been symptom-free and afebrile since February 26. The sedimentation rate has fallen from an initial level of 1.4 to the slightly elevated level of 0.55 mm. per minute.

CASE 5. R. F. is an 11-year-old girl who, on admission to the hospital on March 6, 1950, was obviously acutely ill with active rheumatic fever, pancarditis and congestive heart failure. Pertinent findings included a temperature of 104°F. by rectum, tender, swollen finger joints, a respiratory rate of 60 to 75, cardiac enlargement, murmurs of mitral regurgitation and aortic regurgitation, nodal rhythm, a ventricular rate of 160, a pericardial friction rub, marked enlargement and tenderness of the liver, and edema of the legs and lower back. Treatment with ascorbic acid was begun on the evening of March 6. The fever and joint symptoms gradually lessened and have been completely absent since March 13. The friction rub has not been audible since March 10. The cardiac rhythm reverted to normal within 24 hours of therapy, and the heart rate is now about 120. Hepatic congestion and peripheral edema have entirely disappeared. The patient now feels well and appears obviously much improved. The sedimentation rate is still elevated.

CASE 6. A. F. is an 18-year-old boy without cardiac involvement whose rheumatic-fever manifestations just prior to ascorbic acid therapy (given from March 7, 1950, to the present) consisted of elevation of the temperature to 101°F. by rectum, objective polyarthritis and elevation of the sedimentation rate. Treatment was followed by somewhat slow but steady improvement. The temperature has been normal since March 10, and the joint manifestations have remained completely subsided since March 11. The sedimentation rate is still elevated.

CASE 7. P. B. is a 5-year-old boy with known rheumatic heart disease who had a recrudescence of rheumatic fever on March 9, 1950. Manifestations included elevation of the temperature to 104°F. by rectum, sinus tachycardia, pallor and pain and tenderness of the right knee and both ankles.

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‖The ascorbic acid for this study was supplied by Hoffmann-La Roche, Incorporated, Nutley, New Jersey.

The joint symptoms subsided spontaneously prior to treatment, but the high fever continued. Ascorbic acid therapy was begun on March 14. Since that time there has been a steady decline in temperature, and fever has been entirely absent since March 17. The heart rate has slowed, and the patient's appearance has greatly improved.

Discussion

In 1933 Rinehart[1] proposed the concept that vitamin C deficiency may play a role in the etiology of rheumatic fever, and since that time he and many other investigators have studied the metabolism of ascorbic acid in rheumatic fever and arthritis and have attempted to use it in the treatment of these diseases. However, as far as we are aware, a beneficial effect of ascorbic acid on the course of active rheumatic fever has hitherto not been demonstrated. These previous therapeutic failures may perhaps be attributed to the fact that practically all investigators were thinking in terms of vitamin C deficiency and, hence, used doses of ascorbic acid considerably smaller than those used by us.

Although ascorbic acid is generally considered innocuous, and although no untoward reactions have been encountered in the course of our limited observations, there is obviously a need for careful toxicity studies. It is possible that individual doses of more than 1 gm. or total daily doses of more than 4 gm., if found harmless, may prove to be therapeutically even more effective.

The mechanism by which ascorbic acid may influence the rheumatic process is not known, but the large amounts required to produce the apparent effect suggest that it is not a simple matter of replacement therapy. The relation of ascorbic acid to the activity of the adrenal cortex[2] and the recently demonstrated value of adrenocortical activity in the treatment of rheumatic diseases[3-5] raise many interesting questions concerning the problem of mechanism that cannot be discussed in this short report.

Finally, it should be emphasized that, although our observations suggest that ascorbic acid when administered in sufficient amounts possesses antirheumatic activity, no final assessments can yet be made regarding the possible therapeutic value of this substance. Data are as yet insufficient to allow for a comparison of ascorbic acid with salicylates, cortisone, ACTH and other antirheumatic agents. Nevertheless, this preliminary report is offered, to stimulate further investigations of the therapeutic potentialities of ascorbic acid, of its relation to the antirheumatic action of adrenal hormones and of its possible local role in the tissue reactions that we call rheumatic fever. It also indicates the need for caution in interpreting apparent antirheumatic effects obtained from combinations of various hormones and ascorbic acid.[6]

Summary

Observations suggesting that ascorbic acid in doses of 4 gm. daily possesses antirheumatic activity are presented.

The possible significance of these observations is briefly discussed, and the need for extending them is emphasized.

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THER STUDIES ON THE DETOXICATION OF THE ARSPHENAMINES BY ASCORBIC ACID

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In a previous study of the detoxication of neoarsphenamine by means of organic acids (1) it was suggested that the organic acid (i.e., usually ascorbic acid) may act a) by preventing or retarding the formation from neoarsphenamine of the much more toxic arsenoxide, or b) by peptizing colloidal neoarsphenamine. Rodman and Wright (2) have shown that the colloidal fractions of neoarsphenamine are about ten times as toxic as the whole drug. However, they have also shown that the amount of colloidal material present in the usual commercial sample is quite small, and its removal or peptization would cause a decrease in toxicity of only 10 to 15 per cent, while ascorbic acid is capable of decreasing the toxicity by about 50 per cent (1). A third possibility is suggested by the work of Probey, et al. (3). They have pointed out that only a part of the toxicity of neoarsphenamine is inherent in its arsenic content, and the remainder is due to the presence of the o-aminophenol groups. In other words, the possibility must also be considered that ascrobic acid acts by protecting these groups from oxidation.

The answer to these questions is of some importance since, if ascorbic acid does act by interfering with the conversion of neoarsphenamine to arsenoxide, it might also diminish the effectiveness of the drug. On the other hand, ascorbic acid might alter the rate, or to some extent the site, at which arsenoxide is formed without affecting the total amount eventually formed, or the efficiency of the drug. Such reports as are available (1, 4, 5) indicate that ascorbic acid does not decrease the therapeutic effectiveness of neoarsphenamine.

In the study of these problems, use has been made of both chemical and biological methods. The first concerns the chemical reactions of the drug, particularly its behavior on oxidation. The purpose has been to see whether ascorbic acid influences the rate of oxidation of neoarsphenamine by air, or alters the course of the reaction in any way. The biological approach has involved: a) a study of the effectiveness of ascorbic acid when given in different quantities, and at different times with respect to the arsenical, with the object of determining the probable site of the detoxication process (whether in the blood stream, or in the tissues where neoarsphenamine is temporarily stored), and b) a study of the effect of ascorbic acid on the retention of arsenic in the tissues.

The extreme susceptibility of neoarsphenamine to oxidation is well known. It is generally supposed that the path of oxidation, both *in vitro* and *in vivo* is as follows: neoarsphenamine → arsenoxide → arsonic acid → arsenic acid + aromatic residue. It is reasonable to suppose that ascorbic acid, since it is a reducing agent, would retard the oxidative breakdown of neoarsphenamine to

arsenoxide, at least to some extent. However, the chemical reactivity of ascorbic acid, in the absence of the proper catalysts, is low (6), and, depending on the conditions, it might be entirely without effect on this process. The most direct approach to the problem would be to study the effect of ascorbic acid on solutions of neoarsphenamine undergoing oxidation by air. In order to do this it is necessary to be able to analyze solutions which contain any or all of the following: neoarsphenamine, arsenoxide, arsenoxide N-methanal sodium sulfonate, and arsonic, ascorbic, and dehydroascorbic acids. Rosenthal's method (7) has been generally used in the past for the determination of arsenoxide in arsenical solutions. His procedure has not seemed satisfactory for these experiments because the color reaction is given by whatever compounds in the mixture contain the o-aminophenol group, and also because the color reagent is reduced by ascorbic acid. Consequently, a different approach involving a new precipitating agent for neoarsphenamine has been used.

Chemical study of oxygenated neoarsphenamine solutions. Since several of the substances mentioned as possibly occurring in the mixtures to be analyzed can be distinguished only by means of their behavior toward oxidizing agents, it is necessary that at least one of them be removed quantitatively, or nearly so; otherwise the problem is too complex for solution. The reagent which has been used in this work for the precipitation of neoarsphenamine is the synthetic detergent, cetyl pyridinium chloride.[1] In dilute alcohol this reagent precipitates neoarsphenamine in a yellow granular form, and nearly quantitatively.

The analysis of the various mixtures described below has been carried out chiefly by means of iodine titrations. The reactions of the arsenicals with iodine have been studied previously (8, 9) but primarily as a means of determining their arsenic content: the reactions are nearly stoichiometric. The procedures and results of typical analyses are given below.

PROCEDURE. To 5 cc. of a 1.5% neoarsphenamine solution was added 5 cc. of 10% aqueous sodium acetate, 0.2 cc. of 1% starch solution, and 5 cc. of glacial acetic acid. The resulting solution was titrated with 0.1 N iodine until the addition of 2 or 3 drops gave a blue color which persisted for at least 5 seconds. For the sample used (arsenic content 20.0% 750 gm. equals one mole) the titer was 10.4–10.6cc., or an excess of 0.4–0.6 cc. over the theoretical value of 10.0. This end point is not entirely satisfactory, particularly in the case of aged solutions, as it fades rather rapidly, but in the case of the other arsenicals studied the values obtained are very close to the theoretical (± 3%) and the end points are more stable. The values (as equivalents of iodine per mole) are: sulfarsphenamine, 8.0; arsphenamine, 8.0; Glyvarsenyl (3,4′-diacetylamino 4-hydroxyarseno-benzene 2′ sodium glycolate), 8.0; phenarsine[2] (3-amino 4-hydroxyphenyl dichlorarsine hydrochloride), 2.0; and 3-amino 4-hydroxy phenylarsonic acid, 0.0. Under these conditions ascorbic acid requires two equivalents of iodine per mole, and dehydroascorbic, by definition, none.

Solutions of neoarsphenamine were not completely precipitated by cetyl pyridinium chloride, but the residue was sufficiently small to make the desired analyses possible. To

[1] Manufactured by the Edwal Laboratories, Chicago, Ill. The author is indebted to Mr. Morris Auerbach of the Winthrop Laboratories for having suggested the possibilities of this compound.

[2] Recently the names dichlorophenarsine and oxophenarsine are to some extent being substituted for phenarsine and arsenoxide, respectively.

10 cc. of a 1.5% solution of neoarsphenamine was added 10 cc. of a 4% solution of cetyl pyridinium chloride in 60% ethyl alcohol. After standing several minutes in a stoppered container, the solution was filtered through a No. 1 Whatman paper. The filtrate was, on some occasions, slightly cloudy, but could be clarified by returning to the filter several times. An aliquot of 10 cc. was taken for analysis; a few cc. of glacial acetic acid were added, then 0.1 N iodine was run in a few drops at a time until a yellow color, stable for at least 30 seconds, was obtained. (Note: An excess of iodine reacts with the excess of precipitating agent to give a yellow product, which thus serves as a satisfactory indicator.) The value obtained was a 0.50 cc. of 0.1 N iodine, or about 5% of the amount required for the neoarsphenamine originally taken. This value is due to a small residue of unprecipitated arsenical, since it is constant for the given conditions, providing the amount of neoarsphenamine taken for analysis does not exceed the combining power of the precipitant; in this particular case a two-fold excess of precipitating agent is used.

Arsenoxide N-methanal sodium sulfonate should also be precipitated under the conditions described above since its sodium ion would be replaced by the quarternary ammonium ion.[3] This compound is undoubtedly the one which would be formed during the oxidation of neoarsphenamine, rather than arsenoxide N-methanal sodium sulfoxylate, since the redox potential of the sulfoxylate group is less than that of the arsenobenzene linkage (see table 4). However, 3-amino, 4-hydroxy phenylarsonic acid is not precipitated under the given conditions.

Neoarsphenamine was separated from arsenoxide as follows: In a 100 cc. graduated cylinder was measured 1.00 gm. each of neoarsphenamine and phenarsine, 870 mgm. of sodium bicarbonate, and water to 100 cc. Of this solution, 5 cc. required 9.8 cc. of 0.1 N iodine when titrated in sodium acetate-acetic acid; theory 10.1 cc. The precipitation was now carried out with cetyl pyridinium chloride as described above. A 10 cc. aliquot of the filtrate required 4.2 cc. of 0.1 N iodine as against 3.45 cc. which would be required for the arsenoxide alone. The difference between the theoretical value and that actually obtained is nearly the same as the blank value found with neoarsphenamine alone (0.5 cc. for 10 cc. of the filtrate).

A mixture containing neoarsphenamine, arsenoxide, and ascorbic acid was analyzed in the following way: A stock solution was prepared which contained, per cc., 10 mg. each of neoarsphenamine, phenarsine, and ascorbic acid, with sufficient bicarbonate to bring the mixture to neutrality. Of this solution, 5 cc. when titrated with 0.1 N iodine in sodium acetate-acetic acid required 15.8 cc., the same as the theoretical value. (When a considerable amount of ascorbic acid is present the end points are more precise.) After precipitation with cetyl pyridinium chloride, 10 cc. of the filtrate required 9.95 cc. of 0.1 N iodine to the yellow end point. The theoretical value (combining power of the arsenoxide plus ascorbic acid) is 9.15 cc. Again, if one subtracts the usual blank value of 0.5 cc. for unprecipitated neoarsphenamine, the two figures are brought into substantial agreement.

The partition between arsenoxide and ascorbic acid may now be determined by an analysis of the filtrate for arsenic. (Note: In the experiments which were subsequently run the arsenic in the filtrate was likely to consist only of unprecipitated neoarsphenamine and arsenoxide, or at least of arsenic in the form R-As = 0, since only prolonged aeration will cause the formation of arsonic acid from arsenoxide (see table 3). It is further unlikely that any arsonic acid would be formed in the presence of unchanged neoarsphenamine.)

[3] In order to study this point a solution of the compound in question was prepared by dissolving 600 mgm. of sulfarsphenamine (arsenic content, 21.5%) in 25 cc. of water, adding 34.5 cc. of 0.1 N iodine, enough bicarbonate to bring the solution to neutrality, and water to 65 cc. Of this solution, 5 cc. should contain 41.3 mgm. of arsenoxide N-methanal sodium sulfonate. The iodine titration for 5 cc. was 5.55 cc. of 0.05 N; theory 5.30. After precipitation with cetyl pyridinium chloride the iodine titration for 5 cc. was 1.0 cc. of 0.05 N. This indicates that about 80% of the compound was precipitated, or that 8 mgm. remained in solution.

A 20 cc. portion of the filtrate, prepared from the solution described immediately above, was taken for analysis for arsenic. The alcohol was first boiled off; about 1 cc. of concentrated sulfuric acid was added, followed by 2.5 gm. of powdered potassium permanganate in small portions. From this point on the procedure was identical with that given in U.S.P. XII. The final titration was made on a 20 cc. aliquot with a microburette, using 0.1 N thiosulfate, and 7.2 cc. was required. The blank value for the reagents used in the arsenic determination was 0.4 cc., leaving 6.8 cc. for 20 cc., or 3.4 cc. for a 10 cc. portion of filtrate. Subtraction of the usual neoarsphenamine blank leaves 2.9 cc. for the arsenoxide in the filtrate. The theoretical value is 3.45 cc., indicating a recovery of 82%. This method of analysis is, therefore, not very exact, but is capable of revealing the presence of significant amounts of arsenoxide in neoarsphenamine filtrates, or an increase in the amount of arsenoxide on exposure to air.

TABLE 1

Results of exposure to air of solutions of neoarsphenamine with and without ascorbic acid

SOLUTION	TIME OF EXPOSURE	TOTAL IODINE EQUIVALENT*	IODINE EQUIVALENT OF ARSENOXIDE PLUS ASCORBIC ACID†	IODINE EQUIVALENT OF ARSENOXIDE†	TOXICITY OF SOLUTION‡ LD_{50} ± S.E.§
	days				
A. 1 cc. = 15 mgm. neoarsphenamine	0	10.3	0	0	418 ±11
	1	10.0	0.70	0	
	2	8.5	0.90	0.15	
	3	7.0	0.80	0.20	
	4	6.0	0.80	0.40	155 ±15
B. 1 cc. = 11.25 mgm. neoarsphenamine plus 4.4 mgm. ascorbic acid	0	10.5	2.40	0	600 ±25
	1	10.1	2.90	0	
	2	8.7	3.00	0	
	3	7.5	2.70	0.10	
	4	6.9	2.80	0.25	190 ± 8

* All iodine equivalents are given in terms of cc. of 0.1 N solution required for a 5 cc. aliquot of the original solution or a 10 cc. aliquot of the filtrate.

† The neoarsphenamine blank value of 0.5 cc. for 10 cc. of filtrate has been subtracted in all cases.

‡ As mgm./kgm. of neoarsphenamine.

§ Standard error, estimated graphically (see Miller, L. C., and Tainter, M. L., Proc. Soc. Exp. Biol. and Med., 57: 261, 1944).

The effect of atmospheric oxygen on solutions containing neoarsphenamine and neoarsphenamine plus ascorbic acid was now studied by allowing the two solutions to stand in the dark, but open to the air. Aliquots were analyzed at daily intervals by the method already described. Solution A contained 750 mgm. of neoarsphenamine in 50 cc. of waters Solution B contained 562 mgm. of neoarsphenamine, 220 mgm. of ascorbic acid, and 158 mgm. of sodium bicarbonate in 50 cc. Solution B was thus made up so that the total combining power for iodine would be the same as for (A) but exactly one-fourth of the neoarsphenamine would be replaced by ascorbic acid. The results are given in table 1.

The data in table 1 may be interpreted as follows: The total oxygen uptake, as indicated by the decrease in iodine titration, is almost in direct proportion to the neoarsphenamine content. From this it would be inferred that the ascorbic acid probably undergoes no change. The constancy of the [ascorbic acid plus

arsenoxide] fraction of Solution B points to the same conclusion. The possibility cannot be entirely eliminated that the ascorbic acid undergoes some change since it is known (10) that dehydroascorbic acid is very unstable above pH 5 and changes over rapidly to a substance more strongly reducing than ascorbic acid itself, but it would be more logical to assume that there is no change.

The amount of arsenoxide formed in both solutions is surprisingly small, and does not account for the total decrease in iodine equivalent, but it could account for the increase in toxicity.[4] Since both solutions darkened considerably, it seems evident that the oxidative change involved primarily the o-aminophenol groups.

The neoarsphenamine solution was, as would be expected, always more toxic than the neoarsphenamine with ascorbic acid. However, the *relative increase* in toxicity of both solutions with time was practically the same. The ascorbic acid may be considered as functioning in one of two ways: 1) by delaying chemical changes in the arsenical during the oxygenation, and, possibly more important, 2) by detoxifying *in vivo* the unchanged neoarsphenamine which remains after the oxygenation. The following experiment was set up to investigate this question.

Solution C was made up to contain 1500 mgm. of neoarsphenamine in 50 cc. of water. Solution D contained the same amount of neoarsphenamine plus 375 mgm. of ascorbic acid and 300 mgm. of sodium bicarbonate. The solutions were left open to the air at 37°C. for three days, then the same quantities of ascorbic acid and bicarbonate were added to solution C, after which both were diluted to 75 cc. for analysis. They were also tested for toxicity in the albino rat, with the results given in table 2.

The data demonstrate once more the fact that the amount of arsenoxide formed during the oxygenation of neoarsphenamine is surprisingly small and that the ascorbic acid, when present, seems to remain unchanged. The principal change of the solutions was a darkening, both finally absorbing about 8 times as much light as a fresh neoarsphenamine solution of the same concentration when measured in a photoelectric colorimeter with filter S54. The color change in solution D was somewhat delayed, but it finally had a deeper color. Solution D was, if anything, slightly more toxic than C, demonstrating that the presence of ascorbic acid during the time of exposure to air did not prevent the chemical changes which result in an increase in toxicity. Therefore, an aged neoarsphenamine-ascorbic acid solution is less toxic than an equally aged neoarsphenamine solution simply because it was less toxic at the outset.

It is difficult to say in the case of neoarsphenamine whether the increase in

[4] The LD_{50} of neoarsphenamine (whole drug) is usually about 400 mgm./kgm., or 240 mgm./kgm. of pure drug since approximately 40% of NaCl is added to the commercial product. The LD_{50} of arsenoxide is 14 mgm./kgm. (computed from phenarsine whose LD_{50} is 20 mgm./kgm.,[5] and the conversion factor of phenarsine to arsenoxide is 0.69). If it is assumed that the molar toxicity of arsenoxide and of the N-substituted compound is the same, the approximate toxicity at any stage of the breakdown of neoarsphenamine to arsenoxide may be calculated. An LD_{50} of 155 mgm./kgm. corresponds approximately to a 12% breakdown. This is of the right order of magnitude for the amount of arsenoxide found, but the decrease in total iodine equivalent at this point would indicate a much more extensive breakdown.

toxicity on exposure to air is due to breakdown to arsenoxide, or to oxidation of o-aminophenol groups, or both, since the process of oxidation is evidently complex. Whether the oxidation of o-aminophenol groups actually results in an increase in toxicity is more clearly seen in the case of arsenoxide itself, a compound of relatively simple structure; an oxidation of the As^{+++} to As^{+++++} is known to decrease the toxicity of the compound. A study of the behavior of arsenoxide on oxygenation was therefore carried out, both in the presence and absence of ascorbic acid.

Solution P contained 500 mgm. of phenarsine and 435 mgm. of sodium bicarbonate in 100 cc. Solution PA contained 500 mgm. of phenarsine, 300 mgm. of ascorbic acid, and

TABLE 2

Chemical analysis and toxicity of solutions of neoarsphenamine and of neoarsphenamine plus ascorbic acid, aged three days

SOLUTION	ORIGINAL COMPOSITION	FOR SOLUTION	IODINE TITER† FOR FILTRATE	FOR ARSENIC‡	TOXICITY	
					Dose, mgm./kgm. neoarsphenamine	Mortality
C	1500 mgm. neoarsphenamine in 75 cc.*	11.4	3.7	0.18	150 225 300	0/5 3/5 4/5
D	1500 mgm. neoarsphenamine, 375 mgm. of ascorbic acid and 300 mgm. bicarbonate in 75 cc.	10.0	3.0§	0.30	150 225 300	0/5 4/5 6/6

* Same quantities of ascorbic acid and bicarbonate as in Solution D were added after aging, before the analyses were made.

† All figures given are as cc. of 0.1 N iodine required for 5 cc. of the solution, or 10 cc. of the filtrate after precipitation with cetylpyridinium chloride. Neoarsphenamine blank value of 0.5 cc. subtracted.

‡ For 10 cc. of filtrate, using U.S.P. XII method, and subtracting 0.5 cc. blank for unprecipitated neoarsphenamine.

§ Theoretical value for the ascorbic acid alone, if unchanged, 2.84 cc.

655 mgm. sodium bicarbonate in 100 cc. Both solutions were neutral to litmus. They were exposed to the air and samples were titrated at intervals with iodine in sodium acetate-acetic acid solution. The solutions were also tested at intervals for toxicity in the albino rat. The results are given in table 3.

This experiment demonstrates two significant points. In solution P two separate types of oxidation were going forward. There was oxidation of o-aminophenol groups, as evidenced by the darkening of the solution, and there was oxidation of As^{+++} to As^{+++++}, as evidenced by the decrease in iodine titer. However, there was a latent period of two days in which no change took place in the valence of arsenic, then during the next three days there was a marked change. In solution PA there was also little change in titer during the first two days, but a rapid

change from the third to the fifth day. In solution PA there was never much color change, indicating that the ascorbic acid was protecting the o-aminophenol groups from oxidation.

There was little difference in the toxicity of the two solutions when freshly prepared (see also footnote 4). During the first two days there was an increase in the toxicity of solution P, coinciding with the period during which oxidation of o-aminophenol groups was occurring. During the next three days there was a decrease in toxicity, coincident with the oxidation of the arsenic. However, it must be stated that the extent of change from As^{+++} to As^{+++++} would not account quantitatively for the observed decrease in toxicity, in spite of the fact that arsonic acid is much less toxic than arsenoxide.

TABLE 3

The effect of exposure to air on solutions of phenarsine and of phenarsine plus ascorbic acid

COMPOSITION OF SOLUTION	TIME OF EXPOSURE	TOTAL IODINE EQUIVALENT*	TOXICITY LD_{50} ± S.E.§
	days		
0.5% phenarsine	0	8.67†	19.3 ±1.60
	1	8.76	—
	2	8.66	15.3 ±0.71
	3	8.33	—
	5	7.60	20.5 ±1.15
0.5% phenarsine, 0.3% ascorbic acid	0	17.30‡	18.0 ±0.73
	1	16.80	—
	2	16.75	19.0 ±0.75
	3	16.20	—
	5	14.30	23.4 ±1.35

* As cc. of 0.1 N iodine required for 5 cc. of solution.
† Theory, 8.65 cc.
‡ Theory, 17.18 cc.
§ As mgm./kgm. of phenarsine + standard error (estimated graphically).

In solution PA there was no change in toxicity during the "latent" period of the first two days, but there was a significant decrease in toxicity during the third to fifth days. Here the change in the valence of the arsenic, as revealed by titration, is sufficient to account for the decrease in toxicity. (It is assumed that the ascorbic acid underwent no change; no reasonable interpretation of the results is possible otherwise.)

Discoloration of arsenical solutions as an indication of increased toxicity. Bundesen et al. (11) have shown that solutions of neoarsphenamine and Mapharsen can be prevented from darkening on exposure to air if sufficient ascorbic acid is present. In the present work it has also been found that 1% neutral solutions, or suspensions, of phenarsine, 3-amino 4-hydroxyphenylarsonic acid, arsphenamine, and o-aminophenol darken on exposure to air for a day or more. These same solutions do not darken even in the course of a week if an equal weight of sodium ascorbate is present. Solutions of sulfarsphenamine and Glyvarsenyl do not

darken noticeably either in the presence or absence of ascorbic acid. Ascorbic acid does not decrease appreciably the toxicity of phenarsine,[5] or of sulfarsphenamine,[6] but it does decrease the toxicity of neoarsphenamine, arsphenamine,[7] and Glyvarsenyl (1). It is, therefore, apparent that the effect of sodium ascorbate in decreasing the toxicity of certain arsenicals is not necessarily related to its property of preventing them from darkening on exposure to air. In general, however, ascorbic acid decreases the toxicity of the preparations which are the most unstable toward oxidation and has little effect on the stable preparations. Glyvarsenyl and phenarsine are exceptions to this generalization. The latter exception may be explained on the basis that although phenarsine can and does undergo oxidation of the o-aminophenol group, more prolonged treatment is required to cause this oxidation to occur extensively than in the case of neoarsphenamine[8] or arsphenamine. Furthermore, the oxidation of As^{+++} to As^{+++++} which occurs at the same time decreases the toxicity. No explanation of the behavior of Glyvarsenyl is apparent at this time.

Oxidation-reduction potentials of significant systems of arsenical compounds. The relationship of ascorbic acid to the oxidation of the arsenical compounds in the blood stream may also be studied by means of oxidation-reduction potentials. The potentials of many systems important to this study have already been determined, but those of the various stages of the oxidation of the arsphenamines do not seem to have been reported. The procedures used were as follows:

Quantities of the two components of the system were taken so that each would contribute 25 mgm. of arsenic. These were dissolved in about 75 cc. of oxygen-free water, with the help of bicarbonate, if necessary. Concentrated HCl, 8.5 cc., was then added and the solution was diluted to 100 cc. This makes a solution containing an equal number of equivalents of the oxidized and reduced form, and normal in hydrogen-ions. In cases where precipitation occurred on acidification, the quantities of both components were reduced equally until a point was reached at which no precipitation occurred. The solution was poured into a beaker, covered at once with mineral oil, and its potential was read on a Beckman electrometer. The electrodes used for this purpose consisted of one gold-plated platinum electrode and a normal calomel electrode. There was usually a rather rapid drift to an equilibrium value, which was reached after one or two minutes. After correction

[5] The LD_{50} of phenarsine determined with groups of 10 rats to a dose was 20 mgm./kgm. The LD_{50} of phenarsine plus ascorbic acid determined with groups of 5 rats to a dose was as follows (with sodium ascorbate added in the molar ratios indicated): 1 mole per mole phenarsine, 21 mgm/kgm.; 3 moles per mole phenarsine, 22 mgm./kgm.; 6 moles per mole phenarsine, 26 mgm./kgm. Similarly, the LD_{50} of phenarsine with sodium isoascorbate added in the ratios of 1, 3, or 6 moles per mole of phenarsine was 18 mgm./kgm. It is not clear why the ascorbate and isoascorbate should not give identical results.

[6] The LD_{50} of the arsenical as determined with groups of 15–20 rats to a dose was 590 ± 20 mgm./kgm. With 120 mgm. ascorbic acid (as the sodium salt) added, the LD_{50} determined with groups of 10 rats to a dose was 625 ± 16 mgm./kgm.

[7] The LD_{50} of the arsenical as determined with groups of 15–20 rats to a dose was 190 ± 5 mgm./kgm. With 100 mgm. of ascorbic acid added (as the sodium salt), a dose of 200 mgm./kgm. killed 2/10 and the LD_{50} was greater than 240 mgm./kgm.

[8] The passage of a rapid current of air through a neutral solution of phenarsine for 2–3 hours causes only a slight discoloration, while neoarsphenamine undergoing the same treatment becomes quite dark.

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for the potential of the calomel electrode (−0.248v.), the E_o could be taken simply as the potential difference, since there were present an equal number of equivalents of the oxidized and reduced forms, in normal acid solution. The readings were made at room temperature (22°C.) and the results are given in table 4.

It is clear that the arsenicals would be most likely to oxidize while being transported in the blood stream. While they are stored in the liver or kidney they should remain unchanged. It does not appear from the data that the oxidation potential of blood is high enough to cause the oxidation of o-aminophenol groups. However, it has already been shown that even atmospheric oxygen is capable of causing this change to occur. The presence of an excess of ascorbic acid would be

TABLE 4

Oxidation-reduction potentials of some systems of interest in the arsenical series

SYSTEM	E_0	rH*
Mammalian liver		2.4 (12)
Mammalian kidney		3.1–6.4 (12)
Neoarsphenamine-½ sulfarsphenamine†	+0.310 v.	10.3
Arsphenamine-arsenoxide	+0.378 v.	12.6
Neoarsphenamine + 4 equiv. H_2O_2‡	+0.380 v.	12.7
Sulfarsphenamine + 2 equiv. H_2O_2‡	+0.390 v.	13.0
Ascorbic acid-dehydroascorbic acid	+0.390 v. (13)	13.0
Glyvarsenyl + 2 equiv. H_2O_2‡	+0.428 v.	14.3
Arsenoxide-arsonic acid	+0.580 v.	19.3
Human blood (pH 7.4)	$E_0' = 0.200$ v. (14)	21.5
o-Aminophenol-o-iminoquinone§	+0.790 v. (15)	26.3

*$rH = \frac{2F}{RT} E_m + 2\,pH$.

† I.e., this is the redox potential of the sulfoxylate group.

‡ The quantities of H_2O_2 added were intended to cause the oxidation of half of the arsenical to the arsenoxide stage.

§ This potential cannot be determined directly because the oxidation product exists only temporarily, if at all. However, the potential of the system p-aminophenol-p-iminoquinone has been measured and its "apparent oxidizing potential" is about the same as this system.

expected to decrease the normal rate of oxidation processes in the blood. It has been shown by Bembé and Dietrich (16) that the presence of an excess of ascorbic acid does, in fact, markedly decrease the oxidation-reduction potential of blood.

Biological investigation of the mechanism of ascorbic acid action. Sandground (17) has studied the mechanism of detoxication of pentavalent arsenicals by p-aminobenzoic acid by investigating the "time factor"; i.e., the change in the detoxifying effectiveness of the acid when it is injected at different time intervals with reference to the arsenical. He found that the acid is effective if injected up to three hours before the arsenical, but is markedly less effective if injected 30 minutes or more after the arsenical. Martin and Johnson (18) reported that ascorbic acid is most effective as a detoxicant for arsenicals if injected two hours

previously, in order to permit complete diffusion in the tissues. We (1) have not been able to confirm this.

In this study the hypothesis was adopted rather early that a) the effectiveness of ascorbic acid depends upon its being present at a high concentration in the blood while the arsenical remains in the circulation, and that b) the detoxication takes place in the blood, not in the tissues. In other words, the extent of the detoxication achieved is a function of the concentrations of ascorbic acid and arsenical measured at the first moment when both are present simultaneously in the blood stream. Support for this hypothesis was obtained in the experiments which follow.

TABLE 5

Effect of injecting neoarsphenamine and ascorbic acid (as the sodium salt), at different time intervals, on the mortality of albino rats

DOSE		TIME BETWEEN INJECTIONS	MORTALITY
Ascorbic acid	Neoarsphenamine		
A. Ascorbic acid followed by neoarsphenamine			
mgm./kgm.	*mgm./kgm.*	*minutes*	
0	425		7/10
100	425	0	0/8
100	425	10	3/10
100	425	30	6/10
150	425	30	9/15
200	425	30	2/11
300	425	30	0/5
B. Neoarsphenamine followed by ascorbic acid			
0	425		5/10
100	425	10	2/10
100	425	30	6/10
200	425	30	7/9
300	425	30	5/8
600	425	30	3/8
1000	425	30	4/7

All animals used as subjects in these experiments were albino rats from the laboratory colony, weighing from 80 to 120 gms., and from 35 to 50 days of age. They were fasted for 16 hours prior to the injections, which were made into the saphenous vein in the usual manner. Eight per cent solutions of neoarsphenamine and four per cent solutions of ascorbic acid (with enough sodium bicarbonate added to form the sodium salt) were injected. The time at which each animal received the first injection was noted, and, at exactly the time interval desired, the second injection was begun. Deaths observed within 6 days are recorded in table 5 (A)[9].

The data show, in brief, that 100 mgm./kgm. of ascorbic acid, if given simultaneously, will protect all animals from a dose of neoarsphenamine which, if given

[9] The author is indebted to Mr. Donald Seppelin, Miss Evelyn Brice, and Mrs. Charles Miller for carrying out the toxicity studies.

alone, would have killed about 60 per cent. The mortality rate is significantly increased if the ascorbic acid is given as much as 10 minutes before or after the neoarsphenamine and, if the time interval between the injections is increased to 30 minutes, this amount of ascorbic acid confers no protection whatsoever. Complete protection (i.e., 100 per cent survival) can be obtained by giving ascorbic acid 30 minutes before the arsenical, providing the dose is raised to a level of between 200 and 300 mgm./kgm. However, if the ascorbic acid is given 30 minutes after the neoarsphenamine, even 1000 mgm./kgm. confers no protection.

The results of part (A) of this experiment can be explained in terms of the blood levels of ascorbic acid which are observed following massive injections, as previously published (1). Thus, the injection of 400 mgm./kgm. of ascorbic acid results in a temporary plasma level of 430 mgm. per cent which has, in 30 minutes, decreased to about 120 mgm. per cent. From this it can be computed roughly that a dose of 100 mgm./kgm. would result in an intitial plasma level of 110 mgm. per cent, which would, after 30 minutes, decrease to between 30 and 40 mgm. per

TABLE 6

Effect of ascorbic acid on the retention of neoarsphenamine by tissues

GROUP	TIME AFTER INJECTION	DOSE		AVERAGE ARSENIC CONTENT OF TISSUES*	
		Neoarsphenamine	Ascorbic acid	Liver	Kidney
	hrs.	*mgm./kgm.*	*kgm./kgm.*		
A	24	200	0	4.72 ±0.27	9.20 ±0.64
B	24	200	50	5.26 ±0.26	11.90 ±0.50
C	24	300	75	6.34 ±0.16	14.88 ±0.75
D	48	200	0	2.86 ±0.22	6.74 ±0.37
E	48	200	50	2.60 ±0.28	5.20 ±0.42
F	48	300	75	2.62 ±0.34	7.56 ±1.34

* Expressed as mgm. of As_2O_3 per 100 gm. of fresh tissue ± standard error of the mean.

cent. Therefore, if the extent of detoxication depends on the blood level of ascorbic acid, the injection of 100 mgm./kgm. of ascorbic acid 30 minutes before the arsenical should result in the same mortality as if 30 mgm./kgm. were injected simultaneously with the arsenical. The latter dose is not sufficient to confer any protection (1), and none results here. However, if 100 mgm./kgm. were injected only 10 minutes before the neoarsphenamine, the blood level would be materially higher than 30–40 mgm. per cent when the arsenical was injected and some protection would be expected and is found here.

On the other hand, it can be reasoned that when ascorbic acid is given after the arsenical its effectiveness should be even more radically decreased. Neoarsphenamine is known to disappear very rapidly from the blood stream (about 75 per cent is actually removed within the first two minutes); consequently, if ascorbic acid acts only in the blood stream, it can act upon only the portion which remains. It appears, in fact, that after the lapse of 30 minutes it would be impossible to give enough ascorbic acid to confer complete protection.

Still another biological approach to this problem has been suggested by the work of Hogan and Eagle (19). They have shown that the amount of arsenic remaining in liver and kidney 24 or 48 hours after the intravenous injection of various substituted arsenoxides or arsonic acids is proportional to their toxicity. The effect of ascorbic acid on the retention and distribution of the arsenic of neoarsphenamine was studied by McChesney et al. (1). Although there was noted a slight tendency toward lower kidney concentrations of arsenic when ascorbic acid was given, the difference was not great enough to be impressive. So far as liver was concerned, the ascorbic acid made no difference in the retention of arsenic. In view of the findings of Hogan and Eagle, it has seemed worth while to repeat these experiments, but using smaller doses in order that, as suggested by these authors, the tissues would not be so altered by the toxic effects of the drug as to result ultimately in the death of the animal. The animals were prepared for the experiment as previously described (1). The tissues which were removed were kept frozen until the digestions could be carried out. Arsenic analyses were carried out by the method of Levvy (20), and the results are given in table 6. It is again demonstrated that the ascorbic acid has not altered the retention or distribution of the arsenic significantly. If the principles stated by Hogan and Eagle applied in this case it would be expected, for example, that the tissue levels of arsenic in groups A and C would be the same, and those of group B would be less than either. (The dose of 300 mgm. of neoarsphenamine plus 75 mgm. of ascorbic acid is about equivalent in toxicity to that of 200 mgm. of neoarsphenamine alone.)

Discussion of results. It has been generally supposed that when solutions of neoarsphenamine are exposed to the air they gradually undergo oxidation to the arsenoxide form. This is based on the observations of Rosenthal (7) and of Schamberg, Kolmer, and Brown (21). Rosenthal did not report how arsenoxide N-methanal sulfoxylate would react in his procedure, as he did not have the compound available. He was of the opinion that the side chain would split off during the oxidation. As has been pointed out, there is nothing specific about the Rosenthal color reaction for arsenoxide since other compounds which contain the o-aminophenol group give the same reaction. The only characteristics of arsenoxide upon which an analytical method can be based are the o-aminophenol group, the trivalent arsenic, and the relatively high solubility throughout the pH range. The validity of any analytical method, therefore, depends largely upon the extent to which a physical separation of arsenoxide from the other probable components of the mixture can be made. The precipitating agent which has been used in this work removes all but a small constant fraction of unchanged neoarsphenamine, most of the arsenoxide N-methanal sulfonate (and, presumably any other compounds containing the N-methanal sulfoxylate or sulfonate radical, since these would combine with the cetyl pyridinium radical). The reagent does not precipitate arsenoxide or arsonic acid, but the latter could be distinguished on the basis that its iodine titration is zero.

The method used in this work indicates that during oxygenation of neoarsphenamine solutions the following changes occur: a) a rather rapid drop in the iodine

titer from 10 equivalents per mole to a value which eventually approaches 4 equivalents per mole (this taken by itself would indicate complete oxidation to the arsenoxide stage since 2 equivalents per mole are required to oxidize the sulfoxylate group to sulfonate, and 4 equivalents to oxidize the arsenobenzene linkage to arsenoxide), b) a change in color from yellow to dark brown, indicating oxidation of the o-aminophenol groups, and c) no striking increase in the amount of arsenoxide; the amount formed is entirely inadequate to account for the change in iodine titer mentioned above. Thus, the oxidation process seems more complicated than can be formulated in terms of simple concepts.

The *in vitro* experiments demonstrate that ascorbic acid does not affect the rate of formation of arsenoxide from neoarsphenamine. It is capable of retarding somewhat the oxidation of o-aminophenol groups, but if the oxidation is continued long enough the end result is the same whether ascorbic acid is present or not. Ascorbic acid also retards the oxidation of the o-aminophenol group in arsenoxide.

The results of the *in vitro* experiments have been confirmed biologically. Thus in a solution of arsenoxide protected by ascorbic acid and exposed to the air there is a continuous decrease in toxicity with time, attributable to the oxidation of As^{3+} to As^{5+}. On the other hand, in a solution of arsenoxide unprotected by ascorbic acid there is an early increase in toxicity due to oxidation of the o-aminophenol group, followed by a decrease in toxicity as the arsenic atom is oxidized. Except at the outset the arsenoxide-ascorbic acid combination is always less toxic and less colored than the arsenoxide alone.

In aged neoarsphenamine solutions the situation is more complex. Initially the neoarsphenamine solution is about 40 per cent more toxic than a similar solution containing ascorbic acid. Both solutions become more toxic on exposure to air, but at about the same rate. If a neoarsphenamine solution is aged for three days in the absence of ascorbic acid, it becomes quite deeply colored and very toxic. However, if ascorbic acid is now added to it, it is no more toxic than a neoarsphenamine solution which contained the same amount of ascorbic acid during the time of the exposure to air. The ascorbic acid, therefore, does not actually prevent to any significant degree the chemical changes which cause neoarsphenamine to become more toxic on exposure to the air, but rather acts by detoxifying *in vivo* the neoarsphenamine which remains unchanged after the oxidation has been stopped. During the exposure to air the ascorbic acid probably remains essentially unchanged.

The *in vitro* experiments thus shed little definite light on the mechanism of ascorbic acid action. They demonstrate that the acid is capable of retarding the oxidation of o-aminophenol groups, but that it does not prevent or retard arsenoxide formation.

The *in vivo* experiments demonstrate that a high blood concentration of ascorbic acid is necessary during the time the neoarsphenamine is in the circulation if the detoxifying effect is to be observed. This may be related to the fact that the presence of an excess of ascorbic acid decreases the redox potential of the blood. However, redox potential is not the only consideration since both lactic acid and cysteine, which have lower redox potentials than ascorbic acid (22, 23), are inferior to ascorbic acid as detoxicants (1).

The *in vivo* oxidation of neoarsphenamine may affect the o-aminophenol groups primarily, if the analogy to the *in vitro* experiments is preserved. However, in the blood catalysts such as copper, iron, and hemoglobin are present and may alter the behavior of the ascorbic acid. About the only generalization which can be made is that ascorbic acid tends to have its greatest detoxifying effect on those arsenicals which are most susceptible to oxidation *in vitro*.

SUMMARY

A study of the *in vitro* and *in vivo* behavior of solutions of arsenicals, with and without ascorbic acid, has been made. Ascorbic acid appears to be capable of retarding oxidation of the arsenicals, particularly as it involves the labile o-aminophenol groups. It is thought that the administration of ascorbic acid with an arsenical decreases the oxidation-reduction potential of blood sufficiently to prevent immediate oxidation of the arsenical. *In vivo* evidence suggests that the site of the detoxication process is the blood stream, not the tissues such as liver and kidney. It is confirmed that ascorbic acid does not affect the retention of arsenic by liver and kidney.

A method for the analysis of a mixture containing neoarsphenamine, arsenoxide, and ascorbic acid is given. It is based on iodine titrations and physical separation of the neoarsphenamine by means of cetyl pyridinium chloride.

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THE RELATION OF VITAMIN C DEFICIENCY TO INTESTINAL TUBERCULOSIS IN THE GUINEA PIG

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Plates 26 to 28

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Intestinal tuberculosis is one of the most frequent and serious complications of phthisis. Up to the present time attempts at prevention and treatment have been empirical and unsatisfactory, because the factors involving the production of the intestinal ulcers have not been well understood. The swallowing of sputum laden with bacilli cannot be the sole cause of the condition since many patients swallow such sputum in large quantities for years without developing intestinal lesions, and the problem resolves itself with the question of why the intestines of some patients are susceptible and others resistant to the bacilli passing through them.

Numerous attempts have been made in the past by many workers to produce intestinal tuberculosis in the guinea pig by feeding either tubercle bacilli or tuberculous material. As a rule, the animals develop enlarged cervical or mesenteric lymph glands and die of generalized tuberculosis without developing ulcers in the intestinal tract, but success has been claimed by a few workers. From the reports of the following investigations it is difficult to determine whether or not the diets of their experimental animals were adequate in vitamins.

Villemin (8) in 1868 fed 40 gm. of tuberculous sputum to guinea pigs and killed the animals 3 months later. Lesions were found in the small bowel and cecum. Klebs (4) in 1870 fed guinea pigs tuberculous matter and found tuberculous ulceration in the intestines in two animals. Redman (7) in 1922 reported that in every instance in which he fed virulent tubercle bacilli to guinea pigs, the mesenteric lymph nodes became involved in 34 to 41 days. There were tuberculous nodules in the cecum and colon at 81 and 86 days. They were not open ulcers and no microscopic examinations were reported. Medlar and Sasano (6) in 1924 inocu-

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lated eight guinea pigs subcutaneously with human tubercle bacilli (No. H 37) and kept them for a period of 65 to 145 days. One animal had several ulcers in the cecum. Serial sections revealed tubercles in the lymphoid tissue of the small intestines in six of the remaining pigs. All the animals had extensive disease of the liver, and the authors felt that bacilli were discharged through the bile and thus infected the lymphoid tissue of the intestines. Griffith (3) in 1924 fed twelve guinea pigs with emulsion made from tuberculous tissue of animals infected with bovine tubercle bacilli. All of the animals were killed between the 43rd and 44th days, and all showed from one to many small caseous areas in Peyer's patches, the majority of which were ulcerated on the mucous suface. In the large intestines they were situated in the solitary follicles of cecum or colon and many were ulcerated.

One of us (5) has shown that patients with intestinal tuberculosis frequently make rapid and permanent improvement of their intestinal condition when given cod liver oil and tomato juice in addition to the regular hospital diet, while similar patients given the hospital diet alone do badly. Furthermore we have noticed that the addition of these vitamin carriers to the regular hospital diet seems to be a valuable prophylactic measure. These observations encouraged us to undertake the experiments on the rôle of certain vitamin deficiencies in the production of intestinal tuberculosis that are the subject of this paper.

Preliminary Experiments.—In February, 1928, we conducted a series of preliminary experiments. Forty-five guinea pigs were given a basic diet of hay, rolled oats, and autoclaved milk *ad libitum*. Of these animals, some were sensitized with non-virulent human tubercle bacilli (No. R 1 Saranac Lake Laboratory) and fed virulent bacilli (No. H 37 Saranac Lake Laboratory) in gelatin capsules; others were fed No. H 37 in capsules without being sensitized; some were confined to the basic diet, while others received in addition small quantities of cod liver oil and tomato juice.

Of twenty-five animals that were fed tuberle bacilli with a diet deficient in vitamins A, C, and D, two developed tuberculous ulcers of the cecum in addition to extensive tuberculosis elsewhere; while none of ten infected guinea pigs that received an adequate diet showed intestinal lesions at necropsy.

We learned that this basic diet was far too severe for our purpose, most of our animals dying of scurvy before the tubercle bacilli had time to produce intestinal lesions. We also found that adult guinea pigs withstood the deficiency of vitamins in their food much better than

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young animals. These preliminary experiments were far from conclusive but were sufficiently suggestive to induce us to continue the investigation.

In the following experiments we used only adult guinea pigs fed on a vitamin-poor basic diet without fruit or fresh vegetables.

This diet was composed of white bread (dried and ground) 20 per cent, chipped beef (ground and dried) 20 per cent, potato chips (ground) 20 per cent, rolled oats 15 per cent, skimmed milk powder 15 per cent, and white cane sugar 10 per cent. The various ingredients were thoroughly mixed and fed to the animals *ad libitum* along with hay and water. This diet was adequate in calories, in protein of good quality, in mineral salts, and in vitamin B_1 and B_2 (G). It was relatively deficient in vitamins A, C, and D. The animals ate the diet with great relish and gained weight for the first 2 or 3 weeks. The appetite then failed and they died in 6 to 8 weeks with evidence of scurvy. If, however, this basic diet was supplemented by 0.3 cc. of cod liver oil and 10 cc. of tomato juice daily, the pigs remained strong and vigorous and lived for months. Young pigs have been reared to maturity on this diet. Some became pregnant but aborted. There were necrotic changes in the placenta, which Evans and Bishop (2) have shown are characteristic of vitamin E deficiency. That there was nothing inherently injurious in this diet is shown by the experiment in which we replaced cod liver oil and tomato juice with cabbage leaves, and the animals gave birth to and reared normal young. By supplementing this basic diet with 2 or 3 cc. of tomato juice daily after the appetite began to fail, the guinea pigs were maintained for months in a prescorbutic state.

Experiment I. Jan. 4, 1929.—Fifty-two adult guinea pigs were used in this experiment.

1. Twenty-six normal adult guinea pigs (Groups A, B, C, and D) were used to determine the effect of various diets on healthy animals.

Group A. Ten normal adult pigs received a basic deficient diet only. These animals died between the 6th week and the 4th month. Two had duodenal ulcers at necropsy. Several developed a pyorrhea-like condition about the teeth; pus from the lesions showed the presence of spirochetes, fusiform bacilli, and cocci. One developed a pulmonary lesion in which these organisms were also present. Five of the ten animals lost one or more incisor teeth. Some of the animals presented evidence of scurvy and some did not.

Group B. Five normal adult pigs received a basic diet plus 0.3 cc. of cod liver oil daily. The oil was given by mouth with a tuberculin syringe. These animals died between the 6th week and the 3rd month. Several had a pyorrhea-like condition of the gums and lost incisor teeth. One animal had a few small necrotic areas in the cecum.

Group C. Five normal adult pigs received a basic diet plus 10 cc. of tomato juice daily. The juice was given with a pipette. These animals did very well for 2 months. Then two of them died immediately after giving birth to young. The

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mothers showed no evidence of scurvy but the new-born pigs showed marked signs of this disease. The remaining three animals were killed in the 5th month at the end of the experiment and found to be normal.

Group D. Six normal adult pigs received a basic diet plus 0.3 cc. of cod liver oil and 10 cc. of tomato juice daily. These animals did well for 2 months. Two pigs died of spontaneous pulmonary disease between the 2nd and the 3rd month, and the remaining four were killed in the 5th month at the end of the experiment and found to be normal.

2. Twenty-six adult guinea pigs (Groups E, F, G, and H) were used to determine the effect of various diets on tuberculous animals. Each pig was sensitized by a subcutaneous injection of non-virulent human tubercle bacilli (No. R1 Saranac Lake Laboratory) and subsequently fed 0.5 cc. of tuberculous sputum with a tuberculin syringe daily.

Group E. Ten of the above pigs received a basic diet and 0.5 cc. of tuberculous sputum daily. These animals died between the 2nd and 5th months. Three animals which died early were found to have a normal intestinal tract. Five animals presented one or more open tuberculous ulcers in the intestines and two animals showed caseous tubercles in the lymph follicles of the ileum which had not ulcerated. The ulcerative lesions were most common in the ileum, with cecum next, and jejunum and colon least frequently involved.

Group F. Six sensitized adult pigs received 0.5 cc. of tuberculous sputum daily, and were fed in addition to the basic diet 0.3 cc. of cod liver oil daily. These animals died between 6 weeks and 4 months. One animal had a normal intestinal tract, and one had non-ulcerated caseous tubercles in the ileum, while the remaining four presented from one to twenty open tuberculous ulcers in the ileum, cecum, jejunum, and colon.

Group G. Five sensitized adult pigs received 0.5 cc. of tuberculous sputum daily, and were fed in addition to the basic diet, 10 cc. of tomato juice daily. These animals all did well and were killed in the 5th month at the end of the experiment. One animal had a caseous tubercle in the ileum which had not ulcerated. The other four animals presented normal intestinal tracts, although there was extensive tuberculosis in the liver, spleen, and lungs.

Group H. Five sensitized adult pigs receiving 0.5 cc. of tuberculous sputum daily were fed in addition to the basic diet 0.3 cc. of cod liver oil and 10 cc. of tomato juice daily. These animals all did well except one which died of unknown causes in the 10th week. The remaining four were killed in the 5th month at the end of the experiment. The intestinal tracts of all five animals were normal although considerable tuberculosis was present in the liver, spleen, and lungs.

Discussion of Experiment I

Normal adult guinea pigs kept on the vitamin poor diet described died in from 6 to 16 weeks; some showed definite evidence of scurvy, while others presented a variety of lesions ascribable to the deficient

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diet. Cod liver oil did not prevent the development of these lesions, but the addition of 10 cc. of tomato juice daily to the basic diet protected the animals completely from all signs of deficiency disease.

Sensitized guinea pigs maintained on the basic diet and fed 0.5 cc. of tuberculous sputum daily developed ulcerative tuberculous lesions in the intestines in five out of ten cases. When cod liver oil was added to the basic diet, four out of six animals developed ulcerative intestinal tuberculosis. When, however, the basic diet was supplemented with 10 cc. of tomato juice daily, the animals remained in good health and only one out of ten showed any trace of intestinal tuberculosis.

To recapitulate—of the sixteen sensitized guinea pigs on diets deficient in vitamin C or in vitamins C, A, and D, which received 0.5 cc. of tuberculous sputum daily, nine presented one or more open tuberculous ulcers of the intestines and three presented caseous non-ulcerative tubercles while only four animals presented normal intestines at necropsy. In contrast, nine out of ten guinea pigs presented normal intestinal tracts when the basic diet was supplemented either with tomato juice or cod liver oil and tomato juice. The remaining animal showed a few non-ulcerative tubercles in the ileum.

Experiment II. Mar. 15, 1929.—Forty-six normal adult pigs were used in this experiment (Groups I, J, K, L, and M). With the exception of ten animals in Group M, all the pigs received three doses of approximately 1 mg. of virulent human tubercle bacilli (No. H 37, Saranac Lake Laboratory) by mouth between Mar. 15 and 20. They were given our regular stock diet until Apr. 15 when a number of the pigs presented palpable glands in the neck. The animals were then divided into five groups.

Group I. Sixteen of these animals were given the basic diet described in Experiment I, and 0.5 cc. of tuberculous sputum daily. Eleven of the sixteen pigs died between the 6th week and the 3rd month. The remaining five were killed at the end of the 4th month. Necropsy showed that thirteen pigs had from one to ten tuberculous ulcers in the intestines, two presented caseous non-ulcerative tubercles in the ileum, and only one intestinal tract was normal. In addition, three of the animals had duodenal ulcers and one a pyloric ulcer, all of which were shown to be non-tuberculous on microscopic examination.

Group J. Five adult guinea pigs received 0.3 cc. of cod liver oil and 0.5 cc. of tuberculous sputum in addition to the basic deficient diet. Three of the five pigs died between the 2nd and 3rd months, the remaining two were killed at the end of the 4th month. One animal had numerous caseous non-ulcerative tubercles in the ileum and cecum, and the other four had from one to fifteen tuberculous ulcers in the intestines. One animal had a small duodenal ulcer that was not tuberculous.

Group K. Five adult guinea pigs received 10 cc. of tomato juice daily and 0.5 cc. of tuberculous sputum in addition to the basic diet. These animals were killed at the end of the experiment. Although they had extensive generalized disease, the intestinal tracts of all of them were normal.

Group L. Ten adult guinea pigs received 0.3 cc. of cod liver oil, 10 cc. of tomato juice, and 0.5 cc. of tuberculous sputum daily as well as the basic diet. One of the ten animals died in the 6th week; this animal had refused to swallow any but a small amount of the tomato juice and necropsy revealed numerous open tuberculous ulcers in the intestines. The remaining nine animals were killed at the end of the 4th month. One animal had an open tuberculous ulcer and one had a few caseous non-ulcerative tubercles in the ileum. The remaining seven guinea pigs presented normal intestinal tracts.

Group M. Ten adult guinea pigs which had not been given No. H 37 by mouth received a limited amount of cabbage leaves and 0.5 cc. of tuberculous sputum in addition to the basic diet. These animals did well and were killed at the end of the experiment. At necropsy, none of them presented open tuberculous lesions. However, five had from one to ten caseous non-ulcerative tubercles in the lymph follicles of the ileum. The remaining five guinea pigs showed normal intestinal tracts.

Discussion of Exeriment II

The non-sensitized animals in Experiment II showed much more extensive generalized disease than the sensitized animals in Experiment I, in which the animals had been previously sensitized by a subcutaneous injection of R1. Intestinal lesions developed more frequently in the non-sensitized than in the sensitized animals when fed tuberculous sputum and maintained on a deficient diet. Of the twenty-one animals which were given a diet deficient in vitamin C or in A, C, and D, seventeen developed open tuberculous ulcers, three caseous non-ulcerative lesions, and the intestinal tract of only one animal was normal at necropsy. It was also more difficult to protect these non-sensitized animals against the lesions in the intestines even when adequate diet was given. Twenty-five guinea pigs received either tomato juice, cod liver oil and tomato juice, or cabbage leaves in addition to the basic diet. Seventeen of these animals had normal intestinal tracts at necropsy. Six presented one or more caseous non-ulcerative tubercles in the ileum, while two presented from one to ten open tuberculous ulcers. Two of the animals with ulcers were known to have consistently refused to take the full amount of tomato juice. Summarizing the results of Experiments I and II,

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we obtain the following figures: of thirty-seven animals on diets deficient in vitamin C or in vitamins A, C, and D, twenty-six developed from one to fifteen open tuberculous lesions of the intestines, six developed caseous tubercles which were not ulcerative, and five presented normal intestinal tracts. In contrast, of thirty-five animals which received either tomato juice, cod liver oil and tomato juice, or cabbage leaves, twenty-six presented normal intestinal tracts at necropsy, seven showed from one to ten caseous tubercles which were not ulcerative, and only two had open ulcers.

Pathology

The gross and microscopic appearance of the tuberculous lesions in the intestines of the guinea pig are similar to the tuberculous lesions described by Gardner (1) in man, and by Medlar and Sasano (6) in both man and the guinea pig. The primary lesion is a submucous tubercle in the lymphoid tissue of the intestinal tract. In the early stages, the mucosa over the lesion is intact (Fig. 2). Later the submucous tubercle becomes caseous, ruptures through the mucosa, discharges its contents, and becomes an ulcer (Figs. 1 and 3). The earliest stage of the process which we have studied is shown in Fig. 4. In gross, this solitary follicle seemed somewhat swollen, but otherwise normal. On section, numerous tubercle bacilli were found in large mononuclear cells, some of which showed evidence of degeneration. No giant or epithelioid cells were present in this lesion (Fig. 5). In Fig. 6 an acute ulcerating lesion is shown in which tubercle bacilli were found in large mononuclear cells and in giant cells. Very little necrosis was present. In a more advanced stage of the process, as shown in Fig. 7, the cells near the surface were necrotic. Beneath this was typical tuberculous tissue with giant cells, epithelioid cells, lymphocytes, leucocytes, and fibroblasts. A more chronic tuberculous ulcer is shown in Fig. 3. The necrotic zone has been expelled, fibroblasts were present, and there was a marked infiltration of the lesion with lymphocytes.

Our object in these experiments was to determine if possible the part played by vitamin C deficiency in the pathogenesis of intestinal tuberculosis in the guinea pig. In order to do this we compared the intestinal lesions due to scurvy with those of tuberculosis. We found

that in the early scorbutic lesions submucosal hemorrhage was the outspoken feature; the mucosa overlying the area of hemorrhage becomes necrotic, sloughs, and forms an ulcer (Figs. 9–11). The initial tuberculous lesion also occurs in the submucosa (Figs. 2, 12) and apparently goes on to ulceration (Fig. 3) when there is not sufficient vitamin C in the diet. Apparently, an adequate supply of vitamin C in the diet protects the guinea pig against ulcerative intestinal tuberculosis, even in the presence of submucous tubercle.

The guinea pig and man appear to be similar in their vitamin C requirements. Patients with intestinal tuberculosis improve when tomato juice is added to their diet, while our tuberculous guinea pigs develop intestinal lesions unless protected by an adequate supply of vitamin C in their food.

Our experiments however, do not explain the clinical value of cod liver oil in intestinal tuberculosis. Man is quite susceptible to vitamin A and D deficiencies, while the guinea pig is not. It is thus possible that a deficiency of vitamins A and D (the vitamins of cod liver oil) plays a part in the development of intestinal tuberculosis in man and not in the guinea pig. Before this part of the problem can be settled it will be necessary to repeat our experiments utilizing an animal such as the monkey which is susceptible to the same vitamin deficiencies as man and also to tuberculosis.

CONCLUSION

1. Seventy-two adult guinea pigs were fed tuberculous sputum daily for periods ranging from 6 weeks to 4 months.

2. Thirty-seven of these were maintained on a diet partially deficient in vitamin C; twenty-six developed ulcerative intestinal tuberculosis.

3. In the remaining thirty-five animals whose diet was supplemented by an adequate amount of vitamin C only two developed tuberculous ulcers in the intestines.

4. From these studies we conclude that the ingestion of tubercle bacilli by the guinea pig is not the sole factor in the production of intestinal tuberculosis.

5. In our opinion, an adequate supply of vitamin C usually protects the guinea pig against ulcerative intestinal tuberculosis.

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EXPLANATION OF PLATES

Plate 26

Fig. 1. Photograph of tuberculous ulcers from the small intestine of a guinea pig. ×2.

Fig. 2. Low power photomicrograph of a submucosal tubercle in the intestine of a guinea pig. The mucosa is still intact. ×35.

Fig. 3. Low power photomicrograph of a chronic tuberculous ulcer in the intestines of a guinea pig. ×35.

Fig. 4. Low power photomicrograph of an early tuberculous involvement of a solitary follicle in a guinea pig. See Fig. 5. ×20.

Plate 27

Fig. 5. Camera lucida drawing of a section from early tuberculous ulceration of a solitary follicle in the intestines of a guinea pig. The tubercle bacilli are present only in monocytes. Some of the monocytes are degenerating. A low power photomicrograph of the section from which this drawing was made is shown in Fig. 4. Approximately ×800.

Fig. 6. Camera lucida drawing of a section from an early tuberculous ulcer in the intestines of a guinea pig. Tubercle bacilli are seen in monocytes and in giant cells. Note the dilatation of the capillaries and the proliferation of fibroblasts. Approximately ×800.

Fig. 7. Camera lucida drawing from a section of an extensive tuberculous ulcer in the intestines of a guinea pig. The superficial portion of the lesion is entirely necrotic. Beneath this layer one sees polymorphonuclear leucocytes in various stages of degeneration. Fibroblasts, lymphocytes, monocytes, and giant cells are seen in the depth of the lesion. Approximately ×800.

Fig. 8. Camera lucida drawing of a giant cell in a submucosal tubercle in the intestines of a guinea pig. Low power photomicrograph of the lesion from which this drawing was made is shown in Fig. 2. Approximately ×800.

Plate 28

Fig. 9. Low power photomicrograph of acute scorbutic ulcer in the ileum of a guinea pig. ×15.

Fig. 10. Low power photomicrograph of acute scorbutic ulcer in the ileum showing hemorrhage in mucosa and submucosa. ×90.

Fig. 11. High power photomicrograph of section shown in Fig. 10. Note marked degeneration of mucosa and absence of cellular reaction. ×390.

Fig. 12. High power photomicrograph of the mucosa overlying the caseous tuberculous lesion seen in Fig. 2. Note the accumulation of well preserved lymphocytes and monocytes in contrast to the ischemic necrosis found in the scorbutic lesion shown in Fig. 11. ×430.

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III. Dietary Aspects

Gingivitis—Ascorbic Acid Deficiency in the Navajo[1]

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THIS DIETARY study was part of a larger study to determine whether the gingivitis seen in the Navajo students at the Intermountain Indian School, Brigham City, Utah, was related to an ascorbic acid deficiency. During routine dental examination of children at this school, dental officers noted the frequent occurrence of a gingivitis which they felt might be related to low ascorbic acid intake. Administration of large doses of ascorbic acid in several cases resulted in dramatic improvement of the gingiva and further strengthened this opinion.

The amount of ascorbic acid ingested by students during the summer months was not known, but was believed to be low. In a study of nutritional status of Navajo Indians on the reservation in 1955, Darby *et al.* reported (1) gingival lesions as the most prevalent stigma. Serum ascorbic acid determinations were low, and the dietary sources of ascorbic acid were limited. They indicated that insufficient ascorbic acid was probably a secondary factor.

In a study of spongy, bleeding gums in New Mexico in 1953 (2), school children responded satisfactorily, but rather slowly, to the administration of 100 mg. ascorbic acid daily. Dietary histories indicated a low intake of foods supplying ascorbic acid.

During the school year the condition of the gums of many Navajo students improved. This paper reports an attempt to determine the ascorbic acid intake the children at the school.

Methods

Dental officers of the School Health Center selected 233 pupils of the Intermountain Indian School to take part in the study. An inflamed gum condition described as hemorrhagic hyperplastic gingivitis was the basis for selecting 186 students. For each two students with inflamed gums, one student without gingivitis was chosen as a control, i.e., a total of 97 controls. Examinations were made during the first week of the 1959-60 school year. Of the approximately 2050 pupils examined, about 10 per cent had the type of gingivitis described. Cases of severe gingivitis due to poor oral hygiene were not included.

During the initial examination, blood samples were taken for determination of ascorbic acid of the white blood cells. Analyses of these and subsequent samples were made at the Agricultural Experiment Station, Utah State University, Logan.

The students with gingivitis were divided into two paired groups by matching age, sex, and gum condition (Groups I and II). The controls were also divided into paired groups (Group III and IV). The students ranged in age from eleven to twenty-two years. For convenience, the four students over nineteen years old were included in the sixteen- to nineteen-year group. Groups I and III were given 300 mg. ascorbic acid in tablet form daily in divided doses for twenty-one days. Groups II and IV received placebos.

The number of students in each group is given in Table 1. The ratio of girls to boys enrolled in school was 9:11. The ratio of girls to boys in the study was 4:3. This preponderance of girls (160 girls to 122 boys) follows the impression that the gingivitis occurs a little more frequently among the girls.

At the end of the three-week period, dental examinations were repeated and a second blood sample taken. Six months later, the students were examined again and a third blood sample taken. Group II received an ascorbic acid supplement for three weeks following the study period. An additional blood sample was taken of those children at the end of that time. All others received ascorbic acid only from food sources during the six months.

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The ascorbic acid available from the foods served in the cafeteria was estimated. Recipes for mixed dishes and changes in the master menu were supplied by the dietitian. Ascorbic acid in the mixed dishes was calculated, using the tables in Agriculture Handbook No. 8 (3). Observation of foods served showed that the amounts placed on individual trays varied, but that common household units were reasonably accurate measures of the average servings.

Students kept dietary records of their individual food intake on the day before the study began and and on five days of the twenty-one-day period, i.e., the first, second, eighth, fifteenth, and last days. The records were turned in to the Health Center on the morning after each day's intake was recorded. All but one of the 283 students completed their dietary records.

Ascorbic acid intake for each day's record was calculated from the values of recipes and the tables in Agriculture Handbook No. 8. Inquiries of the Consumer and Food Economics Research Division, U.S. Department of Agriculture about some unlisted food items and ascorbic acid losses in storage and preparation of certain foods brought information and comments (4) on more recent analyses. This unpublished information indicated that ascorbic acid content of foods with less than 5 mg. per serving could be omitted in these evaluations. The foods omitted were: milk, carrots, string beans, baked beans, corn pudding, beets, canned pears, sliced onions, stewed dried fruits (peaches, apricots, apples), fruit pies (apple, cherry, pineapple), fruited gelatin, and ice cream.

Later, values of the foods on the menus were checked using Home and Garden Bulletin No. 72, issued September 1960 (5). Approximately half of the values remained the same as those given in Agriculture Handbook No. 8. The other half differed less than 3 mg., except for raw oranges, (3 in. diam.) which were 11 mg. less. The trend was two to one toward slightly lower values. Most of the changes involved foods which had values of less than 10 mg. per serving, and most of these had already been dropped from the estimates. The remaining differences produced no demonstrable effect on the findings. Loss of ascorbic acid due to length of storage, storage conditions, holding of foods, or additional cooking of potatoes, cabbage, canned fruits and vegetables in the situation at Intermountain is not known.

Food Consumption

Students came from all sections of the Navajo Reservation. A child might eat at home one day and arrive for breakfast at Intermountain the following day, or he might spend from one to three days at a collection point on the Reservation before leaving for Intermountain. The collection points are boarding schools where the children are fed and housed until time for the bus to leave.

The ascorbic acid in foods consumed at home varied, but it is presumed to have been low and irregular. An estimate of the ascorbic acid content of the menus served at the collection points was made from the master menus supplied by the food service supervisor of the Gallup Area Bureau, Indian Affairs. This estimate showed an average of 50 mg. ascorbic acid daily with a range of 27 to 76 mg.

The master menu at the Intermountain School was adjusted during the arrival period so that the ascorbic acid level would remain approximately the same until all students had been examined and the subjects for the study selected. During the seven days just prior to the study, mean ascorbic acid in single average portions of the foods served in the school cafeteria was 35 mg. daily, with a range from 20 to 50 mg.

THE STUDY PERIOD

Most of the foods eaten by the students came from the school cafeteria. A master menu with a four-week cycle is prepared by the dietitian and scheduled for the entire school year. Changes are made in individual meals to take advantage of seasonal food and for other reasons. "Seconds" (foods left over from a preceding meal) are served at times. Students may take "seconds" in addition to or instead of foods on the menus.

The menus as served in the cafeteria between August 26 and September 15 provided an average of 86 mg. ascorbic acid daily, with a range of 36 to 146 mg. Of the twenty-one days, eight exceeded

TABLE 1 Ascorbic acid intake as estimated from five one-day dietary records kept by students

GROUP	ASCORBIC ACID INTAKE								
	Girls			Boys			All students		
	Number	Range	Mean	Number	Range	Mean	Number	Range	Mean
		mg./day	*mg./day*		*mg./day*	*mg./day*		*mg./day*	*mg./day*
I	54	24–95	62	38	28–120	79	92	24–120	69
II	56	29–97	63	37	47–113	80	93	29–113	70
III	24	25–87	58	23	23–113	75	47	23–119	67
IV	26	32–84	62	24	10–112	75	50	10–112	68
Total . . .	160	24–97	62	122	10–120	78	282	10–120	69

100 mg. ascorbic acid; on two days, between 75 and 99 mg. were supplied; and on eleven days, less than 75 mg. were provided. On just over half the study days, ascorbic acid in the menu was below the recommended allowance (6) for any student.

Foods from other sources contributed to the ascorbic acid of the diet. Students could buy food at the coffee shop operated by the Home Economics Department and at the canteens. At food parties in classrooms and in food preparation classes, various foods were eaten. At times, groups of students harvested fruit or tomatoes at nearby orchards or farms, usually eating some of the fruit they picked. Some worked part-time on jobs where they ate a meal away from school. A fruit stand and market was also located across the street from the campus.

A total of 1335 individual dietary records was collected during the five days of the study period. Of these, 757 were from girls; 578 from boys, representing 95 per cent participation. Mean ascorbic acid intake for each child was estimated from the five one-day dietary records. When a student failed to turn in a record, mean intake was estimated from the records available. These findings are given by groups in Table 1.

Comparison of the average mean intakes by groups showed no significant differences. For all children, it was approximately 17 mg. less than the estimated amount of ascorbic acid available in the master menu.

Mean intake of ascorbic acid for each student was compared with his recommended allowance (Table 2). More boys consumed diets supplying between two-thirds and 100 per cent of the allowance for ascorbic acid and more girls had intakes below two-thirds of the recommendation.

Because the recommended allowance for ascorbic acid varies by age and sex, mean intakes of the students were studied by age groups. The ten- to twelve-year-old girls and boys had intakes which were relatively close together, 71 and 77 mg., respectively. Girls in the thirteen- to fifteen and sixteen- to nineteen-year brackets had intakes of 60 and 61 mg., while boys of these ages had intakes of 77 and 78 mg. This divergence with age is more apparent when plotted in Figure 1.

The percentage of students with diets furnishing less than two-thirds of the allowance for ascorbic acid increased with age, going from 9 per cent in the ten- to twelve-year group to 25 per cent in the thirteen- to fifteen-year-olds, to 27 per cent in the oldest group. The number and percentage of students by sex in each age group and the extent to which the allowance was met are shown in Figure 2.

Mean intakes were used to determine the ascorbic acid intake in relation to weight of each child. There were no significant differences between groups.

Clinical and Laboratory Findings

BLOOD ANALYSIS (7)

Initial mean white cell-platelet ascorbic acid values were significantly lower for students with gingivitis than the values for the control groups. All groups had mean white cell ascorbic acid values well below normal; more students with gingivitis had values within the deficient and scorbutic levels.

Response to 300 mg. ascorbic acid daily for three weeks as a dietary supplement is shown graphically in Figure 3. Mean ascorbic acid content of the white cells of students in Groups I and III increased from 14 and 17 mg. to 25 mg. Groups II and IV, deriving their ascorbic acid only from food sources, had values which increased from 12 and 16 mg. to 17 and 18 mg. In Group II, who consumed the school diet for three weeks and then received 300 mg. ascorbic acid supplement daily for three weeks, mean white cell ascorbic acid value reached 32 mg. This high level could not be maintained on the cafeteria diet.

Six months after the initiation of the study, all groups had normal white cell ascorbic acid values that were similar.

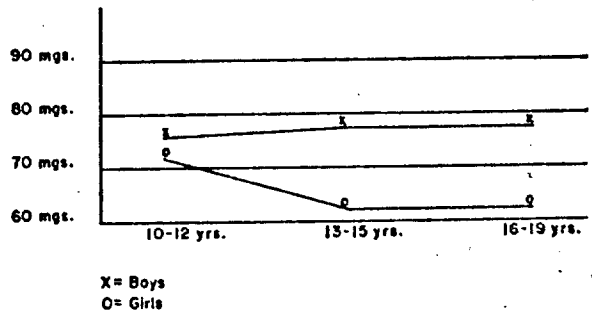

FIG. 1. *Mean intakes of ascorbic acid by age group and sex.*

TABLE 2 Extent to which individual mean intakes from five one-day dietary records of students met the recommended allowances for ascorbic acid

SEX	100 PER CENT OF RECOMMENDED ALLOWANCE		67 to 99 PER CENT OF RECOMMENDED ALLOWANCE		66 PER CENT OR LESS OF RECOMMENDED ALLOWANCE	
	Number	Per cent of students	Number	Per cent of students	Number	Per cent of students
Girls	20	13	93	58	47	29
Boys	29	24	74	61	19	15
Total	49	17	167	59	66	24

GUM CONDITIONS (8)

Gum conditions of paired Groups I and II were similar on initial examination. At the end of three weeks, during which Group I received 300 mg. ascorbic acid supplement daily, the gum conditions in both groups had improved, but the improvement in Group I was markedly greater. Six months later, the gum conditions in the two groups were almost identical. Results indicate that the daily ascorbic acid supplement to the diet contributed to the earlier response and healing of the hemorrhagic hyperplastic gingivitis in approximately 10 per cent of these Indian students.

GENERAL HEALTH STATUS

The students in this study were participating in the regular school activities and were considered as healthy as most school children, having the usual number of colds, respiratory and skin infections.

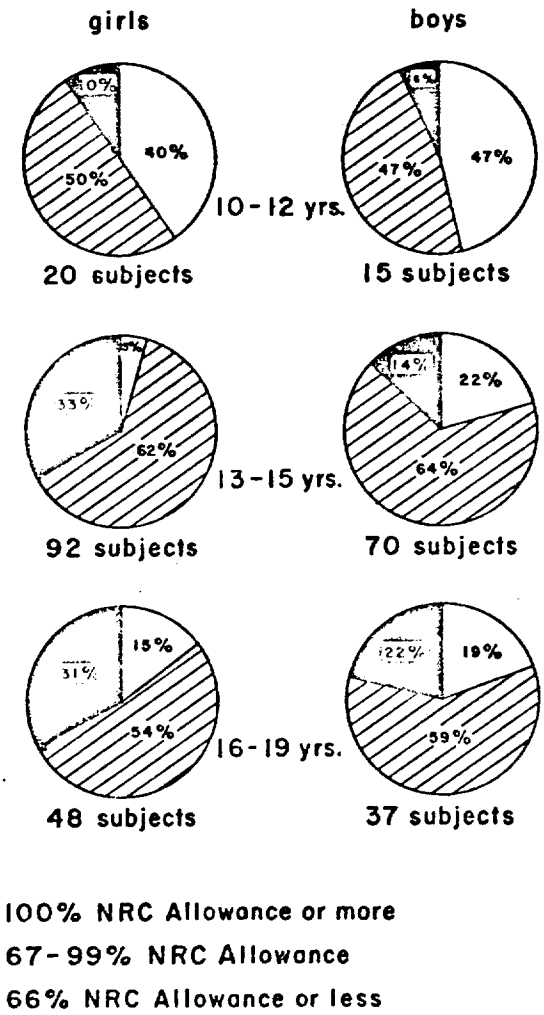

FIG. 2. *Mean ascorbic acid intake of students of three age groups compared with recommended allowances for age and sex.*

The charts at the School Health Center revealed no significant difference between any groups in the study during the three-week period.

Discussion

In classifying the dietary intakes of ascorbic acid, three levels (9) were used as standards: those providing 100 per cent or more of the recommended allowance; those supplying between two-thirds and 99 per cent; and those below two-thirds. The mean intakes indicated on the dietary records of the Navajo students were placed in these categories and compared with other studies. The percentage of students below the two-thirds mark was approximately the same as reported elsewhere, but the percentage with intakes of 100 per cent or more was somewhat less. Mean intake of ascorbic acid for girls (62 mg.) and for boys (78 mg.) differed only slightly from reports for the nation as a whole (girls, 70 to 72 mg.; boys, 75 to 90 mg.) (10, 11).

Dietary records showed that the Navajo boys tended to eat larger servings of food, to take "seconds" more often, and to acquire more foods from sources outside the cafeteria than the girls. The

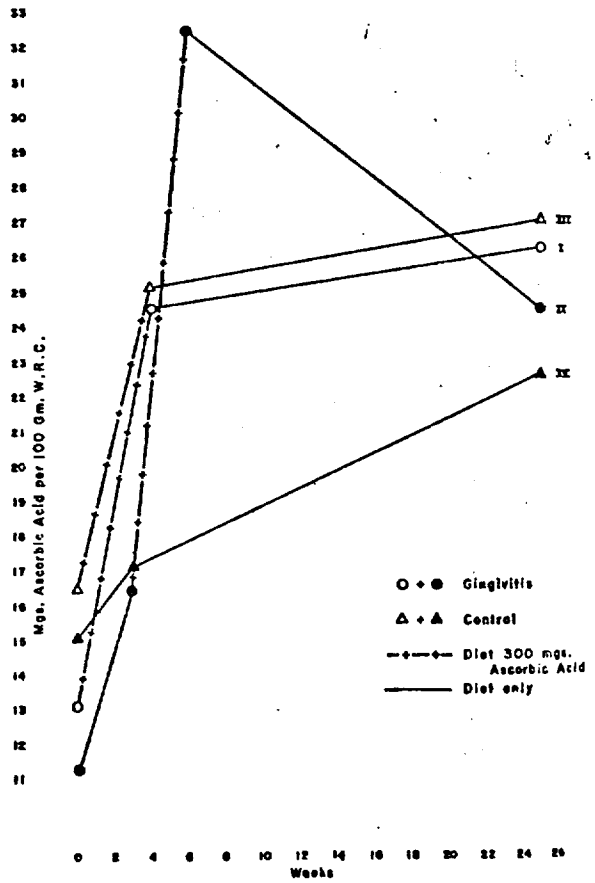

FIG. 3. *Effect of different levels of ascorbic acid intake on mean ascorbic acid blood levels (data taken from reference 7).*

larger servings and food from other sources increased their ascorbic acid intake. Most "seconds" contained little ascorbic acid. In spite of their generally better showing, 22 per cent of the older boys had ascorbic acid intakes below the two-thirds level.

The use of standard tables of food composition (3, 5) without omitting the low value and less reliable food sources of ascorbic acid, may be a source of some error in estimating dietary intakes of ascorbic acid. Mean ascorbic acid intake of our subjects, estimated as described, was 69 mg. This same diet, using standard tables, shows an intake of 82 mg. ascorbic acid daily—a difference of 13 mg. This average of 69 mg. is below the recommended allowance for the youngest group (75 mg. for ten- to twelve-year-olds), yet our subjects showed improvement in gum condition and maintained a normal ascorbic acid level on this diet. Low intake prior to this study and the liberal "margin of safety" incorporated in the recommended allowances may be an explanation for the difference. Variations in the use of food tables may be another explanation for some of the poor correlations reported between calculated dietary intakes and clinical and biochemical findings.

This study provides further evidence that ascorbic acid is one of several nutrients essential to good health. Findings in Figure 3 suggest that dietary interrelationships or general nutritional status influenced the response to ascorbic acid supplementation. Group II, with the lowest initial white cell ascorbic acid level, moved in three weeks from 12 mg. to 17 mg. on foods from the cafeteria. These foods, in addition to supplying ascorbic acid, provided protein, carbohydrates, fats, minerals, and other vitamins in amounts probably much greater than the students had been consuming during the summer. During the second three-week period, these students rceived a supplement of 300 mg. ascorbic acid daily, at the end of which time, white cell ascorbic acid values averaged 32 mg., a saturated level.

Group I, which was paired with Group II and had an initial white cell ascorbic acid level of 14 mg., received the same supplementation, but during the first three weeks of school. They reached a level of 25 mg., but did not achieve saturation. It should be noted that the saturated state of Group II could not be maintained on their usual food intake alone, but that all groups had normal white cell ascorbic acid values at the end of six months, including Group IV who received ascorbic acid only from food sources.

Summary

A series of five one-day dietary records kept by 282 Navajo Indian students at Intermountain Indian School were evaluated to help determine whether the gingivitis seen in 10 per cent of the students was related to an ascorbic acid deficiency.

Mean dietary ascorbic acid intake was 69 mg. The average for the 160 girls was 62 mg.; for the 122 boys, 78 mg. There were no significant differences in intake between any study groups.

Seventeen per cent of the students had food intakes meeting their recommended allowance for ascorbic acid. Twenty-four per cent consumed less than two-thirds of their allowance. More children with adequate intakes were in the ten- to twelve-year group. The percentage of subjects having deficient intakes of ascorbic acid increased with age, reaching 27 per cent in the sixteen- to nineteen-year group. More boys met their allowance than girls.

Dietary ascorbic acid intake was estimated after omitting from the calculations those foods which provided less than 5 mg. ascorbic acid per serving. Comparison of these findings with estimates for the same diet using standard tables of food composition revealed an average difference of 13 mg. daily. Neither set of figures allowed for loss of ascorbic acid in storage, due to cooking practices, by holding foods on steam table, or by reheating foods. Use of standard tables, without adjustment, for estimation of dietary intakes may yield falsely high levels. This, along with the liberal "margin of safety" in the recommended allowance and the inherent errors in reporting and calculating data, offers a possible explanation of some of the poor correlations reported between dietary and clinical and biochemical findings.

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RESEARCH

ASCORBIC ACID CONTENT OF TOMATOES AS AFFECTED BY HOME CANNING AND SUBSEQUENT STORAGE, AND OF TOMATO JUICE AND FRESH ORANGE JUICE AS AFFECTED BY REFRIGERATION

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THE widespread use of canned tomatoes as a source of vitamin C in the diet occasions interest in the stability of this vitamin under various treatments and canning methods. The work published prior to the development of a method for chemical determination of ascorbic acid was based principally on biological assays using guinea pigs. The length of these biological tests made storage of the food an unavoidable factor, and in addition the error introduced by the animal variations was likely to be greater than the effect of the treatment used on the food assayed. The introduction of Tillmans's reagent (1, 2, 3) as an indicator for determining ascorbic acid has made it possible to secure values with a high degree of accuracy and within a short time after the receipt of the sample.

Several reports have appeared in the literature giving values for the ascorbic acid content of tomatoes and various tomato products, determined by chemical methods. Maclinn, Fellers, and Buck (4) have published the results of an extensive study concerning the ascorbic acid content of fresh tomatoes of different varieties and strains.

Bailey (5) has reported that the vitamin C value of commercially canned tomato juices representing the brands approved by the Council on Foods of the American Medical Association varies from 0.13 to 0.29 milligram per gram. A study (6) recently completed in this laboratory shows that commercially canned tomatoes may vary in ascorbic acid content from 0.13 to 0.22 milligram per milliliter, and commercially canned tomato juice from 0.08 to 0.26 milligram per milliliter. The variation was shown to be as great from sample to sample of the same brand as between brands.

Tressler and Curran (7) have conducted experiments on the relation of head space to the destruction of ascorbic acid in commercially bottled tomato juice. They found that the destruction of the vitamin in storage was proportional to the amount of oxygen in the container.

Daniel and Rutherford (8) reported that both home canning and subsequent storage caused a significant loss in the ascorbic acid content of tomatoes and tomato juice preserved in tin and in glass containers. Hauck (9) has recently reported that the reduced ascorbic acid of freshly opened tomato juice canned in tin was more stable than samples from the same lot of juice canned in glass. The former lost approximately one fourth of its reduced ascorbic acid when stored in the refrigerator for 4 days after opening.

The present experiments were an attempt to obtain further information regarding any losses in ascorbic acid which might take place as a result of (1) different methods

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in the home canning of tomatoes, (2) storing canned tomatoes exposed to light and protected from light, and (3) permitting loosely covered containers of tomato juice and of orange juice to stand in the refrigerator.

The ascorbic acid determinations were made according to the method recommended by Musulin and King (10) in which 2 per cent of metaphosphoric acid is added to the extracting solution and the extract titrated in the usual manner employing the indophenol indicator. Averages of three determinations are reported.

Effect of home-canning procedures: The tomatoes used in these tests were of the Bonny Best variety, and all of them were obtained from the same plot in Arlington County, Virginia. They were purchased in lots of 1½ and 2½ bushels over a period of about 5 weeks.

To avoid differences due to random sampling, half of each tomato was placed in the fresh sample for immediate analysis and the other half in the corresponding sample for canning and subsequent analysis. Preliminary treatment consisted of washing the tomatoes, scalding from 45 to 60 seconds, dipping in cold water, removing skins, and trimming for uniformity.

The tomatoes were canned by the hot-pack and cold-pack methods, in pint glass fruit jars with glass caps and wire clamps. The jars were tempered in boiling water, and ½ teaspoon of salt was placed in each before filling with tomatoes. The clamps were adjusted to seal the jars partially before processing, and the seals were completed immediately after processing. The partial seal was used to permit the escape of air from the head space during processing. Special care was taken throughout the experiment to pack the tomatoes closely into jars, work out entrapped air, and fill the jars to within ¼ to ½ inch of a straight edge across the top to reduce head space to a minimum. This was regarded as a minimum of space to allow for expansion of the food during processing.

In the cold-pack method, the halves of tomatoes were packed raw and cold into the tempered jars and placed in the boiling-water bath. One group of the jars was processed 30 minutes; one group, 45 minutes; and a third, 60 minutes, at 212°F. Timing was started when the water bath came again to the boil after the tomatoes had been placed in it. The halves of tomatoes for the hot-pack canning were quartered, brought to boiling in an open kettle, stirred sufficiently to prevent scorching, packed at once into the jars, and processed 5 minutes at 212°F. Each sample of tomatoes, whether cold packed or hot packed, consisted of 9 pint jars.

The fresh samples were analyzed immediately after peeling. The corresponding canned samples were analyzed the following day; in each case the contents of one set of 9 jars were composited into one sample for analysis. All samples, fresh and canned, were prepared for analysis by grinding in a meat chopper and straining through cheesecloth. Ten milliliter samples of the juice thus obtained were extracted and titrated for ascorbic acid.

The results (table 1) show that the ascorbic acid present in tomatoes is not affected by either the hot- or the cold-pack canning methods as applied in this study. Increasing the time of processing of cold-packed tomatoes to 60 minutes, a longer processing period than would ever be recommended for practical purposes, did not affect the ascorbic acid content.

The average ascorbic acid content (0.17 milligram per milliliter) obtained for the fresh fruit in this study is lower than any value reported by Maclinn, Fellers, and Buck (4) for the Bonny Best variety. This may have been due to the fact that the tomatoes were picked toward the end of the growing season when there was

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TABLE 1

Ascorbic acid content of tomatoes home canned by different methods and analyzed 24 hours after canning

METHOD OF CANNING	LOT NUMBER*	ASCORBIC ACID PER MILLILITER			
		Fresh	Canned	Difference	Description of fresh samples
		mg.	mg.	mg.	
Cold-packed, processed 30 minutes	1	0.20	0.17	−0.03	Mature, firm
	2	0.20	0.20	0.00	Mature, firm
	4	0.22	0.22	0.00	Mature, firm
	6	0.17	0.18	+0.01	Slightly immature, firm
	8	0.14	0.14	0.00	Slightly immature, firm
	10	0.11	0.11	0.00	Mature, soft
	12	0.15	0.16	+0.01	Slightly immature, soft
	14	0.18	0.18	0.00	Slightly immature, firm
	16	0.16	0.15	−0.01	Immature, firm
Cold-packed, processed 45 minutes	1	0.21	0.20	−0.01	Mature, firm
	2	0.20	0.19	−0.01	Mature, firm
	4	0.23	0.23	0.00	Mature, firm
	6	0.15	0.15	0.00	Slightly immature, firm
	8	0.14	0.14	0.00	Slightly immature, firm
	10	0.12	0.12	0.00	Mature, soft
	12	0.14	0.14	0.00	Slightly immature, soft
	14	0.17	0.17	0.00	Slightly immature, firm
	16	0.15	0.16	+0.01	Immature, firm
Cold-packed, processed 60 minutes	1	0.21	0.20	−0.01	Mature, firm
	3	0.19	0.18	−0.01	Mature, firm
	5	0.12	0.13	+0.01	Slightly immature, firm
	7	0.19	0.17	−0.02	Slightly immature, firm
	9	0.11	0.11	0.00	Slightly immature, firm
	11	0.14	0.14	0.00	Slightly immature, soft
	13	0.13	0.13	0.00	Slightly immature, soft
	15	0.18	0.16	−0.02	Slightly immature, firm
	16	0.13	0.13	0.00	Immature, firm
Hot-packed, processed 5 minutes	1	0.21	0.19	−0.02	Mature, firm
	3	0.19	0.18	−0.01	Mature, firm
	5	0.13	0.12	−0.01	Slightly immature, firm
	7	0.17	0.16	−0.01	Slightly immature, firm
	9	0.12	0.11	−0.01	Slightly immature, firm
	11	0.17	0.17	0.00	Slightly immature, soft
	13	0.14	0.13	−0.01	Slightly immature, soft
	15	0.20	0.19	−0.01	Slightly immature, firm
	16	0.16	0.15	−0.01	Immature, firm

* Lot numbers refer to tomatoes purchased at the same time.

considerable rain and the fruit was not always completely ripened.

Effect of storing under different conditions. The samples for the storage tests were canned in glass containers by the hot-pack method previously described. In this case one quarter of each tomato was reserved for the fresh sample; the remaining three quarters were processed together. On the day after the canning was done, one third of the jars were opened and the contents analyzed for ascorbic acid. The remaining jars of tomatoes were divided into two lots and held 6 months before

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TABLE 2

Changes in ascorbic acid content of canned tomatoes under different conditions of storage

SAMPLE NUMBER	ASCORBIC ACID PER MILLILITER				DESCRIPTION OF FRESH SAMPLES
	Fresh	Canned, stored 24 hours	Canned, stored 6 months at room temperature (approximately 70° to 80° F.)		
			On open shelves	In dark cupboard	
	mg.	*mg.*	*mg.*	*mg.*	
1	0.20	0.20	0.13	0.12	Slightly immature, firm
2	0.17	0.17	0.12	0.11	Slightly immature, firm
3	0.18	0.17	0.11	0.11	Slightly immature, firm
4	0.16	0.17	0.09	0.09	Slightly immature, firm
5	0.19	0.17	0.08	0.09	Slightly immature, firm

analysis. Half were stored on open shelves facing an east window in direct sunlight part of the day; the rest were placed away from direct sunlight on adjoining shelves which were shielded from light by black photographic paper. The analyses were carried out in the same manner as for the preceding tests. The results (table 2) show that tomatoes canned by the hot-pack method described here lose from 30 to 50 per cent of their ascorbic acid when stored for 6 months at room temperature, approximately 70° to 80°F. Light appeared to have no effect under the conditions of this experiment.

In a former study from this laboratory, Daniel and Rutherford (8) obtained a slightly greater loss in ascorbic acid content of tomatoes stored in jars wrapped in photographic black paper. They offered as a possible explanation of these findings a slight difference in temperature of the fruit in the wrapped and unwrapped jars due to absorption of heat by the black paper. Hauck (9) more recently has suggested that light may aid in the preservation of ascorbic acid, presenting data on two samples of the same tomato juice canned in glass, one exposed to the light and one stored in the dark. At the present time there seems to be no satisfactory explanation of the divergent results of these three studies.

Effect of storing in open containers in a refrigerator. It is the custom in some households to open a large can of tomato juice or to prepare from canned or fresh tomatoes more than the amount of juice required for one meal and to store the unused portion in a refrigerator in a loosely covered container. Tests were made to determine the effect of such treatment on the ascorbic acid content. Since orange juice is frequently handled in the same manner, tests on this juice were also included.

To prepare the juice from whole canned tomatoes, one portion of a mixture of two cans of commercially canned fruit was forced through cheesecloth; the other portion was put through an ordinary wire strainer such as is used by most housewives. Juice from the fresh tomatoes was extracted by forcing the peeled, chopped fruit through either cheesecloth or a wire strainer. Commercially canned tomato juice was given no treatment. The orange juice was obtained by extracting on a glass reamer and removing the seeds by either a wire strainer or cheesecloth.

The different juices were placed in the same kind of glass jars with loosely fitting lids and were stored in a refrigerator at a temperature of approximately 40° to 45°F. Ascorbic acid determinations were made immediately after preparation of the

TABLE 3

Changes in ascorbic acid content of tomato juice and orange juice stored in unsealed containers in refrigerator, 40° to 45° F.

NUMBER AND DESCRIPTION OF SAMPLE	ASCORBIC ACID PER MILLILITER AFTER STORAGE FOR VARIOUS NUMBERS OF DAYS					
	0	1	2	3	4	5
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Tomato juice, commercially canned						
1	0.18	0.18	0.18	0.18	0.18	0.17
2	0.17	0.16	0.16	0.16	0.16	0.16
3	0.14	0.14	0.14	0.14	0.13	0.12
4	0.23	0.23	0.22	0.22	0.20	0.18
5	0.16	0.16	0.16	0.16	0.15	0.14
Juice from commercially canned tomatoes						
1*	0.20	0.18	0.17	0.14	0.12	—
2*	0.20	0.19	0.17	0.14	0.12	—
3†	0.21	0.19	0.16	0.14	0.10	—
4†	0.21	0.19	0.17	0.14	0.10	—
Juice from raw tomatoes						
1*	0.16	0.17	0.15	0.12	0.10	—
2*	0.16	0.17	0.15	0.12	0.09	—
3*	0.16	0.15	0.14	0.10	—	—
4*	0.16	0.15	0.14	0.10	—	—
5†	0.15	0.15	0.14	0.13	—	—
6†	0.15	0.14	0.14	0.13	—	—
Orange juice						
1*	0.38	0.37	0.36	0.35	0.34	—
2*	0.38	0.37	0.36	0.35	0.34	—
3*	0.38	0.37	0.36	0.36	0.35	—
4†	0.37	0.37	0.35	0.36	0.35	—
5†	0.37	0.36	0.35	0.35	0.35	—
6†	0.37	0.37	0.36	0.36	0.36	—

* Strained through wire strainer.
† Strained through cheesecloth.

juice and on samples taken from each jar at 24-hour intervals for a period of from 3 to 5 days.

The results of these tests (table 3) show that there was no significant loss of ascorbic acid in the commercially canned tomato juice stored in loosely covered containers at or below 45°F. for 4 days. Juice from commercially canned tomatoes and from fresh tomatoes showed a slight loss on the second day of storage under similar conditions and a more rapid destruction of the vitamin on the following days of the study. Freshly extracted orange juice stored in the same way showed a gradual though slight loss of ascorbic acid, beginning about the second day. There was no difference in the results when the juice was strained through cheesecloth or through a wire sieve.

A previous report from this laboratory (11) indicated that orange juice kept in a refrigerator 6 hours might lose as much as 10 per cent of its ascorbic acid value while tomato juice might lose 3 per cent in only 1 hour. In view of the cumulative evi-

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dence concerning ascorbic acid determinations, this slight initial loss during the first day is not significant since the differences reported are within the limits of accuracy of the chemical method when acetic acid alone is used as the extracting liquid. The earlier study reported 40 per cent loss in 4 days' storage of tomato juice; in the present study tests were made at regular intervals during the storage, and a very gradual destruction of ascorbic acid was found. In the light of present findings, the interpretation of the effect of overnight storage of juices is more lenient than that given in 1936.

Hauck (9) has shown that tomatoes home canned in tin and stored in a refrigerator after opening lose ascorbic acid at about the same rate as the commercially canned tomatoes used in this study, but that those canned in glass lose their potency at a faster rate.

Summary. Canning tomatoes by either the hot-pack or cold-pack method as described in this paper caused no significant loss in ascorbic acid content of the tomatoes. Increase in processing time in the cold-pack method did not affect this vitamin.

Tomatoes canned in glass jars and stored for 6 months at room temperature, either on open shelves receiving considerable sunlight or on darkened shelves, lost approximately the same amount of ascorbic acid, namely from 30 to 50 per cent.

Commercially canned tomato juice showed no significant loss in ascorbic acid value when stored in loosely covered containers in a refrigerator for 4 days. Juice prepared from commercially canned tomatoes and from fresh tomatoes and stored under the same conditions showed significant losses of this vitamin after 2 days of storage. Freshly extracted orange juice in the same study showed slight but gradual loss of ascorbic acid, beginning about the second day. None of the four juices tested lost appreciable amounts of ascorbic acid during the first 24 hours.

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Studies on Malarial Parasites. V. Effects of Ascorbic Acid on Malaria (*Plasmodium knowlesi*) in Monkeys.*

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Several different investigators[1-3] have reported that in human cases of tertian malaria there was an increased demand for ascorbic acid and the administration of additional vitamin was necessary to prevent a mild state of scurvy. In spite of these observations no one has reported a causal relationship between malaria and ascorbic acid deficiency.

During the course of our malaria studies with *P. knowlesi* in monkeys (*Macaca mulatta*)[4] certain observations were made which led us to suspect that ascorbic acid might have some influence on the course of parasitemia in the animal host. Using the method of Mindlin and Butler[5] for determining plasma ascorbic acid and of Butler *et al.*[6] for whole blood levels of the vitamin it was found that the average plasma level of ascorbic acid for 20 parasitized monkeys was only 0.24 mg %. Nonparasitized animals were found to have an average plasma level of 0.56 mg %. Also during the course of our studies on 120 monkeys, 7 animals inoculated with parasites showed no plasma ascorbic acid and less than 0.1 mg % in the whole blood. This condition was present in spite of the fact that the animals were receiving a well balanced diet containing generous daily portions of raw potatoes and oranges. Such a condition has been previously reported in both humans[7] and monkeys.[8] This may be explained on the basis of an abnormal intestinal flora since it has been shown by various workers[9,10] that under certain conditions some intestinal bacteria destroy ascorbic acid and may be responsible for a state of scurvy.

The 7 animals having extremely low blood ascorbic acid values showed abnormal courses of parasitemia. Instead of the usual rapid increases in numbers of parasites and death of the animals within 6 or 7 days after inoculation there was only a slow rise in percentage of parasites, and a gradual spontaneous control of the infection. Into 4 of these animals ascorbic acid was injected intramuscularly on 3 successive days (starting 7, 7, 8 and 9 days following parasite inoculation in the 4 monkeys) and in all cases a significant increase in numbers of circulating parasites was observed. One of these animals (given ascorbic acid 7 days after parasite inoculation) then showed a normal rise in parasite count and died in the usual manner with a high percentage of the erythrocytes parasitized. The other 3 animals (given ascorbic acid 7, 8 and 9 days after parasite inoculation) however, showed a subsequent drop in parasite count with spontaneous control of the infection, presumably due to the development of an immunity prior to the injection of ascorbic acid. The remaining 3 monkeys out of the total 7 were given no vitamin treatment and all 3 completely controlled their infections. In Ta-

* This work was done under a contract recommended by the Committee of Medical Research, between the Office of Scientific Research and Development and the President and Fellows of Harvard College.

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TABLE I.
Effect of Ascorbic Acid on Parasitemia of Ascorbic Acid Deficient Monkeys (Spontaneously Appearing).

Days after parasite inoculation	Total parasite count		
	Ascorbic acid deficient animals		
	No. 59	No. 101	No. 109
4	—	0.1	—
5	0.1	—	—
6	0.3	2.4	occasional parasite in thick film
7	0.5	0.8*	—
8	0.5	8.5*	0.004
9	—	20.6*	0.004*
10	2.0	13.5	1.1*
11	2.4	dead	0.05*
12	0.4		—
13	occasional parasite in thick film (chronic)		0.01 (chronic)

* 80-100 mg Merck's crystalline ascorbic acid intramuscularly. All 10 animals referred to were approximately the same size with an average weight of about 2.5 kilos.

ble I are shown the parasite changes of 3 representative animals of the 7.

Three monkeys were then placed on a synthetic diet deficient in ascorbic acid similar to that of Shaw and coworkers.[11] This diet which was freshly mixed every 5 or 6 days and stored in the cold room at +2°C consisted of the following percentages of ingredients: sucrose 65, casein 15, Phillips and Hart IV salts 4, corn oil 4, cod liver oil 2, and yeast 10.[†] Vitamins were supplemented in the indicated numbers of milligrams per kilogram of diet: biotin 0.1, thiamine 5, riboflavin 5, pyridoxine 5, calcium pantothenate 15, nicotinic acid 25, choline 250, inositol 500, and paraaminobenzoic acid 500. When early deficiency symptoms appeared (disappearance of plasma and whole blood ascorbic acid, slight loss of body weight and increased capillary fragility), the animals were inoculated intravenously with 6 million parasites per kg of body weight. A control animal on a normal diet containing adequate ascorbic acid was likewise inoculated with the same dosage of parasites. To the 3 experimental animals, No. 120, 119 and 117, a solution of crystalline ascorbic acid was injected, as shown in Table II, starting, respectively, immediately before, 4 days after and 14 days after parasite inoculation. The control and the animal No. 120 showed immediate parasite increases with normal courses of parasitemia and deaths on the 7th day with, in each case, 31% of the erythrocytes parasitized. Animal No. 119 showed no parasite increase until after ascorbic acid was given but then had a normal course of parasitemia, with death on the 6th day following vitamin administration. Animal No. 117 showed only a small parasite increase prior to ascorbic acid administration, but died 2 days after receiving vitamin with only 8% of the erythrocytes parasitized. This animal did not die of the malaria infection alone, but manifested a complicating anemia perhaps due to the more advanced state of the vitamin deficiency and a greatly lowered food intake during the last week of the animal's life. (There appeared also to be some resistance to parasite increase indicative of an immune reaction).

Whether this action of ascorbic acid administration to the vitamin-deficient animals is a direct effect on the parasites or if it is an indirect one through the animal host we do not know. We suspect that it may be the latter since attempts to demonstrate the need of ascorbic acid for the *in vitro* growth and multiplication of *P. knowlesi* in

[11] Shaw, J. H., Phillips, P. H., and Elvehjem, C. A., *J. Nutrition*, 1945, **29**, 365.

[†] Generous quantities of Strain K Dried Brewer's Yeast were kindly supplied by Anhauser-Busch, Inc., St. Louis, Mo.

TABLE II.
Effect of Ascorbic Acid on Parasitemia of Ascorbic Acid Deficient Monkeys (Produced by Dietary Deficiency).

Days after parasite inoculation	Total parasite count			
	Control animal (normal diet)	Ascorbic acid deficient animals (Vitamin C deficient diet) No. 120	No. 119	No. 117
0	—	—‡	—	—
3	0.3%	0.3%‡	350/mm³	50/mm³
4	2.0 "	2.7 "‡	270/ "‡	120/ "
5	10.0 "	8.0 "‡	0.2 %‡	30/ "
6	12.6 "	14.9 "‡	0.15 "‡	150/ "
7	31.3 "*	31.0 "*	0.8 "‡	100/ "
8			3.2 "‡	300/ "
9			26.0 "	580/ "
10			144.7 "*†	2000/ "
11				0.5%
12				1.2 "
13				0.9 "
14				5.5 "‡
15				8.8 "*‡

† 84% of the red cells contained parasites with many double and triple infections per cell.
* Animal died within 18 hours following this parasite count.
‡ 80-100 mg Merck's crystalline ascorbic acid intramuscularly.

24-hour cultures have so far been inconclusive, although in the absence of the vitamin the usual protoplasmic mass is not achieved. This growth deficiency and the degenerate appearance of the parasites are also very striking in the vitamin-deficient host. These studies on the effect of ascorbic acid and of ascorbic acid analogs are being continued with simian and human plasmodia.

Conclusions and Summary. 1. Plasma and whole blood ascorbic acid levels were significantly lower in parasitized monkeys than in normal animals.

2. There was an abnormal course of parasitemia in 7 of our monkeys which showed spontaneous ascorbic acid deficiency and in 2 other animals which were made ascorbic acid deficient by removing the vitamin from their diet.

3. Following the administration of the pure ascorbic acid, there was a normal course of parasitemia in one of the monkeys spontaneously ascorbic acid deficient and in those made ascorbic acid deficient by dietary means.

Withholding ascorbic acid therapy for more than 7 days appeared to give the spontaneously deficient monkeys time to produce an immunity and to control the infections. If ascorbic acid treatment was withheld from the animals made vitamin C deficient there was a prolongation of the course of infection but eventually the parasitemia overwhelmed the animal.

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Toxicity and Therapeutic Activity of Tyrothricin by Oral Administration.

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Since penicillin by oral administration has been found effective in the prophylaxis and treatment of certain infections, the matter of the toxicity and possible therapeutic effectiveness of tyrothricin by this route of administration is not without interest in relation to antibiotic therapy.

In these experiments a 2% alcoholic solu-

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ASCORBIC-ACID METABOLISM AFTER ADMINISTRATION OF CORTICOTROPHIN

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THE high concentration of ascorbic acid in the adrenal cortex and its disappearance in many species when corticotrophin is injected (e.g., Sayers et al. 1946) suggest an important relationship between ascorbic acid and adrenal cortical activity. It is uncertain whether the ascorbic acid which disappears is consumed within the cortex or extruded into the plasma; Vogt (1948) could find no evidence for the latter process in dogs and cats.

In addition there is evidence that the adrenal cortical hormones affect ascorbic-acid metabolism throughout the body: Hess et al. (1951) have reported changes in the blood-glutathione level in response to adrenal cortical activity, and other reports by Prunty and Vass (1943a) and Stewart et al. (1953) indicate that ascorbic-acid metabolism is probably related to that of glutathione. Changes in the tuberculin sensitivity of guineapigs have been interpreted by Long et al. (1951) as showing that cortisone exerts its effects by increasing the conversion of ascorbic acid to dehydro-ascorbic acid.

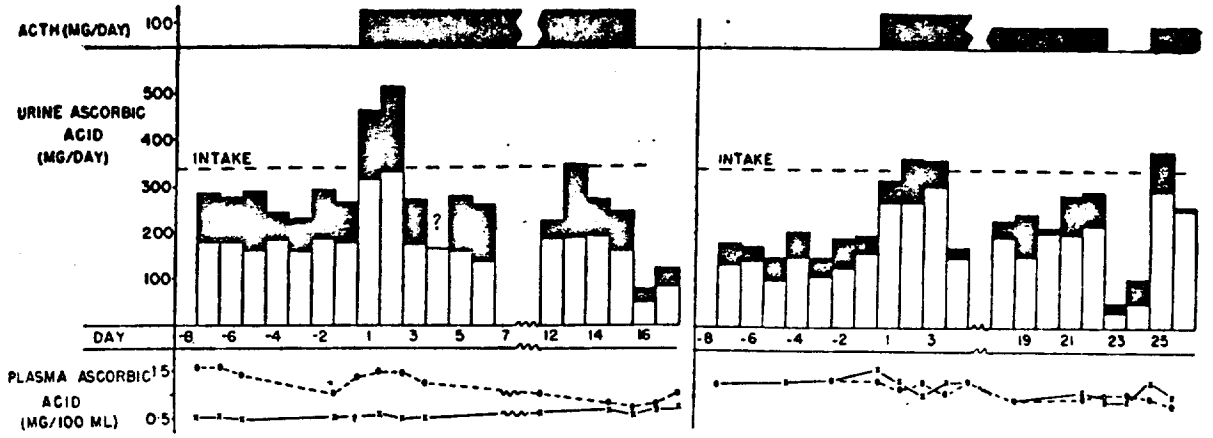
We therefore studied changes in the quantity of reduced ascorbic acid and its immediate oxidation products in the plasma and urine of patients treated with corticotrophin and in the urine of guineapigs injected with corticotrophin. Control studies were made on patients not treated with corticotrophin. We thought it possible that any considerable changes in the over-all demand for ascorbic acid within the adrenal cortex and elsewhere might be reflected by inverse changes in the quantity of ascorbic acid in plasma and urine, and that any increased conversion of ascorbic acid to its immediate oxidation products might be accompanied by parallel changes in plasma and urine.

Methods

CHEMICAL

Reduced ascorbic acid was determined by titration with dichlorophenolindophenol ("dye"). For plasma the procedure used by Prunty and Vass (1943b) was followed. For urine a suitable volume (1–5 ml.) from a twenty-four-hour save, collected over metaphosphoric acid, was directly titrated with 0·05% dye.

Mindlin and Butler (1938) and Evelyn et al. (1938) showed that urine, but not plasma, contains substances other than ascorbic acid which react with the dye and give falsely high results. Those interfering substances were further investigated and partly characterised by



Case 1, a man aged 43, with rheumatoid arthritis

Case 2, a woman aged 58, with rheumatoid arthritis

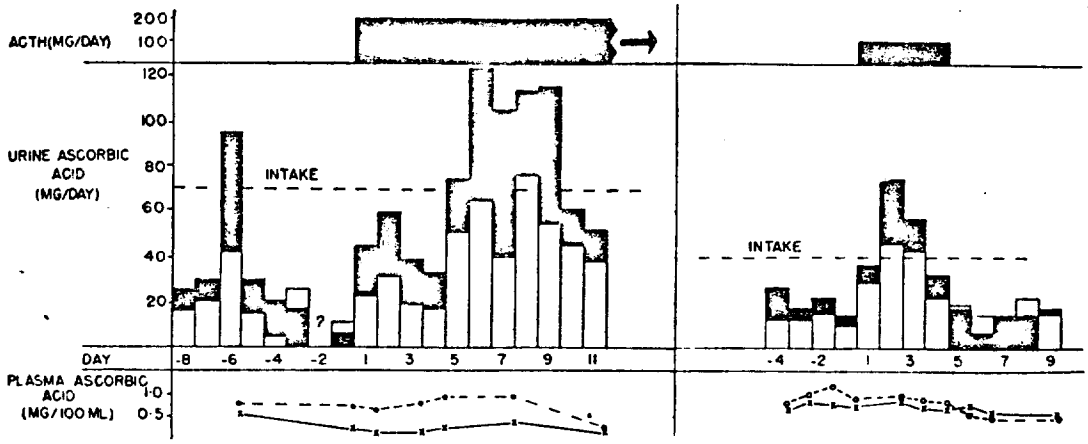
Fig. 1—Effect of corticotrophin in 2 patients with rheumatoid arthritis and high ascorbic-acid saturation, showing levels of ascorbic acid in plasma and in urine before, during, and after corticotrophin therapy.

	Urine	Plasma
Reduced ascorbic acid	┌─┐	x——x
Total ascorbic acid	▬	o - - - o

Lugg (1942) and Snow and Zilva (1944) while evolving a method for eliminating them; they may be divided into two general classes, after typical members of each class: "–SH-like" and "reductone-like." The former class is quickly removed by formaldehyde at pH 1·5–3·5; the latter class reacts slowly or not at all with formaldehyde. Ascorbic acid, on the other hand, is slowly removed by formaldehyde at pH 1·5 and rapidly at pH 3·5. In a modification (McSwiney and Prunty unpublished) of Snow and Zilva's method, suitable volumes of urine are adjusted to pH 1·5 and pH 3·5 by electrometric titration with 5*N* hydrochloric acid or with citrate buffer, and to each sample formaldehyde is added to produce a final concentration of 0·29 *M*. After a time which depends on temperature (see Snow and Zilva 1944) a portion of the reaction mixture is titrated with 0·05% dye. It has been found that, after reaction at pH 1·5, 1% of the ascorbic acid has been removed together with the "–SH-like" interfering substances, and at pH 3·5 88% of the ascorbic acid plus the "–SH-like" are removed, whereas the reductone-like substances are mostly unaltered. The difference between the two titration figures therefore represents 87% of the true reduced ascorbic acid originally present. In some urine the ascorbic-acid level is so low that the method cannot be applied with accuracy. This was true of most of the urine from case 6 (fig. 3); in all the other cases the urine-reduced ascorbic acid was determined by the "formaldehyde correction" procedure.

The immediate oxidation products of ascorbic acid are dehydro-ascorbic acid and diketogulonic acid. The former was estimated in urine by reducing it with hydrogen sulphide and titration by the procedure used by Prunty and Vass (1943b). The total of reduced ascorbic acid plus dehydro-ascorbic acid plus diketogulonic acid was determined by the method of Roe and Kuether (1943) as modified by Geschwind et al. (1951). There are again unidentified substances which can interfere with this reaction, as Penney and Zilva (1945) have shown; these substances are not known to be present in human tissues or fluids.

In the light of present knowledge, therefore, it is reasonable to conclude that measurements can be made of: (1) reduced ascorbic acid (dye titration directly for plasma filtrate, with formaldehyde correction for urine); (2) dehydro-ascorbic acid (treatment with hydrogen sulphide); and (3) reduced ascorbic acid plus its immediate oxidation products (Roe and Kuetherer's method).



Case 3, a man aged 43, with rheumatoid arthritis

Case 4, a man aged 24, eunuchoid

Fig. 2—Effect of corticotrophin in patients with "intermediate" ascorbic-acid saturation. Symbols as in fig. 1.

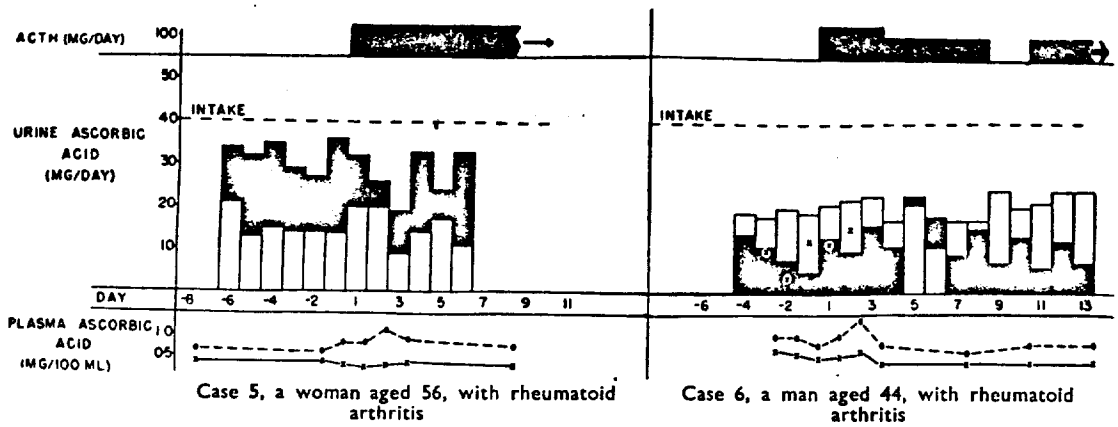


Fig. 3—Effect of corticotrophin in patients with "low" ascorbic-acid saturation. In case 6 formaldehyde correction was impossible for most urines, and white blocks indicate uncorrected titration values; formaldehyde corrections are indicated by crosses.

ASCORBIC-ACID INTAKE

In six patients treated with corticotrophin ascorbic-acid intake was controlled by administering a constant diet free from fresh fruit and calculated to contain a maximum of 40 mg. of ascorbic acid a day; this was supplemented in cases 1 and 2 with 300 mg. a day, and in case 3 by 30 mg. a day, of oral ascorbic acid in divided doses. To avoid changes due to recent absorption, the plasma-ascorbic-acid was determined twelve hours after the last oral supplement of ascorbic acid and two hours after a breakfast which contained less than 5 mg. of ascorbic acid.

Three control patients not treated with corticotrophin also received a constant diet with a maximum of 40 mg. a day of ascorbic acid; the purpose in these patients was to study the effect of raising the intake from 340 to 640 mg. a day, and they therefore received oral ascorbic acid in appropriate dosage. Cases 7 and 8 received 300 mg. a day until stabilised on this dosage, and the dosage was then doubled (fig. 5). In case 9 the preliminary stabilising period was shortened by giving 900 mg. of ascorbic acid daily as a priming dose for the first three days.

The guineapigs ate a diet free from ascorbic acid. Since oral ascorbic acid is destroyed in the guineapig intestine, they were injected subcutaneously with ascorbic acid or sodium ascorbate twice daily, the total dose being 10 mg. per 100 g. of body-weight. For sodium ascorbate, ascorbic acid was neutralised to pH 7 with 4·5% sodium bicarbonate; this procedure was adopted because some of the guineapigs showed local irritation with ascorbic acid, and it was thought that this might be responsible for the irregular excretion during the control periods. This, however, was not so.

In all the cases an adequate stabilising period was allowed before either corticotrophin or increased oral ascorbic acid was given.

Results

EFFECTS OF CORTICOTROPHIN

Six patients received corticotrophin; the diagnoses and the dosage are indicated in figs. 1–3; all responded well to the hormone as judged by clinical improvement, decrease in eosinophil-counts, and increases in urine steroid output.

From the results obtained they may be divided into three pairs; cases 1 and 2 had initial plasma-ascorbic-acid levels (Roe and Kuether method) greater than 1 mg. per 100 ml. and were considered to have a "high" saturation of ascorbic acid; cases 3 and 4 had initial

INTAKE AND OUTPUT OF ASCORBIC ACID (MG. PER DAY)

Case no.	Intake (mg. daily)	Substance	Urinary excretion: Base-line before corticotrophin therapy		Outputs on individual days of corticotrophin treatment											Stabilised on corticotrophin		Output in excess of pre-treatment base-line	
			No. of days	Amount (mg. per day)	1	2	3	4	5	6	7	8	9	10	11	No. of days	Amount (mg. per day)	Days of treatment	Amount (mg. per day)
1	340	Reduced A.A.	8	177 ± 9·3	316	334	177	..	..	..	..	..	..	..	..	12	171 ± 29·8	..	29[illegible]
		D.H.A.A.	8	3·1 ± 2·8	12	3	0	..	..	..	..	..	..	..	..	12	8·8 ± 7·3	1 and 2	..
		Total A.A.	8	263 ± 29·3	461	516	271	..	..	..	..	..	..	..	..	12	285 ± 56·1	..	45[illegible]
2	340	Reduced A.A.	8	130 ± 18·8	266	266	302	146	..	..	..	..	..	..	..	8	203 ± 24·2	..	43[illegible]
		D.H.A.A.	8	3·4 ± 1·5	4	6	4	6	..	..	..	..	..	..	..	8	5·6 ± 4·2	1–3	..
		Total A.A.	8	176 ± 20·5	316	362	357	168	..	..	..	..	..	..	..	8	254 ± 28·8	..	50[illegible]
3	70	Reduced A.A.	7	22 ± 9	23	32	19	17	51	65	41	76	55	45	38	..	..	..	22[illegible]
		D.H.A.A.	7	3·4 ± 1·4	2	0	0	0	4	1	0	2	20	..	1	..	..	1–11	..
		Total A.A.	7	29 ± 30	45	60	39	33	74	123	105	113	115	61	52	..	..	..	57[illegible]
4	40	Reduced A.A.	4	12·8 ± 1·8	29	46	43	22	..	..	..	..	..	..	..	..	..	..	8[illegible]
		D.H.A.A.	4	1·7 ± 0·8	4	7	8	7	..	..	..	..	..	..	..	..	..	1–4	..
		Total A.A.	4	20·3 ± 4·8	37	75	57	32	..	..	..	..	..	..	..	..	..	..	121
5	40	Reduced A.A.	6	15·9 ± 2·8	21	20	9	14	..	..	..	..	..	..	..	8	13·7 ± 5·1	..	−18
		D.H.A.A.	6	3·7 ± 2·2	6	3	1	0	..	..	..	..	..	..	..	8	1·7 ± 2·0	1–8	..
		Total A.A.	6	32·2 ± 3·2	32	26	19	33	..	..	..	..	..	..	..	8	33·3 ± 10·5	..	9
6	40	Reduced A.A.	3	9·0 ± 3·5	12	15	..	..	..	..	..	..	..	..	..	4	14·3 ± 4·7	..	37
		D.H.A.A.	3	5·3 ± 5·0	2	3	..	..	..	..	..	..	..	..	..	4	2·0 ± 2·2	1–7	..
		Total A.A.	3	8·0 ± 2·5	13	10	..	..	..	..	..	..	..	..	..	4	15·3 ± 4·8	..	51

plasma-ascorbic-acid levels of 0·75–1·0 mg. per 100 ml. and were considered to have an "intermediate" saturation of ascorbic acid; and cases 5 and 6 had initial plasma-ascorbic-acid levels of less than 0·6 mg. per 100 ml. and were considered to have a "low" saturation. Those with "high" (cases 1 and 2, fig. 1) and "intermediate" (cases 3 and 4, fig. 2) ascorbic-acid saturation showed effects which were qualitatively similar, and will be called group 1, whereas those with "low" (cases 5 and 6, fig. 3) ascorbic-acid saturation form group 2.

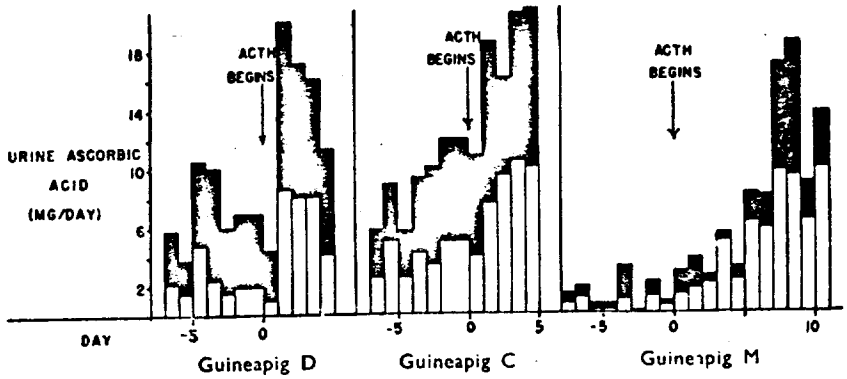


Fig. 4—Effect of corticotrophin on urinary ascorbic acid in guineapigs.

The urinary output of ascorbic acid is shown in the accompanying table and figs. 1–3. The figures show outputs for individual days, and for the sake of clarity the table gives average outputs for the base-line periods and, where possible, for the periods when patients had stable outputs during their course of corticotrophin. Cases 3 and 4 did not become stable. The outputs on various days of treatment with corticotrophin are also shown.

In group 1 corticotrophin increased the urinary output of ascorbic acid usually for the first two or three days only. The amount of ascorbic acid excreted over and above the pre-treatment output has been calculated by deducting the average pre-treatment output from the raised outputs, and the results are shown in the table. In cases 1–3 there was an excess excretion of more than 450 mg. of total ascorbic acid.

An important feature is that the output measured by Roe and Kuether's method rises above the intake. At the same time there was in cases 1, 3, and 4 an increase in the amount but not in the proportion of oxidation products.

When corticotrophin was withheld, in cases 1 and 2, there was a reversal of these changes; because of clinical reactions withdrawal was never continued until the patients returned to a stable urinary output, and it is not known whether the retention of ascorbic acid at this phase would be equal to the excess excretion during the first days of treatment with corticotrophin.

Urinary dehydro-ascorbic acid was significantly increased by corticotrophin ($P = 0·01$) in case 4 but not in cases 1–3.

In group 2 changes in the amount and state of ascorbic acid in the urine are not statistically significant.

In five guineapigs corticotrophin increased the reduced ascorbic acid and oxidation products in the urine (fig. 4). Guineapigs C and D were given corticotrophin 6·66 I.U. hourly for nine hours daily. Guineapig M and two others were given 'Acthar gel' 20 I.U. b.d. Preliminary experiments had indicated that these relatively large doses were necessary to produce changes in the urinary excretion of ascorbic acid in guineapigs.

Guineapigs C and D received ascorbic acid, and the others sodium ascorbate. The increases in excretion were more prolonged than in man and usually persisted throughout the period of administration of corticotrophin. Exceptions were seen in guineapigs on long courses of corticotrophin, when the urinary excretion of ascorbic acid became irregular in the last few days before death (from unknown causes).

Plasma-ascorbic-acid levels were studied in the patients receiving corticotrophin, and the results are shown in figs. 1–3. There is no consistent change with corticotrophin, but there is a tendency in both groups for the level of oxidation products to rise at first and then to fall. The plasma levels estimated by both methods show a fall with continued therapy.

Renal Clearance of Ascorbic Acid.—With the reservation that single determinations may not reflect diurnal changes in plasma, the twenty-four-hour renal clearances of ascorbic acid were increased by at least 50% over the pre-treatment levels during the administration of corticotrophin.

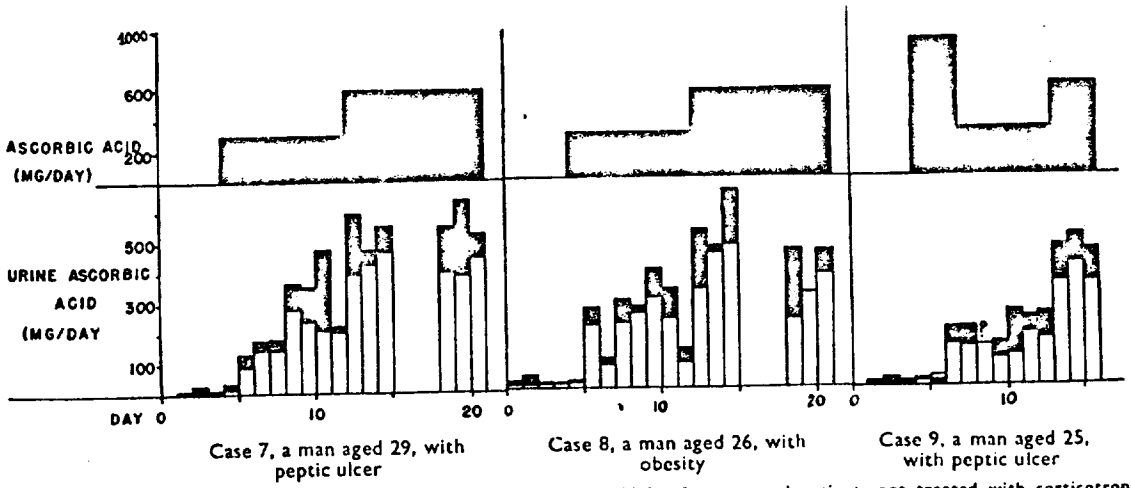


Fig. 5—Effect of changes in ascorbic-acid intake on urinary ascorbic acid in three control patients not treated with corticotrophin.

EFFECTS OF INCREASING ASCORBIC-ACID INTAKE IN CONTROLS

Since the observed changes in the amounts of oxidation products in urine during corticotrophin therapy might be due solely to increased output of ascorbic acid, three patients (cases 7–9) on constant diet were studied at different levels of ascorbic-acid intake. The results (fig. 5) indicate that, when the urine output is increased by raising the intake, the alterations in urine-ascorbic-acid levels are qualitatively similar to those accompanying the administration of corticotrophin; in particular there is an increase in the amount of oxidised ascorbic acid.

Discussion

Our results confirm those of Beck et al. (1951), who found that both corticotrophin and cortisone increased the urinary excretion of ascorbic acid measured by the Roe and Kuether method. The daily intake of ascorbic acid in most of their patients would place them in our "high" and "intermediate" categories.

This increase could be due to diminished utilisation or to excretion of ascorbic acid already present within the cells or on the extracellular fluid. It is unlikely that corticotrophin would produce only a temporary decrease in utilisation, since its other effects are notably increasing in intensity during the stage at which the ascorbic-acid excretion diminishes to the base-line level, and this possibility is certainly excluded as the sole cause in those cases where the urinary output exceeds the intake. The extracellular fluid may be taken to have expanded, and its level of ascorbic acid has usually increased during the stage of increased excretion; hence it will have accumulated ascorbic acid and cannot have been the source of the increase in urinary ascorbic acid. It is therefore most probable that the cells provide the excess ascorbic acid which is excreted. The adrenal glands cannot have been the sole source of the increased excretion of ascorbic acid, since the total amount of ascorbic acid in two human adrenals is about 10 mg.*—far less than the excess of 454–578 mg. excreted in cases 1, 2, and 3 (see table).

Further tentative calculations may be made:

(1) If the concentration of ascorbic acid in saturated human tissues (other than adrenals) is similar to that of the guineapig, it may be taken to be about 200 mg. per litre of intracellular fluid (see Yavorsky et al. 1934). With corticotrophin therapy probably about a litre of water is transferred from intracellular to extracellular fluid (Prunty et al. 1953) and might passively transfer 200 mg. of ascorbic acid; in our highly saturated patients there was an additional approximate 300 mg. of ascorbic acid which would appear to have been actively mobilised from the reserves within the cells by some independent mechanism, presumably dependent on adrenal cortical activity. Support for this view comes from the abrupt decrease in urinary ascorbic acid with cessation of corticotrophin therapy in cases 1 and 2; the magnitude of the change is again greater than could be expected simply from shift of fluid into the cells. It is also of interest that patients with "low" saturation do not show considerable changes in urinary excretion, perhaps because they have no reserves to mobilise.

(2) In group 1 there is evidence for increased conversion of ascorbic acid to the immediate oxidation products judged by the increased difference between Roe and Kuether and dye figures for urine and plasma; only case 4 shows a significant increase in dehydro-ascorbic-acid output (from 2 to 8 mg. a day). The similarity between these changes and those induced by raising the oral intake of ascorbic acid in cases 7, 8, and 9 makes it probable that corticotrophin had produced a "flooding" of the extracellular fluid with ascorbic acid similar to that induced by the increased oral intake; in other words, the increased production of oxidation products does not appear to be a specific effect of corticotrophin or of adrenocortical steroids.

These results do not indicate any change in the amount of ascorbic acid utilised in the adrenal cortex or elsewhere.

*Two determinations of ascorbic acid in human adrenal in this laboratory are: (1) normal adrenal obtained at operation, 80 mg. of ascorbic acid per 100 g.; and (2) adrenal obtained three hours after death, 104 mg. of ascorbic acid per 100 g.

Summary

The amounts of reduced ascorbic acid and its immediate oxidation products in the plasma and urine of patients treated with corticotrophin were determined.

In patients with adequate ascorbic-acid saturation there was an increased urinary output, usually temporary. This increase was probably due to three causes: an increased renal clearance of ascorbic acid; a probable shift of ascorbic acid from cells to extracellular fluid along with the usual shift of water; and another unidentified mechanism causing an additional transfer of ascorbic acid from the cells to extracellular fluid.

In patients with adequate ascorbic-acid saturation there was evidence for increased production of the immediate oxidation products of ascorbic acid. Control studies in patients not receiving corticotrophin indicated that this increase was not a specific effect of corticotrophin or of adrenal cortical steroids.

In patients with low ascorbic-acid saturation there were no such changes when corticotrophin was administered.

In guineapigs with adequate ascorbic-acid saturation corticotrophin produced effects similar to those in adequately saturated patients.

Our thanks are due to the nurses of Holden Metabolic Ward; to the departmental technical staff for analytical assistance; and to the governors of St. Thomas's Hospital for financial assistance from endowment funds. The corticotrophin used in the experiments on guineapigs was supplied by the Medical Research Council.

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A Color Reaction of Ascorbic Acid with Nicotinamide and Nicotinic Acid.

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Pelham Manor, New York.

Since reactions between different vitamins are always of interest, it appears worthwhile to report the following observations. In the course of performing a number of spot tests, it was observed that a yellow color was produced when a mixture of ascorbic acid and nicotinamide was moistened with water and stirred into a thick paste. The formation of the canary yellow color was almost immediate. Nicotinic acid gave a similar color reaction with ascorbic acid although the reaction proceeded more slowly. None of the other vitamins that were tested gave this reaction; these included pyridoxine hydrochloride, thiamine, calcium pantothenate, and para-amino benzoic acid.

Failure to note this reaction previously in any of the numerous mixtures of vitamins used as dietary supplements probably was due to the common practice of including riboflavin in such preparations. The deep yellow color of the riboflavin would mask that of the reaction.

Whether the reaction alters the biologic activity of the vitamins or represents one of the functions of ascorbic acid in the body is not known.

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Killing and Lysis of Gram-negative Bacteria Through the Synergistic Effect of Hydrogen Peroxide, Ascorbic Acid, and Lysozyme

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A mixture of hydrogen peroxide and ascorbic acid has been found to generate an antibacterial mechanism which is active against gram-negative bacteria. It results in bacterial death and renders the organism sensitive to lysis by lysozyme. Under the conditions used, horseradish peroxidase did not augment the antibacterial effect. It is suggested that the effector mechanism involves the generation of short-lived free radicals which disturb the integrity of the cell wall. This effect alone might kill bacteria by interfering with selective permeability, but in the presence of lysozyme a further bactericidal activity is accomplished by complete disruption of the cell. It is proposed that a transient antibacterial system such as that described could exist within phagocytic cells. Free radicals would be formed through the interaction of certain oxidizable substances and hydrogen peroxide, which is produced during the enhanced metabolic activity that accompanies ingestion of bacteria. Such a system would help to explain why macrophages, which are apparently devoid of preformed bactericidins, are nonetheless very efficient in killing most phagocytosed bacteria.

Elie Metchnikoff, a pioneer in the field of immunology, was the first to emphasize the importance of phagocytes in the host-parasite relationship. He demonstrated that after the ingestion of a living microorganism by phagocytic cells a digestive and destructive action accomplished the complete dissolution of the microbe (11). He was not able to characterize the digestive ferment acting on the microbes or even to determine whether enzymic attack was the process responsible for their death.

Cohn, in his studies on bacterial inactivation within macrophages, demonstrated that death preceded digestion. He also noted that lysozyme-sensitive bacteria were degraded more readily in both polymorphonuclear leukocytes and macrophages (3). Lysozyme is the bacteriolytic enzyme shown by Fleming in 1922 (10) to lyse suspensions of certain bacterial species. Its lytic action is brought about by the enzymatic hydrolysis of the β-1,4-glycosidic linkages in the peptidoglycans which are responsible for the rigidity of the bacterial cell (14, 32). This substrate is known to exist in the cell walls of both gram-positive and gram-negative bacteria, but not always in an accessible location. Since the cell wall of the gram-positive *Micrococcus lysodeikticus* is composed almost exclusively of the peptidoglycan, this organism is exquisitely sensitive to lysis by lysozyme. In the case of gram-negative bacteria, however, the surface layers, containing lipopolysaccharides, proteins, and lipids, so obscure the substrate as to make it inaccessible to the enzyme and, therefore, resistant to lysis. It is known, however, that in some circumstances gram-negative bacteria may be rendered sensitive to lysozyme. Thus, under extreme conditions of H-ion concentration, such as *p*H 3.5 (32) or 9.0 (34), or in conjunction with the chelating compound ethylenediaminetetraacetate (29), lysozyme exerts a lytic effect on gram-negative bacteria. These, however, are artificial conditions which are unlikely to be employed by phagocytes in their attack on bacteria. A more natural bacteriolytic system, consisting of specific antiserum, complement, and lysozyme, was shown to degrade lysozyme-resistant gram-negative bacteria (1, 23). However, destruction of bacteria within phagocytes can occur in the absence of a classical antibody molecule (2, 19).

From this brief review, it is obvious that a need exists for a better understanding of the mechanisms which would explain the degradation of bacteria within phagocytic cells (3). One

approach to this problem is to create a lytic system in vitro which makes use of conditions and substances that are known to exist in phagocytic cells. The system would have to be capable of acting on surface layers of gram-negative bacteria so as to uncover the substrate of lysozyme. For this purpose, hydrogen peroxide and ascorbic acid were selected for study because they have been shown to exert a degradative effect on lipids, carbohydrates, and proteins (12, 15, 22, 24, 37); moreover, they are known to be present in leukocytes (13, 27, 28, 36).

As stated earlier, it is known that degradation of bacteria within phagocytic cells follows the bactericidal event (3). It was of interest, therefore, to investigate whether the act of uncovering the lysozyme substrate of these gram-negative bacteria was in itself bactericidal. The antibacterial activity of hydrogen peroxide as a topical antiseptic has been recognized for decades (7). Also, the in vitro bactericidal activity of hydrogen peroxide in conjunction with ascorbic acid has been reported (8, 9). Recently, hydrogen peroxide has been implicated in the killing of bacteria inside phagocytic cells (13, 16, 21). According to a current hypothesis, the synthesis of hydrogen peroxide is coupled with the oxidation of reduced nicotinamide adenine dinucleotide phosphate produced through the hexose monophosphate shunt (30), a metabolic event which is augmented after the ingestion of bacteria by the cell (20). However, the exact mechanism of bacterial inactivation in all the above cases is far from clear.

This paper demonstrates that a mixture of hydrogen peroxide and ascorbic acid generates an antibacterial mechanism which appears to depend upon free radical formation. It results in bacterial death and renders the organism sensitive to lysis by lysozyme.

MATERIALS AND METHODS

Determination of lytic activity. The components used in the system were added to Bausch and Lomb Spectronic-20 colorimeter tubes. They were incubated at 37 C and, at intervals, the optical densities (OD) were measured at 540 nm.

Determination of bactericidal activity. The components used in this system were added to Falcon plastic tubes containing bacteria at a final concentration of 10^8 to 10^9/ml. The mixtures were incubated for 30 min at 37 C. They were then diluted in Hanks' balanced salt solution and were plated on Trypticase soy-agar plates.

Materials. The hydrogen peroxide used was a 30% (v/v) solution of s. p. grade Superoxol from Merck & Co., Inc., Rahway, N.J. L-Ascorbic acid was obtained from Eastman Organic Chemicals, Rochester, New York, and from Merck and Co. Horseradish peroxidase (HOPD grade) and lysozyme (Lys) were obtained from Worthington Biochemical Corp., Freehold, N.J. Sodium thiosulfate was obtained from J. T. Baker Co., Phillipsburg, N.J.

The gram-negative organisms employed in this study were *Salmonella pullorum*, *S. enteritidis*, and an untyped *Escherichia coli* from a human isolate. They were grown in Trypticase soy broth (BBL). Organisms in the log phase of growth were washed and suspended in the buffer to be employed for the experiment at a concentration which gave the desired OD at 540 nm when added to the reaction mixtures.

RESULTS

Lytic effect of H_2O_2, ascorbic acid, and lysozyme. In the following experiments, the reaction mixtures contained combinations of acetate (*p*H 5.0) or phosphate buffers (*p*H 6.2 or 7.0) at 50 to 100 μmoles/ml: *S. pullorum*, 2×10^8 to 6×10^8/ml; H_2O_2, 1.0 to 10.0 μmoles/ml; ascorbic acid (AA), 0.1 μmole/ml; and Lys and horseradish peroxidase (HRP), both at 10 μg/ml. The various mixtures were incubated at 37 C for 60 min. OD at 540 nm was measured after 0, 30 and 60 min.

The results of Fig. 1 reveal that little or no change occurred in OD in any of the control mixtures. In contrast, the combination of H_2O_2, AA, and Lys showed a significant decrease in OD indicative of lysis. The inability of HRP to enhance this effect was unexpected, since it is known to catalyze the reaction between H_2O_2 and AA (40). The same lytic phenomenon was also found with *S. enteritidis* and *E. coli*.

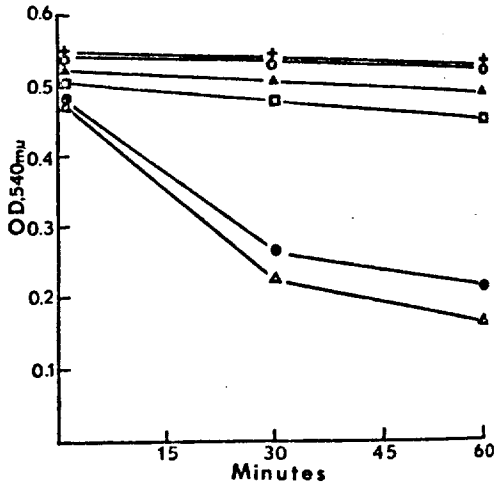

FIG. 1. *Changes in OD of a suspension of S. pullorum incubated at 37 C in the presence of buffer control (+); Lys and AA (○); H_2O_2 and Lys (▲); H_2O_2 and AA (□); H_2O_2, AA, Lys, and HRP (●); H_2O_2, AA, and Lys (△).*

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Effect of pH on the lytic activity of H_2O_2, AA, and Lys. In the foregoing experiment, lysis of bacteria occurred at *p*H 6.2. However, the H-ion concentration within phagocytes might be expected to range between neutrality and the acidic *p*H which develops within digestive vacuoles. Accordingly, the lytic activity of H_2O_2, AA, and Lys were compared at *p*H 5.0, 6.2, and 7.0. Since the previous experiment had suggested a slight decrease in OD with H_2O_2 and AA, this mixture was also tested at the same H-ion concentrations (Fig. 2).

At *p*H 5.0, no lytic activity was observed in the H_2O_2 + AA + Lys mixture. In contrast, lysis was virtually complete within 15 min at *p*H 7.0; whereas at the intermediate *p*H, progressive lysis occurred over the entire period of incubation.

In the absence of Lys, H_2O_2 + AA produced a small but maximal change in OD at *p*H 7.0. From this data, it was not readily apparent whether this decreased OD indicated partial lysis or a change in the shape of the bacterial cells. The latter might be expected if the surface components of the cell are modified under the influence of H_2O_2 + AA, so that Lys-substrate becomes exposed. This possibility was examined in the following experiment.

Uncovering the lysozyme substrate with H_2O_2 and AA. Reaction mixtures were set up containing bacteria and H_2O_2 or bacteria, H_2O_2, and AA. The concentration of each was identical to those used in the previous experiments. The mixtures were incubated for 30 min, centrifuged to remove reagents, and resuspended in buffer containing Lys at 10 μg/ml. A normal reaction mixture was also prepared at this time; it contained bacteria, Lys, H_2O_2, and AA. The reaction mixtures were then reincubated for 60 min at 37 C. OD was read at intervals of 15 min.

The results in Fig. 3 show that bacterial cells were modified by pretreatment with H_2O_2 and AA. Upon resuspension, they were found to have been rendered susceptible to the enzymic action of Lys. H_2O_2 alone did not sensitize the cells in this way.

It will be noted that OD at zero-time were not uniform. This was due to a dilution effect in tubes from which AA had been omitted, but in those containing H_2O_2 and AA it was due to the rapidity with which lysis occurred after the addition of Lys.

Relationship between bacteriolytic and bactericidal activities at neutral and acid pH. The results in Table 1 demonstrate that a powerful bactericidal effect is generated in a mixture consisting of H_2O_2, AA, and Lys. It was much greater at *p*H 7.0 than at *p*H 5.0, and it was impaired, rather than enhanced, by the addition of HRP, as was noted previously in bacteriolysis. For this reason, HRP was omitted from all subsequent

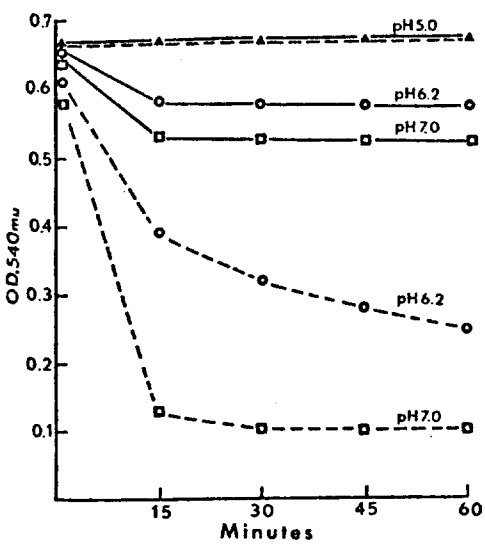

FIG. 2. *Effect of pH on the lysis of S. pullorum incubated at 37 C in the presence of: H_2O_2 and AA (solid lines); H_2O_2, AA, and Lys (broken lines).*

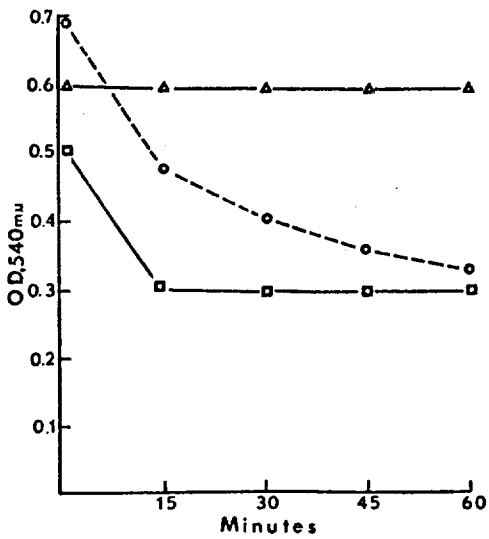

FIG. 3. *Sensitization of S. pullorum by H_2O_2 and AA to Lys activity. Solid lines indicate bacteria pretreated with the following reagents for 30 min at 37 C: H_2O_2 alone (△); H_2O_2 and AA (□). Cells were then resuspended in buffer containing Lys. A control lytic tube (○) contained H_2O_2, AA, and Lys added at zero-time.*

experiments. With one exception, the data of Table 1 show that control mixtures were bactericidally inert. The control which showed activity contained HRP, AA, and H_2O_2. It, too, was more effective at *p*H 7.0. Since HRP did not augment either the bacteriolytic or bactericidal (Table 1) activities of Lys + AA + H_2O_2, it was of interest to study bacterial survival in the presence of AA and H_2O_2, even though this combination was shown to be devoid of bacteriolytic activity.

The data in Table 2 indicate that the combination of H_2O_2 and AA results in a powerful antibacterial system which is further accentuated by the addition of Lys, especially at *p*H 7.0. Figure 4 shows that a positive correlation exists between H-ion concentration and the bactericidal or bacteriolytic activities of the complete reaction mixture. At the higher *p*H, both lytic and cidal activities were more intense. It seemed inconsistent, however, that no bacteriolysis occurred at a *p*H 5.0, despite the presence of a marked bactericidal effect. If the "uncovering" of the lysozyme substrate is involved in the bactericidal event, the absence of a lytic effect at *p*H 5.0 could perhaps be explained in terms of the *p*H optimum for Lys. The following experiment was performed in an attempt to clarify this point.

TABLE 1. *Bactericidal activity of H_2O_2, AA, and Lys at acidic and neutral pH values*

Reagent[a]	No. of viable salmonellae per milliliter at	
	*p*H 5.0	*p*H 7.0
None	5.5×10^8	4.5×10^8
HRP + Lys + AA	5.1×10^8	4.6×10^8
HRP + Lys + H_2O_2	2.1×10^8	4.4×10^8
HRP + AA + H_2O_2	5.6×10^7	3.2×10^4
HRP + Lys + AA + H_2O_2	1.1×10^7	2.2×10^4
Lys + AA + H_2O_2	2.4×10^5	9.0×10^2

[a] Concentrations were as stated in text; incubations were at 37 C for 30 min.

TABLE 2. *Enhancing effect of Lys on the bactericidal activity of H_2O_2 and AA*

Reagent[a]	No. of salmonellae per milliliter at	
	*p*H 5.0	*p*H 7.0
None	2.8×10^8	3.2×10^8
H_2O_2 + AA	9.6×10^4	1.3×10^4
H_2O_2 + AA + Lys	3.9×10^4	3.0×10^2

[a] Incubation was at 37 C for 30 min.

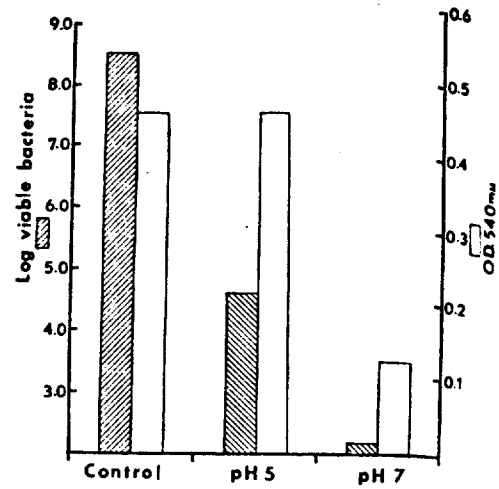

FIG. 4. *Effect of pH on bactericidal (hatched bars) and bacteriolytic (open bars) activities of mixtures containing H_2O_2, AA, and Lys at concentrations indicated in the text. The control tubes contained the bacteria in buffer alone. Incubation was at 37 C for 30 min.*

Three different reaction mixtures were prepared. Tubes 1 and 2 contained bacteria in acetate buffer at *p*H 5.0; tube 3 contained H_2O_2 and AA at the same *p*H. All tubes were incubated for 30 min at 37 C. The bacteria were then removed by centrifugation and resuspended at *p*H 7.0 as follows: tube 1 received Lys as a control; tube 2 received H_2O_2, AA, and Lys; and tube 3 received Lys alone. All tubes were then reincubated at 37 C for an additional 30 min. OD at 540 nm was measured at 0, 15, and 30 min. All reagent concentrations were those used previously.

The results in Fig. 5 demonstrate that sensitization of bacteria occurred at *p*H 5.0 in the presence of H_2O_2 and AA, but owing to the inactivity of Lys at this *p*H, bacteriolysis was absent. This is evidenced by the fact that the sensitized bacteria underwent immediate lysis when resuspended in buffer containing Lys at *p*H 7.0.

The foregoing experiments suggest a common basis for the bacteriolytic and bactericidal effects produced by H_2O_2, AA, and Lys insofar as the act of bacterial sensitization to Lys is identical in both.

Nature of the products responsible for bacterial death or sensitization to lysozyme. The results to this point have demonstrated that bacterial lysis occurs when the interaction of H_2O_2 and AA has rendered the cell sensitive to Lys. However, they do not indicate the nature of the products responsible for this sensitization. The following experiments were performed in an attempt to resolve this question.

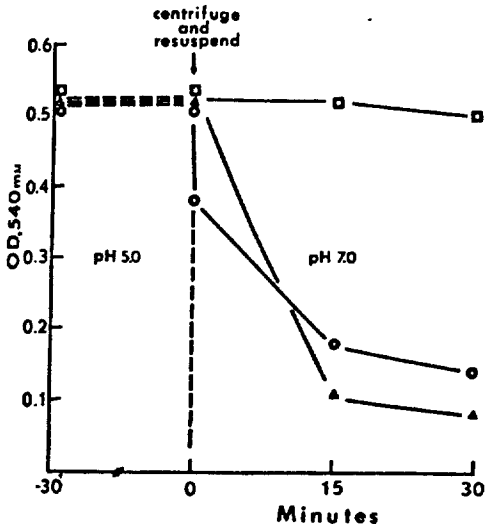

FIG. 5. *Salmonellae sensitized by H_2O_2 and AA at pH 5 to enzymatic lysis by Lys at pH 7. Pretreatment period was for 30 min at 37 C. The experimental conditions were pretreatment of bacteria in buffer at pH 5, followed by Lys in buffer at pH 7 (□); buffer at pH 5, followed by H_2O_2, AA, and Lys at pH 7 (▲); H_2O_2 and AA at pH 5, followed by Lys at pH 7 (○).*

A reaction mixture containing H_2O_2 and AA would be expected to produce end products of water and the oxidized form of AA, dehydroascorbic acid. Therefore, a test was made to determine whether dehydroascorbic acid at the equivalent molar concentration would serve to sensitize bacteria to the lytic action of Lys. It was completely inactive in this respect, suggesting the involvement of some other product of the reaction between H_2O_2 and AA. The preincubation of H_2O_2-AA-Lys, before the addition of bacteria, would be expected to reveal the presence of this sensitizing substance if it were stable. However, the bacteria were shown not to be sensitized under these conditions. The same results were obtained when Lys and bacteria were added to a preincubation mixture of H_2O_2 + AA. Likewise, the data in Table 3 demonstrate that the preincubated mixture of H_2O_2, AA, and Lys were devoid of bactericidal activity. The aforementioned experiments strongly suggested the involvement of some unstable intermediary product of the interaction between H_2O_2 + AA. It has been shown by Yamazaki et al. (40) that unstable free radicals are generated in mixtures of AA and H_2O_2. The possible involvement of free radicals in the sensitization process was investigated in the following experiment.

Inhibition of the bactericidal system by a free radical scavenger. Although free radicals can be directly demonstrated by electron paramagnetic resonance spectroscopy (40), proof of their involvement in reactions described in this study requires a more indirect approach. For example, one criterion of a reaction which depends upon free radical formation is its inhibition by radical trapping agents (35). Sodium thiosulfate, which has been used as a free radical scavenger (37), was therefore included in the mixture of H_2O_2, AA, and Lys at a concentration of 0.2 μmoles/ml. The results in Table 4 indicate that bactericidal activity was completely inhibited by sodium thiosulphate, presumably by the trapping of free radicals before they could act upon the bacterial surface.

DISCUSSION

These studies suggest that the oxidation of ascorbic acid by hydrogen peroxide gives rise to a very potent antibacterial mechanism which is mediated by free radicals. This is manifested by bacterial death or by bacteriolysis in the presence of lysozyme.

It is known that free radicals are quite nonspecific in their attack (18, 31). In this case, it is assumed that they modify the outermost layer of the bacterial cell, allowing lysozyme to reach its substrate. It is consistent with this view that *n*-butyl alcohol, a lipophilic solvent, also renders gram-negative bacteria susceptible to lysozyme

TABLE 3. *Lack of bactericidal activity upon preincubation of reagents before addition of bacteria*

Reagent[a]	No. of viable salmonellae per milliliter
None	4.7×10^8
Lys + AA	5.3×10^8
Lys + H_2O_2	4.5×10^8
AA + H_2O_2	6.6×10^8
Lys + AA + H_2O_2	5.7×10^8

[a] Reagent mixtures without bacteria were incubated for 30 min at 37 C. The salmonellae were then added and incubated for 30 min at 37 C.

TABLE 4. *Inhibition of H_2O_2-AA-Lys bactericidal system with a free radical scavenger (ST)*[a]

Reagents	No. of viable salmonellae per milliliter
None	4.4×10^8
H_2O_2 + AA + Lys + ST	5.3×10^8
H_2O_2 + AA + Lys	5.5×10^2

[a] ST = sodium thiosulfate.
[b] Incubation was 37 C for 30 min.

(25, 26). It is possible, therefore, that free radicals produce a breach in the cell wall, with resulting loss in selective permeability and consequent death. The electron microscope may be able to reveal the physical changes which occur in cells exposed to H_2O_2 and AA, and an internal radioactive label, such as ^{32}P, may be useful in detecting a permeability defect. These techniques have been used to great advantage in analyzing the immune bacteriolytic system (1, 23) by Spitznagel and Wilson (33, 39).

Although the bactericidal activity of hydrogen peroxide and ascorbic acid has been reported by earlier workers (8, 9), it was necessary to reexamine this action to relate it to the bacteriolytic effect described in this paper. There is, moreover, an apparent discrepancy between these observations and those reported recently by other workers (16, 17, 20, 21), who have shown optimal bactericidal activity of H_2O_2 and myeloperoxidase at acidic reactions, whereas in the presence of lysozyme, optimal bacteriolytic and bactericidal activities were found at neutral *p*H in this study. This conflict was partially resolved by the finding that sensitization, but not bacteriolysis, occurred at *p*H 5.0. This was unexpected, since lysozyme is said to be active from *p*H 3.5 to pH 10.0 (26). However, Fleming (10) and Thompson (38) have reported optimal lysozyme activity at neutral *p*H. It is possible, therefore, that the more vigorous bactericidal activity of the hydrogen peroxide-myeloperoxidase system at low H-ion concentrations (16, 21) may be accounted for by the *p*H optimum of this enzyme rather than that of lysozyme.

As stated earlier, the primary purpose of these studies was to provide an in vitro model that would explain the digestion of gram-negative bacteria inside phagocytes (3). In the model studied, bacterial inactivation consisted of two steps. The organism was first rendered sensitive to lysozyme by H_2O_2 and AA; the second step involved bacteriolysis in the presence of Lys. Of great interest was the observation that the sensitization step was accompanied by bacterial death. Thus, death preceded digestion in the system, a sequence of events which is consistent with Cohn's conclusions regarding bacterial inactivation within phagocytes (3). Cohn also demonstrated that lysozyme-sensitive bacteria were degraded more readily in both polymorphonuclear leukocytes (PMN) and macrophages. But, unlike the PMN, not all mononuclear phagocytes contain lysozyme (4, 5), nor have they been shown to contain any of the other well-characterized antibacterial substances (6). Presumably, they also lack peroxidase (4), the catalyst of an antibacterial system involving H_2O_2 (16, 17, 21). Nonetheless, both types of phagocytes are capable of killing a broad spectrum of ingested bacteria (2, 3, 19). Since bacterial death was shown to occur in the present studies in the absence of Lys or peroxidase, a similar process of bacterial inactivation could occur in macrophages even if both of these enzymes are lacking. This does not preclude the possibility that the same mechanism also operates in granulocytes which do contain Lys and peroxidase. It could be argued that phagocytosis of the bacterium results in the synthesis of excess H_2O_2 as a result of the enhanced metabolic activity which is known to accompany particle uptake (28, 30). In the presence of AA or any other oxidizable substance which can liberate free radicals, the integrity of the bacterial cell wall would be lost. This alone might account for the intracellular death of a majority of organisms. The additional influence of enzymic attack on substrates exposed in a disorganized cell wall could serve to increase the vulnerability of the remainder.

ACKNOWLEDGMENT

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Ascorbate-Nitrite Reaction: Possible Means of Blocking the Formation of Carcinogenic N-Nitroso Compounds

Abstract. *The formation of carcinogenic N-nitroso compounds by the chemical reaction between nitrous acid and oxytetracycline, morpholine, piperazine, N-methylaniline, methylurea, and (in some experiments) dimethylamine was blocked by ascorbic acid. The extent of blocking depended on the compound nitrosated and on the experimental conditions. Urea and ammonium sulfamate were less effective as blocking agents. The possibility of in vivo formation of carcinogenic N-nitroso compounds from drugs could be lessened by the combination of such drugs with the ascorbic acid.*

Most *N*-nitroso derivatives of secondary amines and amides are powerful carcinogens, which can act in several species (*1*). These derivatives might possibly be produced in the human stomach by the acid-catalyzed reaction between nitrite (*2*), which is present in some foods, and *N*-nitrosatable compounds,

which may be ingested as drugs, food additives, or natural constituents of food (*3*). As an experimental model for this hypothesis, tumors were induced in rats and mice by the long-term feeding of nitrite together with certain secondary amines (including the vermicide piperazine), alkylureas, and *N*-alkylcarbamates (*4*). The biological results are fairly well correlated with kinetic studies on the widely varying rates of the nitrosation reactions (*5*).

Since many drugs contain secondary amine, alkylurea, or *N*-alkylcarbamate groups, oral administration of these drugs might constitute a human health hazard, if nitrite-containing food were ingested at the same time. Examples of such drugs are piperazine, phenmetrazine, primaquine, pamaquine, physostigmine, synephrine, sulfanilylurea, and neohydrin. This possible hazard might be reduced by administering the drug together with a substance that preferentially reacts with and destroys any nitrite occurring in the stomach. We now present chemical data suggesting that ascorbic acid might be used for this purpose.

In addition to the classes of compound already mentioned, Lijinsky *et al.* (*6*) recently reported that certain drugs which contain tertiary amine or dialkylamide groups can be nitrosated to give dialkylnitrosamines. For example, reaction of the dimethylamino compound oxytetracycline with 0.30*M* nitrite at *p*H 4 and 37°C for 4 hours was reported to give a 65 percent yield of dimethylnitrosamine. We attempted to repeat this study on a commercial sample of oxytetracycline (Terramycin) and obtained negative results, but when pure oxytetracycline was used, we confirmed the published results. Our sample of Terramycin was compounded as a mixture containing 80 percent ascorbic acid, and when the same proportion of ascorbic acid was added to a sample of pure oxytetracycline and this was treated with nitrite, no nitrosation was observed. Further experiments were conducted in the presence and absence of ascorbate, and these confirmed that ascorbate almost completely blocked the nitrosation under conditions similar to those used by Lijinsky *et al.* (Table 1, experiments 1 and 2).

We then examined the effect of ascorbate on the nitrosation of five representative compounds whose nitrosation kinetics we have studied (*5*) (Table 1, experiments 2 to 21). Using a 2 : 1 ratio of ascorbate to nitrite, we found more than 98 percent blocking of nitrosation for morpholine and piperazine at all *p*H values tested. Blocking was only partial for *N*-methylaniline, but was fairly complete for methylurea. When the relatively slow nitrosation of dimethylamine was studied under conditions similar to those used for the other amines (reaction at 25°C with excess amine), blocking by ascorbate was fairly effective at *p*H 3 and 4, but at *p*H 2 there was little effect, and at *p*H 1, where the yield without ascorbate was only 0.042 percent, ascorbate increased the nitrosation to 0.076 percent (experiments 15 to 18). When dimethylamine was nitrosated under similar conditions to those used for the nitrosation of oxytetracycline, that is, prolonged reaction at 37°C with excess nitrite, the yield without ascorbate was much larger (2 to 82 percent) and more than 90 percent blocking was observed in the presence of ascorbate (experiments 19 to 21).

Dahn *et al.* (*7*) studied the kinetics of the reaction between nitrous acid and ascorbic acid, in which dehydroascorbic acid and nitric oxide are formed, probably via the nitrite ester of the 3-hydroxy group. In 0.1 to 0.5*N* perchloric acid the main nitrosating species is protonated nitrous acid ($H_2NO_2^+$), whereas in more dilute acid (*p*H 1.5 to 5.0) the nitrosating species is nitrous anhydride (N_2O_3). These are also the nitrosating species for second-

Table 1. Effect of ascorbate on the yield of *N*-nitroso compounds. A solution of oxytetracycline (250 mg), $NaNO_2$ (500 mg), and acetic acid (1 ml) in water (to 23 ml) was adjusted to the desired *p*H with HCl and kept for 22 hours at 37°C in a 100-ml flask fitted with a loosely stoppered condenser; it was then made alkaline with solid NaOH and extracted twice with 75 ml of CH_2Cl_2. The CH_2Cl_2 solution was extracted with 14 ml of 0.1*N* HCl and analyzed for dimethylnitrosamine by gas-liquid chromatography (*6*). For the experiments with secondary amines (*14*), 5 ml of a solution of $NaNO_2$ in 0.05*M* sodium citrate was acidified to the desired *p*H with perchloric acid and was added at 25°C to a solution of the amine plus sodium ascorbate in 15 ml of 0.05*M* citrate (previously adjusted to the same *p*H with perchloric acid). The 20-ml reaction mixture was kept in a stoppered 50-ml conical flask for 5 to 60 minutes at 25°C in the dark. The reaction was stopped by making it alkaline, and solid NaCl was added (in the case of some amines). The nitroso derivative was extracted with CH_2Cl_2 or ether, and its absorbance at 358 to 369 nm was measured. When the absorbance was less than 0.10, the extract was dried over Na_2SO_4, some of the solvent was removed by a rotary evaporator, and another absorbance reading was taken. Results were calculated from the molar absorptivity (ϵ) of the nitroso derivative and corrected for losses due to incomplete extraction. Each experiment was performed in duplicate with and without blocking agent. The full spectra of the extracts at 320 to 450 nm were identical to those of the nitroso derivative. When amine, nitrite, or ascorbate were separately run through the complete procedure, the extract showed no absorbance. When an acidic solution of nitrite plus ascorbate was made alkaline, it turned yellow and then red, but extracts showed no absorption above 320 nm. Methylnitrosourea formation at 25°C was determined by absorbance of the aqueous solution at 400 nm (ϵ, 79) (*5*).

Exp. No.	Base (m*M*)	Nitrite (m*M*)	Ascorbate (m*M*)	*p*H	Time (min)	Yield from amine or urea (%)		Blocking (%)
						Without ascorbate	With ascorbate	
				Oxytetracycline				
1	21.6	315	475	2.8	1320	44	0	100
2	21.6	315	475	3.8	1320	63	0.045	99.9
				Morpholine				
3	25	50	100	2	30	20	0.48	98
4	25	50	100	3	30	65	0	100
5	25	50	100	4	30	34	0	100
				Piperazine				
6	10	10	20	1	60	35	0.58	98
7	10	10	20	2	20	44	1.2	98
8	10	10	20	3	10	56	0.33	99
9	10	10	20	4	15	54	0.24	99
				N-Methylaniline				
10	2.5	2.5	5	3	5	68	27	60
11	2.5	2.5	5	4	10	16	8.8	45
				Methylurea				
12	5	10	20	1	10	85	6.9	92
13	5	10	20	2	10	58	7.6	87
14	20	20	40	3	30	27	0	100
				Dimethylamine				
15	500	50	100	1	60	0.042	0.076	−80
16	500	50	100	2	60	0.27	0.29	−7
17	500	50	100	3	60	0.76	0.20	74
18	500	50	100	4	60	0.92	0.03	97
19	20	300	500	2	1440	2.2	0.17	92
20	20	300	500	2.8	1380	20	1.7	91
21	20	300	500	3.8	1380	82	1.4	98

ary amines and alkylureas (*5*) [in the presence of bromide, iodide, and thiocyanate ions, the corresponding NOX species are active nitrosating species for N-nitrosation (*8*)]. Therefore, the blockage of *N*-nitrosation by ascorbate was probably due to competition for available nitrite, or to be precise, for N_2O_3 and $H_2NO_2^+$. Ascorbate anion is 240 times more rapidly nitrosated than ascorbic acid (pK_a, 4.29), due presumably to the greater nucleophilic activity of the anion. At *p*H 3 to 5, where the proportion of anion is significant, reaction with nitrite is so rapid that the formation of N_2O_3 is rate-limiting. This explains why ascorbate is more effective as a blocking agent at *p*H 3 to 4 than at *p*H 1 to 2.

Ascorbate had no effect on decomposition of the *N*-nitroso derivatives of methylurea and the amines listed in Table 1, indicating that its action in reducing or eliminating the formation of *N*-nitroso compounds is solely a consequence of the reaction with nitrite.

Nitric oxide produced by the ascorbate-nitrite reaction may combine with atmospheric oxygen to form nitrogen dioxide, some of which would reenter solution and react with water to generate equimolar amounts of nitrous and nitric acids. Thus up to half of the nitric oxide could potentially be reconverted to nitrite. The presence of excess ascorbate in our experiments served to minimize the effect of such reconversions on the extent of *N*-nitrosation.

The nitrosation kinetics for the amines studied is in accord with the relative effectiveness of ascorbate in blocking the nitrosation of these compounds. For example, *N*-methylaniline, like ascorbate, is nitrosated so rapidly that N_2O_3 formation is rate-limiting (*9*), so that the amine competes rather successfully with ascorbate for N_2O_3, and blocking is only partial. Morpholine and piperazine are nitrosated less rapidly (*5*), N_2O_3 formation is not rate-limiting, and it is therefore not surprising that these nitrosations were effectively blocked by ascorbate. Dimethylamine, which is nitrosated much slower than the other amines tested (*5*), was not effectively blocked by ascorbate under those conditions in which relatively little nitrosamine was formed even in the absence of ascorbate. When the reaction conditions were adjusted to increase the yield of nitrosamine, the blocking action of ascorbate was more pronounced. The reason for this behavior is not clear. Blocking of methylurea nitrosation was perhaps stronger than might be expected at *p*H 1, where the $H_2NO_2^+$ mechanism is predominant and methylurea is rapidly attacked (*5*).

Table 2. Effect of the blocking agents urea and ammonium sulfamate on the yield of nitrosomorpholine and mononitrosopiperazine. The experimental procedure followed that described for Table 1, except that urea or ammonium sulfamate was substituted for ascorbate.

Base (m*M*)	Nitrite (m*M*)	Blocking agent (m*M*)	*p*H	Time (min)	Yield from amine (%): Without blocking agent	Yield from amine (%): With urea	Yield from amine (%): With ammonium sulfamate	Blocking (%): Urea	Blocking (%): Ammonium sulfamate
					Morpholine				
25	50	100	1	45	7.3	0.4	0	95	100
25	50	100	2	30	25	18	0	24	100
25	50	100	3	30	56	55	0.6	2	99
25	50	100	4	30	32		9.3		71
					Piperazine				
10	10	20	1	60	35	11	0	68	100
10	10	20	2	20	36	31	0	14	100
10	10	20	3	10	56	57	14	−2	75

The kinetics of oxytetracycline nitrosation have not yet been examined. Studies on other tertiary amines (*10*) suggest that nitrous acid first reacts with oxytetracycline to give dimethylamine, which is then nitrosated to give dimethylnitrosamine. Under similar conditions, ascorbate blocked the formation of dimethylnitrosamine more effectively from oxytetracycline (experiments 1 to 2) than from dimethylamine (experiments 19 to 21), suggesting that ascorbate reacts with nitrite sufficiently rapidly to prevent the formation of dimethylamine from oxytetracycline.

Since urea and ammonium sulfamate (*11*) also react rapidly with nitrite, the effect of these substances on the nitrosation of piperazine and morpholine was examined (Table 2). Urea was relatively ineffective, especially at higher *p*H. Ammonium sulfamate was even more effective than ascorbate at *p*H 1 and 2, but was relatively ineffective at *p*H 3 and 4. The *p*H of the fasted stomach can reach 0.9, but when a meal is ingested the *p*H of the food is lowered gradually over 1 to 2 hours until it reaches 1.5 to 2.0 (*12*). Hence ascorbate has the advantage over the other agents in that it acts efficiently at the higher *p*H values, and it could react with nitrite in the stomach before the *p*H is lowered to where extensive reaction with amines or ureas might occur.

Although under some conditions the nitrosation of dimethylamine was actually increased by ascorbate, the chemical results on the whole support the suggestion that drugs which can be *N*-nitrosated could be combined with ascorbate before they are administered. This would apply whenever nitrosation of the drug in vivo was likely to occur and combination with ascorbate would reduce this potential hazard without affecting the drug action. Furthermore, addition of ascorbate to certain foods containing nitrite or nitrosatable compounds might be worth considering. In fact, small amounts of ascorbate are often added to nitrite-preserved meat to increase the nitric oxide available for formation of the red nitrosomyoglobin color (*13*).

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Ascorbic Acid and Ethylene Diamine Tetraacetate as Antidotes in Experimental Vanadium Poisoning. (20831)

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It has been recently shown that the calcium disodium salt of ethylenediamine tetra-acetic acid ($CaNa_2EDTA$) affords protection against lethal doses of vanadium in mice(1). In a study of vanadium toxicity by Proescher *et al.*(2), it was suggested that the mode of detoxication of vanadium might be reduction in the body, from the evidence that the lower valence states of vanadium appeared to be less toxic than the higher valence states. It was therefore thought possible that ascorbic acid, a reducing substance, might also decrease the toxicity of vanadium. Accordingly, comparative tests of antidotal effectiveness of ascorbic acid and $CaNa_2EDTA$ were made in 3 animal species and the probable detoxication mechanism of the former antidote has been elucidated by polarography.

Methods. Mice of 20-29 g body weight of the Webster† strain and rats, 100-150 g body weight of a Wistar-Carworth† derived strain were used. Dogs were male mongrels, 6-12 kg in weight. The form of vanadium for administration was $NaVO_3 \cdot H_2O$‡ in isotonic saline solution. As used for mice, the concentration was 4 mg/ml (pH 7); for rats, 1% solutions (pH 7.85), and 3% solutions (pH 7)§ for the dogs. Calcium disodium ethylenediamine tetraacetate was chosen as the EDTA salt because of the reduced tendency of this salt to combine with the blood calcium(3). Ascorbic acid solutions (ca. pH 2.5) prepared without neutralization seemed to be well tolerated. Mice were injected intraperitoneally with ascorbic acid, 20 minutes prior to vanadium administration by the same route. Rats were injected with vanadium subcutaneously and were given the antidotes intraperitoneally 5 minutes later when signs of toxicity had become manifest. An observation period of 7 days was used in all mouse and rat tests. Dogs were injected intramuscularly with vanadium and the antidotes administered intraperitoneally after the first vomiting episode. Polarographic studies were made with a visible recording Sargent Model XXI instrument. Phosphate buffer, pH 7.4, gave no evidence of significant complex formation with vanadium. Each determination was made at 30°C on a 20-ml aliquot containing 0.01% gelatin as a maxima suppressor.

TABLE I. Protection of Mice by Ascorbic Acid against the Toxicity of Vanadium. Mortality ratios expressed as number of animals that died (numerator) to those used (denominator).

mg/kg $NaVO_3 \cdot H_2O$	mg/kg as V	Controls, no ascorbic acid	Ascorbic acid (1 g/kg), 20 min. before vanadium inj.
30	10.9	14/20	1/20
40	14.6	19/20	0/20

Results. Mice. Table I shows the protection afforded mice by ascorbic acid, intraperitoneally administered in a dose of 1 g/kg, 20 minutes prior to vanadium administration. Protection was essentially complete at doses corresponding to the LD_{70} and LD_{95} levels of $NaVO_3 \cdot H_2O$ (30 and 40 mg/kg, respectively). Ascorbic acid injected alone produced no signs of toxicity in 20 mice other than slight transitory depression. In subsequent tests, doses as low as 125 mg ascorbic acid/kg body weight afforded essen-

TABLE II. Antidotal Action of $CaNa_2EDTA$ and Ascorbic Acid in Vanadium-Poisoned Rats. Antidotes administered 5 min. after vanadium. Mortality ratios expressed as number of animals that died (numerator) to those used (denominator).

mg/kg $NaVO_3 \cdot H_2O$	mg/kg as V	Controls	$CaNa_2$-EDTA, 1 g/kg	Ascorbic acid, 250 mg/kg
60	21.9	20/20	7/20	6/20

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† Obtained from Hamilton Laboratory Animals, Inc., Cincinnati, O.

‡ Obtained from Fisher, Eimer and Amend.

§ The more concentrated 3% metavanadate solutions required neutralization with HCl to bring to a more physiologic pH value.

tially complete protection against an LD_{70} of $NaVO_3 \cdot H_2O$.

Rats. Table II shows the dosage-mortality data for rats when either $CaNa_2EDTA$ or ascorbic acid was administered after vanadium had given rise to manifest signs of toxicity. The 60 mg/kg dose of $NaVO_3 \cdot H_2O$ was MLD_{100} by subcutaneous administration. When $CaNa_2EDTA$ (1 g/kg) was administered 5 minutes after the vanadium, 65% of the rats survived. Ascorbic acid (250 mg/kg) administered in the same manner resulted in 70% survival.

Dogs. The intramuscular MLD of 10 mg $NaVO_3 \cdot H_2O$/kg was found to produce death within 18-36 hours with survival in only an occasional animal after a 7-day semicomatose state. Using this dose, 3 dogs were given $CaNa_2EDTA$ (100 mg/kg) intraperitoneally, after the first vomiting episode had occurred. Two more doses of EDTA at the same level and by the same route were administered at 2 and 4 hours after the first injection. Twenty-four hours later the dogs appeared to be in fair condition, and within 48 hours, the dogs were eating and showed few or no signs of vanadium toxicity. Similar responses were obtained after 3 more dogs were given 10 mg $NaVO_3 \cdot H_2O$/kg, followed by 50 mg ascorbic acid/kg‖ after the first toxic signs. Two subsequent injections of ascorbic acid were given, of the same dose and by the same route, at 2 and 4 hours after the initial injection. The ascorbic acid-treated dogs appeared to recover more quickly and showed fewer signs of toxicity than the EDTA-treated dogs. The dogs that had been treated with the antidotes appeared to be in good health and remained so for an observation period of 8 weeks after the V injection.

Polarographic studies. Ascorbic acid produced an anodic wave at an observed half-wave potential ($E_{1/2}$) of −0.01 volt. Current-voltage at different V^{+5} concentrations produced ill-defined, double waves measurable quantitatively within ±20%. A potential of −0.17 volt was taken as a reproducible $E_{1/2}$ for V^{+5} at pH 7.4 and was used as a reference for subsequent work. Similar findings have been previously described for V^{+5} in oxalate solutions(4). To determine the effects of ascorbic acid on V^{+5}, a polarogram was made of a solution containing 77 μg $NaVO_3 \cdot H_2O$/ml of phosphate buffer at pH 7.4; subsequently ascorbic acid, sufficient to give a concentration of 385 μg/ml, was added to the oxygen-free solution. Polarograms of this solution made at intervals of 15 minutes indicated that after the first interval approximately 50% of the V^{+5} had disappeared, and after one hour, 100%. This solution, buffered with acetate to pH 5.6 gave a double anodic wave characteristic of V^{+2}, thus suggesting that V^{+5} is reduced by ascorbic acid to V^{+2}. Hydrogen peroxide, an associated oxidation product of ascorbic acid, was believed not to be the agent involved in this reduction because the polarographic waves did not simulate those developed from the addition of ascorbic acid to V^{+5} solution. After reduction of V^{+5} with ascorbic acid bubbling air through the solution for several hours oxidized all the ascorbic acid, and the V returned to the pentavalent state as evidenced by the reappearance of the characteristic V^{+5} curve.

To observe the effect of an acid without strong reducing powers, gluconic acid was added to a fresh solution of $NaVO_3 \cdot H_2O$. The resulting curve resembled the V^{+5} curve, but was greatly expanded, and the observed $E_{1/2}$ was shifted slightly, thus suggesting the formation of a vanadium complex(5). The failure of V^{+5} to disappear in this experiment showed that gluconic acid does not reduce vanadium as does ascorbic acid.

$CaNa_2EDTA$ added to a solution of $NaVO_3 \cdot H_2O$ did not change the wave pattern, but shifted the $E_{1/2}$ slightly. ($CaNa_2$-EDTA concentration, 1540 μg/ml; $NaVO_3 \cdot H_2O$ concentration, 77 μg/ml.) The observed response suggests a very loosely-bound complex of vanadium with the chelating agent. The $E_{1/2}$ of a vanadyl sulfate (V^{+4}) solution was shifted slightly when treated with $CaNa_2EDTA$ indicating the probable formation of a weak complex similar to that found with V^{+5}.

Discussion. In mice and rats, signs of

‖ This dose of ascorbic acid for dogs was approximately 5 times that of the metavanadate on a mg basis; that for rats (250 mg/kg) and mice (125 mg/kg) approximately 4 times.

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vanadium toxicity were usually present for 2 hours or more after $CaNa_2EDTA$ was administered to vanadium-poisoned animals; however, recovery gradually occurred in the majority of the animals. Dogs behaved similarly, with signs of vanadium toxicity being present for some time after $CaNa_2EDTA$ administration, with gradual, but eventual, recovery.

When ascorbic acid was administered to vanadium-poisoned animals, the signs of vanadium toxicity appeared to be arrested within one-half hour and recovery appeared to be more rapid than with $CaNa_2EDTA$. It has been reported(6) as well as indicated from the present studies, that EDTA forms a loose complex with vanadium which might explain why a short period of time is required before the signs of toxicity begin to subside in vanadium-poisoned animals, after EDTA administration. On the other hand, ascorbic acid was found to react rapidly with $NaVO_3 \cdot H_2O$ *in vitro* from polarographic studies, in which approximately 50% of the pentavalent vanadium disappeared from solution within 15 minutes after addition of ascorbic acid. Unfortunately it was not possible to decide from these determinations alone whether ascorbic acid formed a complex with the vanadium it reduced. Only 50% ascorbic acid was recoverable, however, by actual analysis from an equimolar mixture of vanadium and ascorbic acid shortly after mixing. A reasonable assumption is that the remainder was oxidized to noncomplexable moieties.

It has been shown(7) that acidity decreases the toxicity of injected vanadium; however, this is not believed to be the chief mode of action of ascorbic acid in these antidotal tests. Relatively large doses of ammonium chloride were required to give only a moderate prophylactic protection, whereas far smaller doses of ascorbic acid afforded high protection against vanadium. Moreover, in the smaller effective doses employed, 125 mg/kg, ascorbic acid would not be expected to alter appreciably the tissue milieu.

Summary. 1. Ascorbic acid at 1 g/kg when administered prior to vanadium antagonizes the toxicity of vanadium as $NaVO_3 \cdot H_2O$ in mice at the LD_{70} and LD_{95} levels. Dosage levels as low as 125 mg/kg ascorbic acid showed a protective action against the LD_{70} of $NaVO_3 \cdot H_2O$ in mice. 2. Antidotal effects of calcium disodium ethylenediamine tetraacetate and ascorbic acid have been demonstrated in vanadium-poisoned rats and dogs, after signs of toxicity had become manifest. 3. Ascorbic acid is probably the more rapidly-acting of the 2 antidotes. Signs of vanadium toxicity were present for a longer period of time in vanadium-poisoned animals after $CaNa_2EDTA$ than after ascorbic acid administration. 4. One possible mode of detoxication of vanadium by ascorbic acid is suggested, on the basis of *in vitro* polarographic studies, to be reduction of the vanadium.

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TOXICOLOGY

Vitamin C as an antidote in cases of poisoning from lethal doses of mercury, Article by Momcito Mokranjac and Ceda Petrovic, presented by René Fabre

Vitamin C under the given conditions may serve as a sure antidote in cases of poisoning from even 100% lethal doese of mercury salts.

We have previously demonstrated(1) that vitamin C may be usefully given as an antidote in cases of poisoning from lethal doses of lead. In this work, we will briefly describe the results which we have obtained using vitamin C as an antidote in cases of poisoning from lethal doses of mercury salts.

In this present article, we will describe the first results of our research, using guinea pigs for our experiments.

In a large series of experiments, we determined the LD_{100} amounts for guinea pigs. These experiments showed us that the amount which is 100% lethal for guinea pigs is 14.3 mg/kg of animal weight. This amount was determined on thirty animals. All the animals died within a period varying generally between 22 and 50 hours (80%) and exceptionally up to 125 hours.

Once the LD_{100} was determined, we began the experiments with vitamin C. These experiments were done in four series.

In the first series of experiments, the guinea pigs received first of all for 6 days 0.2 g of vitamin C orally. The sixth day we gave to all the animals in the experiment (20) amounts of mercury chloride which we

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(1) M. Mokranjac, S. Radmic and D. Soldatovic, Acta Pharm. Jugosl. 12, 1962, p. 3.

had first determined as being 100% fatal for guinea pigs. After the poisoning of the animals, we continued giving them 0.2 g of vitamin C per day and observed them. The first two or three days after the poisoning, the animals lost a little weight, but continued to eat and to behave almost normally. The animals were continuously given 0.2 g of vitamin C per day for 20 days after the poisoning. All the animals treated in this way survived. On the 20th day, the experiment was stopped, all the animals of the experiment being considered as saved. However, their observation was continued, and now, several months after the poisoning, they are still well.

Next, we wanted to see what would happen if the animals were not treated with vitamin C before poisioning. For this, we directly gave the guinea pigs the lethal dose of mercury salts and immediately afterwards 0.2 g% of vitamin C, and continued to give them vitamin C in the days following. Of the 25 animals treated in this way, 9 died between the third and the eighth days. The others survived the poisoning.

The third series of experiments had to show us whether a single dose of vitamin C given after the poisoning could save the animal. The animals of this series received without any preparation the lethal dose of mercury salts and immediately afterwards, vitamin C (1 g). But in the following days, we did not continue giving the animals the vitamin. Of the 10 animals of this experiment, 8 died between 43 and 250 hours after the poisoning. Thus, the preparation of the animals by the preliminary treatment with vitamin C played an essential role in the treatment of animals poisioned by mercury chloride.

In the following series of experiments, we wanted to clarify even further the significance of the preliminary treatment with the vitamin

before poisoning. Twenty guinea pigs were given 0.2 g% vitamin C for 6 days. The sixth day the animals were poisoned with a 100% lethal dose of mercury chloride, but vitamin C was not continued to be given. Of all these animals, only 2 died from poisoning and this was between 45 and 124 hours.

For each series of the experiments, we had a control lot of animals.

In trying to explain theoretically the not only favorable, but the essential effect of vitamin C in poisoning from lethal doses of mercury, since, as was seen in the first experiment, 100% of the animals poisoned by lethal doses of mercury salt were saved, we thought first of all that it involved an oxidation-reduction reaction; in other words, that vitamin C caused a reduction of the mercuric salt into mercurous salt (calomel) insoluble and consequently, not toxic. But the second and third series of our experiments which we related above did not support this explanation. We are pursuing experiments in order to try to explain this effect of vitamin C.

But in any case, the results revealed in this article show that vitamin C may serve under fixed conditions as an absolutely sure antidote in poisoning from lethal doses of mercury salts.

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C. R. Acad Sci (Paris) 258: 1341-42, 1964.

TOXICOLOGIE. — *La vitamine C comme antidote dans les cas d'intoxication par les doses mortelles de mercure.* Note (*) de MM. **Momčilo Mokranjac** et **Čeda Petrović**, présentée par M. René Fabre.

La vitamine C peut servir, dans les conditions données, comme un antidote sûr, même dans les cas d'intoxication par des doses 100 % mortelles de sels de mercure.

Nous avons antérieurement démontré (1) que la vitamine C peut être utilement appliquée comme antidote dans les cas d'intoxication par des doses létales de plomb. Dans ce travail nous exposerons brièvement les résultats que nous avons obtenus en utilisant la vitamine C comme antidote dans les cas d'intoxication par des doses mortelles de sels de mercure.

Dans la présente Note, nous exposerons les premiers résultats de nos recherches faites en utilisant pour nos expériences les cobayes.

Nous avons d'abord, dans une grande série d'expériences, déterminé les doses DL_{100} pour les cobayes. Ces expériences nous ont montré que la dose 100 % mortelle pour les cobayes est de 14,3 mg par kilogramme du poids d'animal. La détermination de cette dose a été faite sur une trentaine d'animaux. Tous les animaux ont succombé dans un délai variant généralement entre 22 et 50 h (80 %) et exceptionnellement jusqu'à 125 h.

Une fois la dose de DL_{100} déterminée, nous avons commencé les expériences avec la vitamine C. Ces expériences ont été faites en quatre séries.

Dans la première série d'expériences, les cobayes recevaient d'abord pendant 6 jours 0,2 g de vitamine C par voie buccale. Le 6e jour nous avons donné à tous les animaux d'expérience (20) les doses de chlorure de mercure que nous avons d'abord déterminées comme étant 100 % mortelles pour cobayes. Après l'intoxication des animaux on a continué à leur donner 0,2 g de vitamine C par jour, et l'on a observé les animaux. Les deux ou trois premiers jours après l'intoxication, les animaux perdaient un peu de poids, mais continuaient à manger et à se comporter presque normalement. On continua toujours à donner aux animaux 0,2 g de vitamine C par jour pendant 20 jours après l'intoxication. Tous les animaux traités de la sorte ont survécu. Le 20e jour on a cessé l'expérience, tous les animaux de l'expérience étant considérés comme sauvés. Cependant, on a continué de les observer encore, et maintenant, plusieurs mois après l'intoxication, ils se portent toujours bien.

Dans la suite nous avons voulu voir ce qui se passerait si les animaux n'étaient pas traités par la vitamine C avant l'intoxication. Pour cela nous avons donné aux cobayes directement la dose mortelle des sels de mercure, et tout de suite après la vitamine C (0,2 g) en continuant de leur donner la vitamine C les jours suivants. De 25 animaux traités de la sorte, 9 ont succombé entre les 3e et 8e jours. Les autres ont survécu à l'intoxication.

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La troisième série d'expériences devait nous montrer si une seule dose de la vitamine C donnée après l'intoxication pouvait sauver l'animal. Les animaux de cette série, ont reçu, sans aucune préparation, la dose létale du sel de mercure, et tout de suite après de la vitamine C (1 g). Mais on n'a pas continué les jours suivants à leur donner de la vitamine. De 10 animaux de cette expérience, 8 ont succombé entre 43 et 250 h après l'intoxication. Donc la préparation des animaux par le traitement préalable par la vitamine C a joué un rôle essentiel dans le traitement des animaux intoxiqués par le chlorure de mercure.

Dans la série suivante d'expériences nous voulions éclaircir plus encore l'importance du traitement préalable par la vitamine avant l'intoxication. On a donné aux 20 cobayes, pendant 6 jours, 0,2 g de la vitamine C par jour. Le 6e jour les animaux ont été intoxiqués par la dose 100 % mortelle de chlorure de mercure mais on n'a pas continué à leur donner la vitamine C. De tous ces animaux 2 seulement ont succombé à l'intoxication et cela entre 45 et 124 h.

Il va de soi que pour chaque série d'expériences on avait un lot d'animaux de contrôle.

En essayant d'expliquer théoriquement l'action non seulement favorable, mais capitale de la vitamine C dans ces intoxications par les doses mortelles de mercure, puisque, comme on l'a vu dans la première expérience, 100 % des animaux intoxiqués par les doses létales de sel de mercure ont été sauvés, nous avons d'abord pensé qu'il s'agissait peut-être d'une réaction d'oxydo-réduction, autrement dit que la vitamine C a provoqué une réduction du sel mercurique en sel mercureux (calomel), insoluble, et par conséquent non toxique. Mais les deuxième et troisième séries de nos expériences, que nous avons relatées plus haut, ne parlent pas en faveur de cette explication. Nous poursuivons les expériences en vue d'essayer d'expliquer cette action de la vitamine C.

Mais, en tout cas, les résultats exposés dans cette Note montrent que la vitamine C peut servir, dans les conditions déterminées, comme un antidote absolument sûr dans les intoxications par les doses mortelles de sels de mercure.

(*) Séance du 6 janvier 1964.

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Indust. Eng. Chem. Anal. Ed. 13, 793-794 1941,

Rapid Photometric Determination of Ascorbic Acid in Plant Materials

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THE method described here is an adaptation and modification of the photometric determination of ascorbic acid in blood serum as reported by Mindlin and Butler (*7*) and modified by Bessey (*1*) to include colored or turbid solutions and plant tissue extracts. The new features reported here involve chiefly the methods of extraction, filtering instead of centrifuging, and various changes in concentration of reagents to permit measurement of a wider range—1 to 14 micrograms—of ascorbic acid in the final aliquots. These modifications were developed especially to permit high-speed work on large numbers of plant samples daily, as in evaluating thousands of individuals among segregating populations incidental to breeding for high vitamin C content in vegetables. By these modifications, an analyst with two assistants can readily run 120 samples daily.

Since the photometric method has been discussed by others (*1, 7*) and the use of the indophenol dye has been reviewed by King (*5*), the principles and the advantages of the basic method need not be reviewed here.

Apparatus and Reagents

The fresh plant tissues were first reduced to a fine pulp in the Waring blender as described by Davis (*3*), using two containers alternately to increase the output of the machine.

The following solutions were required: (1) 3 per cent metaphosphoric acid; (2) sodium citrate buffer of 211 grams of citric acid in 2 liters of 1 *N* sodium hydroxide; (3) a buffer at pH 3.6 (±0.1); mixture of 3200 ml. of solution 1 and 868 ml. of solution 2; and (4) a solution of 2,6-dichlorophenolindophenol containing 34.4 mg. (Eastman Kodak Co. preparation) in 1 liter of water. Solutions were used within 5 days and always stored in the ice chest overnight. Control of pH was accomplished by means of the McGinnes (*6*) glass electrode. The purity of the ascorbic acid used for preparing the calibration curve, and for the recovery experiments, was tested by titration with standard iodine solution as described by Bessey and King (*2*). Quantitative titration values were obtained within 2 per cent of theory.

The photoelectric colorimeter described by Evelyn (*4*) was used with green filter No. 520 (transmission limits 495 to 550 millimicrons). Six dozen 17.5 × 2.2 cm. (7 × 0.875 inch) absorption test tubes were selected which agreed to within 0.25 galvanometer unit.

Procedure for Calibrating Standard Curve

A fresh solution of ascorbic acid is prepared by dissolving 25 mg. in 250 ml. of buffer at pH 3.6 (solution No. 3). (After 2 days, at 4° C., this solution lost 5 per cent of its reducing power.) One- to 14-ml. amounts, by 1-ml. increments, are added to 100-ml. volumetric flasks, which are then made to volume with the buffer at pH 3.6. Five-milliliter portions of the dye solution are added to each of a series of colorimeter test tubes with a pipet reserved for this purpose, thus assuring as near the same quantity of dye as practicable in all experiments.

The colorimeter is set at 100 per cent transmission, using a tube containing 5 ml. of buffer (pH 3.6), 5 ml. of the dye, and a few crystals of ascorbic acid for complete decoloration; this is conducted in triplicate to assure a correct galvanometer setting. The center setting (without any tube in the instrument) is then recorded and maintained constant for the subsequent determinations: five milliliters of a sample solution are quickly delivered from an Ostwald pipet into 5 ml. of the dye and shaken vigorously about 5 seconds; a reading is taken 15 seconds after initial mixing and again at 30 seconds. After observing the values for all 14 samples, a blank reading in triplicate is obtained using 5 ml. of the buffer. The differences between the 15- and 30-second readings did not exceed 0.5 galvanometer unit and the average of the two was used.

TABLE I. CALIBRATION DATA FOR PHOTOMETRIC DETERMINATION OF ASCORBIC ACID[a]

Micrograms/Ml.[b]	Galvanometer Readings[c]	Photometric Density (2 − log G)[d]	Log G Sample − Log G Blank: Observed	Calculated[e]	Ratio[f] (Observed/Calcd. × 100)
0 (blank)	20.50	0.6830	....	....	....
1.006	23.63	0.6220	0.0610	0.0581	105.0
2.012	26.13	0.5750	0.1080	0.1024	105.5
3.018	28.13	0.5435	0.1395	0.1467	95.0
4.024	31.63	0.4930	0.1900	0.1912	99.4
5.030	35.63	0.4425	0.2405	0.2355	102.1
6.036	39.00	0.4060	0.2770	0.2799	99.0
7.042	42.63	0.3680	0.3150	0.3243	97.2
8.048	47.38	0.3220	0.3610	0.3686	98.0
9.054	54.00	0.2656	0.4174	0.4130	96.1
10.060	60.50	0.2182	0.4648	0.4573	101.6
11.066	66.00	0.1805	0.5025	0.5017	100.3
12.072	74.25	0.1293	0.5537	0.5461	101.3
13.078	81.25	0.0901	0.5929	0.5904	100.3
14.084	87.75	0.0568	0.6262	0.6348	98.7

[a] 5 ml. of dye (34.44 mg. of 2,6-dichlorophenolindophenol per liter) mixed with 5 ml. of sample solutions containing 1 to 14 micrograms of ascorbic acid per ml.
[b] Ascorbic acid stock solution contained 25.18 mg. dissolved in 250 ml.
[c] Galvanometer was read to nearest quarter division and mean of 15- and 30-second readings used.
[d] Corrected for slight deviations from true linearity of relation between current and galvanometer deflection.
[e] Calculated from best line (derived by method of least squares) satisfying observed points.
[f] Coefficient of variation = 1.1%.

In Table I the data obtained for the calibration are presented. By the method of least squares, the equation for the line best satisfying the experimental points was found to be: $Y = 0.0441\ X + 0.0137$, where X = micrograms of ascorbic acid per ml. and Y = the log of the galvanometer readings for samples minus that for the blank (Figure 1). The coefficient of variation for the deviations from the line was only 1.1 per cent. A repetition of the calibration curve after a period of 5 weeks resulted in a similar series of points varying by only about 1 per cent from the original calibration.

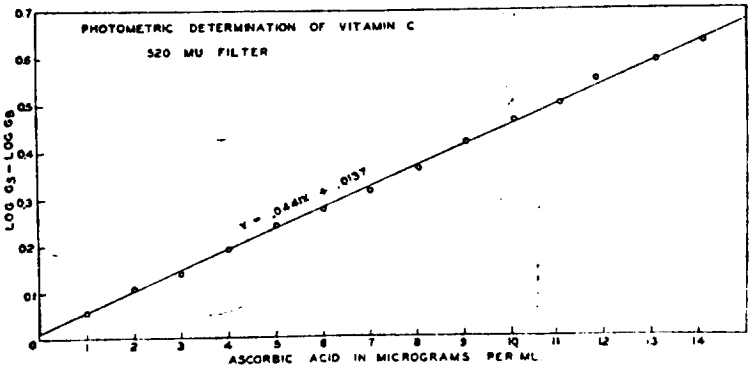

FIGURE 1

Procedure for Plant Materials

APPLICATION TO BEANS AND CABBAGES. From a dispensing buret 100 ml. of 3 per cent metaphosphoric acid are added to the container of the blender; 25 grams of fresh tissue, weighed to the nearest 0.1 gram, are added and mixed at high speed for 2 minutes. The fresh vegetable tissues are thus reduced to an extremely fine pulpy suspension, and a temperature rise of about 5° C. occurs. About half of the contents is then filtered through a dry fluted No. 12 Whatman filter paper. Since the filtrate comes through cloudy at the start, about 10 ml. are discarded. About 10 ml. of clear filtrate are collected in a dry Erlenmeyer flask.

The aliquot of filtrate taken for dilution will depend upon the approximate vitamin content of the sample. A volume is selected which is expected to contain between 200 and 600 micrograms, so that after dilution to 50 ml. the final concentration in the aliquots analyzed may conveniently lie between 4 and 12 micrograms per ml. For cabbage, which may contain about 40 to 90 mg. of ascorbic acid per 100 grams of fresh tissue, a 3-ml. aliquot is adequate, whereas for snap beans, containing about 20 to 40 mg. per 100 grams, a 5-ml. aliquot is taken.

Aliquots of the clear filtrates are transferred to 50-ml. volumetric flasks and a sufficient quantity of sodium citrate buffer (solution No. 2) is added to bring the pH to approximately 3.6; in the case of cabbages, beans, and several other vegetables studied, 0.25 ml. of buffer per ml. of aliquot was required. The mixture is then made to volume with the citrate-phosphate buffer (solution No. 3). The final pH should be 3.6 ± 0.1. When many samples of similar material are analyzed in groups of 24, a pH determination on occasional samples will permit adequate control.

In calculating the ascorbic acid content of plant tissue the water in the sample must be taken into account. In the present investigation the results obtained with the recovery experiments indicated that the vitamin is distributed in the liquid phase of the mix, with no measurable amount absorbed.

As a test of the accuracy of the method, the recovery of ascorbic acid was studied. Since a great deal of air is whipped into the extracting solution during the 2-minute stirring period, the possibility existed that oxidation might necessitate the use of an empirical correction factor. This was shown unnecessary, however, by quantitative recoveries when buffered solutions of ascorbic acid, at various concentrations, were carried through all the steps in the analysis. The possibility that ascorbic acid oxidase might partially destroy the vitamin during the analysis was also excluded by the quantitative recoveries obtained. Recovery experiments are complicated by the difficulty in obtaining uniform 25-gram samples of plant tissue. Four separate series, using individual cabbages, were conducted in the following manner:

> The head was sliced along its polar axis and 25-gram sections were cut for analysis; five alternate sections were supplemented with different amounts of pure ascorbic acid and the average value for the unsupplemented five sections was used in the recovery calculations. A fresh solution containing 1 mg. of ascorbic acid per ml. was added—4, 6, 8, 10, and 12 ml., respectively—to each section before conducting the extraction.

The average recovery for the 20 separate analyses was slightly high, 103 ± 1.3 per cent. However, in these experiments a difference in total vitamin concentration, between supplemented and unsupplemented extracts, is being measured.

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AM. J. CLIN. NUTR. 27: 696-699, 1974

Studies on the mechanism of the "thiamin-sparing" effect of ascorbic acid in rats[1, 2]

D. S. Murdock[3], *M. L. Donaldson*[4] *and C. J. Gubler*[5]

ABSTRACT The effects of supplementation of thiamin-deficient diets with ascorbic acid on the rate of development and severity of some thiamin-deficiency symptoms were studied in rats. With a 5% oral supplement of ascorbic acid, thiamin-deficient rats had a near normal rate of growth and only a slightly subnormal level of thiamin in the feces as compared with rats on the same diet but receiving injected or oral supplements of thiamin. When the ascorbate was injected subcutaneously, it had little positive effect. *Am. J. Clin. Nutr.* 27: 696–699, 1974.

A number of agents, such as high fat (1–4), sorbitol (4–6), antibiotics (4, 7, 8), and others (9) have been reported to cause an apparent "thiamin-sparing" effect in rats maintained on thiamin-deficient diets. As there is no evidence to indicate that any of these agents acts as a direct precursor or positive effector in thiamin biosynthesis, they presumably function by one of the following mechanisms: *1*) They decrease the need for thiamin by supplying acetylSCoA units by non-thiamin-requiring pathways. This may apply to a high fat diet. However, this thiamin-sparing effect of high fat diets, originally reported by Evans and Lepkovsky (1), has been shown to break down in carefully controlled studies with purified diets, except in deficiency produced by oxythiamin treatment (6, 10, 11). *2*) The rats may profit from increased thiamin production or a decrease in thiamin utilization by the microflora of the gut. This is undoubtedly the mechanism with sorbitol and other poorly absorbed carbohydrates and with antibiotics (4, 5, 7, 8, 12). The effects of high fat diets may also be partly mediated through this mechanism (13).

Large supplements of ascorbic acid to thiamin-deficient diets can also prevent and delay the onset of or alleviate the symptoms of thiamin deficiency (14, 15). Rats on a thiamin-deficient diet supplemented with 5% ascorbic acid maintain an essentially normal growth rate and have nearly normal levels of thiamin in the liver and carcass (9, 14), in contrast to those with no supplement. Scott and Griffith (9) studied the effects of different levels of ascorbic acid in rats on thiamin-deficient diets and proposed that ascorbic acid stimulates thiamin biosynthesis in the gut. Bánhidi (15) attributed this sparing effect to a reduction of thiamin disulfide in the tissues to thiamin, thus making it available to the tissue. However, it appears more likely that ascorbic acid, like penicillin (7), may alter the intestinal flora to select for thiamin-producing species over those using thiamin. This extra thiamin produced in the cecum would be largely unabsorbed and hence unavailable to the rat unless the feces were eaten (coprophagy) (3, 7). Barnes (16) estimated that normal rats ingest 50 to 65% of their feces.

It is obvious that the mechanism of the so-called thiamin-sparing effects of ascorbic acid is not yet clear. The object of this study was to explore the mechanism of this effect in rats.

Materials and methods

White, male Sprague-Dawley strain rats were used for all experiments. They were fed a normal chow diet for 1 week after arrival, after which they were randomly separated into groups, placed in individual

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stainless steel cages with raised screen bottoms, and started on the standard thiamin-deficient diet (17). Thiamin, when given as a supplement, was injected subcutaneously at 10 μg/100 g body weight per day or added to the drinking water at the level of 1.3 μg/ml. Water and food were given ad libitum and rats were weighed three times weekly.

In experiment I, attempts were made to determine the effects of prevention of coprophagy on growth rates of thiamin-deficient rats with and without 5% ascorbic acid supplements in the diet. Feces catchers were first made as described by Ryer and Walker (18). These worked well on thiamin-supplemented control rats, but the deficient rats, both with and without ascorbic acid, seemed to have an inordinate compulsion to get at their feces and soon removed or chewed through the catchers. A number of other types of catchers were also tried with the same result. It was thus not possible to prevent coprophagy for a sufficient time to determine the effects on growth.

Experiment II was a preliminary study to determine optimum levels for oral and injected ascorbic acid. Rats were divided into three groups: a control group (I) was fed the thiamin-deficient diet and injected with 10 μg thiamin in 0.2 ml 0.9% saline/100 g body weight per day; a second group (II) received the thiamin-deficient diet containing 1% added ascorbic acid at the expense of the sucrose; and a third group (III) was fed the thiamin-deficient diet and also was injected subcutaneously with 0.1 g ascorbic acid per 10 g food consumed. After 19 days, both deficient groups of rats looked unkempt and were losing weight rapidly and therefore were given a single injection of 10 μg of thiamin/100 g body weight. In addition at 19 days, the ascorbic acid level was increased to 2% in the diet in group II and, in group III, to 0.1 g administered subcutaneously twice daily.

In experiment III, ascorbic acid was injected into the rats to determine whether the effect was in the gut or was a systemic thiamin-sparing effect. Ascorbic acid was neutralized to pH 7.0 with NaOH for injection. It was not possible to inject subcutaneously amounts greater than 0.1 g/injection without causing undesirable side effects (necrosis, et cetera). Hence, in this experiment, 0.1 g sodium ascorbate/10 g food consumed per day was injected subcutaneously. This represented the equivalent of approximately 1% on the basis of diet. Four groups were carried as follows: control group A, thiamin-deficient diet plus 1.5 μg thiamin/ml drinking water; group B, thiamin-deficient diet containing 5% ascorbic acid; group C, thiamin-deficient diet alone; group D, thiamin-deficient diet plus 0.1 g ascorbic acid/10 g food consumed, injected subcutaneously.

In experiment IV, rats were divided into three groups: *1*) control, thiamin-deficient diet plus 1.5 μg thiamin/ml in drinking water; *2*) thiamin-deficient, thiamin-deficient diet, no supplement; *3*) thiamin-deficient plus ascorbic acid, thiamin-deficient diet containing 5% ascorbic acid. The rats were maintained for 2 weeks on the respective treatments, after which the feces were collected for 2 days and were stored frozen until assayed. For assay the feces were thawed and desiccated under vacuum. Samples were taken for dry weight. For thiamin assay, appropriate amounts of dry feces were homogenized in 15 ml of ice cold 0.2 M $HClO_4$ per gram of sample. The homogenate was then treated and assayed for total thiamin by the method of Murdock and Gubler (19).

Results and discussion

The growth curves for the rats in experiment II are shown in Fig. 1. As can be seen, rats receiving 1% ascorbic acid with the diet still became deficient, but grew better and survived longer than rats that received the equivalent amount injected. When the amount was increased to 2% it further improved the growth rate if given orally, but had little effect when injected. From these data a level of 5% in the diet was decided upon for subsequent studies. Because 0.2 g injected caused necrosis, even

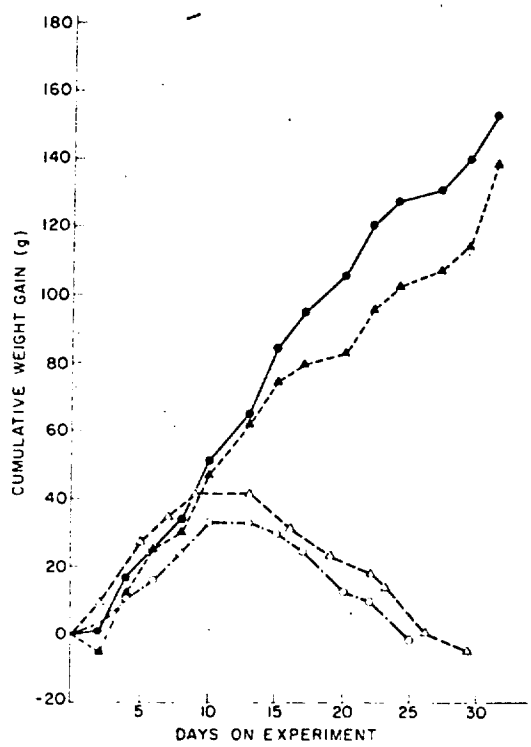

FIG. 1. Effects of oral and parenteral supplements of ascorbic acid on rate of growth of thiamin-deficient rats in experiment II: Group I, ● – ●, control rats; thiamin-deficient diet plus 10 μg thiamin/100 g body weight per day subcutaneously. Group II, ○–•–•○, thiamin-deficient diet supplemented with 1% ascorbic acid in diet. Group III, △– – –△ thiamin-deficient diet plus 0.1 g ascorbate/10 g food eaten per day injected subcutaneously. At ↑ the ascorbic acid was increased to 2% in diet or 0.1 g injected twice daily. At ↓ a single injection of 10 μg thiamin was given to prevent too early death.

when divided into two doses, 0.1 g was chosen for the succeeding experiment.

In Fig. 2, the mean weight gains for each of the four groups of rats of experiment III are presented. The thiamin-deficient rats with 5% ascorbic acid in the diet (group B) show a weight gain only slightly inferior to that for the control group (A) receiving thiamin, whereas those injected with ascorbic acid (group D) grew only slightly better than the thiamin-deficient rats with no supplement (group C). This supports the results shown in Fig. 1.

In Table 1, the dry weight of feces and thiamin content are presented for experiment IV. Not only was the fecal weight greater with the oral ascorbic acid supplement, thus indicating either a greater food intake or more prolific growth of gut microflora or both, but the thiamin content was considerable higher than in the corresponding group without ascorbic acid. If we assume that rats ingest 50% of their feces, as suggested by Barnes (16), the ascorbic acid-supplemented rats would have available approximately 7 μg of thiamin per day as compared with only 1 μg without ascorbic acid. This is slightly under the 10 μg/day required for optimum growth (9) and is reflected in the somewhat suboptimal growth rate shown in Fig. 2 for this group. Scott and Griffith (9) reported that rats fed a thiamin-deficient diet containing 5% ascorbic acid grew at a rate equivalent to rats that received 7.9 μg thiamin/day orally. In view of the great compulsion of these rats to obtain their feces, the degree of coprophagy was probably greater than 50%. In addition, an unknown quantity of thiamin may be available by direct absorption from the lower gut, although it has been claimed (8) that absorption in this area of the gut is unlikely.

From the data presented here and that from the earlier studies cited, it appears that the thiamin-sparing effect of ascorbic acid is due primarily to stimulation of thiamin biosynthesis by the gut microflora which then becomes available to the rats largely via coprophagy. There is no evidence of a direct systemic thiamin-sparing effect.

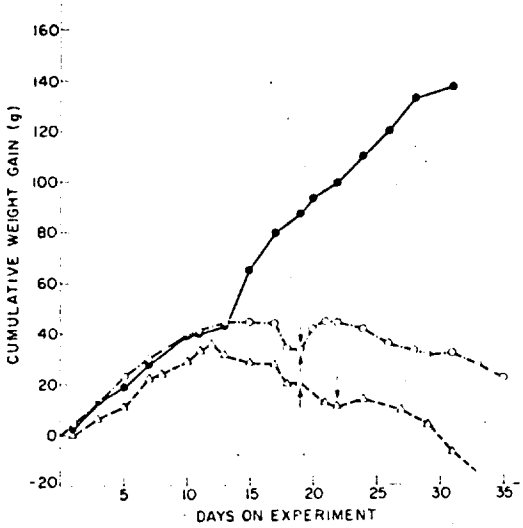

FIG. 2. Effects of thiamin and ascorbic acid supplements on the growth of rats in experiment III: ●–●, control group A, 1.3 μg thiamin/ml in drinking water; ○– – –○, thiamin deficient plus vitamin C (0.1 g/100 g food eaten per day, injected subcutaneously, group B; ○–•–•–○, thiamin deficient, no supplement, group C; ▲– – – –▲, thiamin deficient plus vitamin C (5%) in diet, group D.

Summary

The effects of supplementation of thiamin-deficient diets with ascorbic acid on the rate of development and severity of thiamin deficiency symptoms were studied in rats. A 5.0% oral supplement allowed a nearly normal growth

TABLE 1
Fecal thiamin output, experiment IV

Dietary treatment	No. of rats	Feces, g/day[a]	Thiamin, μg/g feces[a]	Available thiamin, μg/day[b]
Thiamin-deficient + thiamin	8	0.69 ± 0.147	18.44 ± 9.780	12.22 ± 5.430
Thiamin-deficient	7	0.48 ± 0.059	2.39 ± 1.880	1.15 ± 0.844
Thiamin-deficient + 5% ascorbic acid	8	0.69 ± 0.050	10.70 ± 2.940	7.40 ± 2.231

[a]On the basis of dry weight, mean ± SE. [b]Based on estimated 50% coprophagy. These levels of fecal thiamin represent 35 to 50% of all the feces excreted, as the rats ingest 50 to 65% of their feces according to Barnes (16).

rate and prevented other thiamin deficiency symptoms, as well as markedly increasing the thiamin content of the feces, as compared with that of a comparable group without the ascorbic acid supplement. The daily injection of an amount of ascorbic acid equivalent to 1% in the diet had little or no sparing effect as compared with a significant effect on growth and longevity with 1% in the diet.

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BIOCHEMICAL CHANGES IN ORGANS OF CHICKS AND RATS POISONED WITH ETHYLENE DIBROMIDE AND CARBON TETRACHLORIDE*

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ABSTRACT

The following biochemical parameters were determined in the livers of rats and chicks which had been previously treated with ethylene dibromide (EDB) and carbon tetrachloride: sulfhydryl groups, ascorbic acid, nicotinamid-adenine-dinucleotides (NAD and NADP) and alkaline phosphatase. Ascorbic acid levels were also determined in adrenals and alkaline phosphatase activity in blood. A marked depression in the level of free sulfhydryl groups was observed in the livers of the two species during the first hours after poisoning. The initial drop in the SH level was followed by a subsequent rise, the rate of which differed with species, sex, and poison. Carbon tetrachloride treatment resulted in a very marked depression of ascorbic acid content in rat liver while EDB caused only a slight depression. The ascorbic acid content of chick liver was less affected by the two poisons as was the liver NAD (P) content of both species. A rise of alkaline phosphatase was found with both poisons in rat liver in contrast to depression of the enzyme activity in chick liver. Rats and chicks responded differently to toxic action of the two poisons. This may be related to the different metabolism of the poisons in the two species.

Introduction

Ethylene dibromide (EDB) is used for insect control alone or in mixtures with other fumigants (carbon tetrachloride, ethylene dichloride, etc.). When fed in small amounts to laying hens in fumigated grain, EDB was found to decrease the size and number of eggs [1]. No such influence was found with carbon tetrachloride under the same conditions [2].

On the other hand, carbon tetrachloride as well as ethylene dibromide caused in rats fatty degeneration and necrosis of the liver [3, 4]. Chicks were found to be considerably less sensitive to carbon tetrachloride than mammals [5].

It was therefore deemed important to study some of the biochemical disturbances following ethylene dibromide and carbon tetrachloride poisoning in chicks, to investigate the toxic effect of EDB on rats, and to compare the results with those obtained after CCl_4 poisoning.

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Pyridine nucleotides NAD(P) [6] and alkaline phosphatase [7, 8] were determined in liver and blood because these substances are known to be severely affected by necrosis of the liver. Ascorbic acid and sulfhydryl groups were also examined because of their important function in oxido-reduction processes and in detoxification mechanisms [9, 10, 11].

Methods

Animals and Treatments

Albino rats and Leghorn chicks of both sexes, weighing between 120 - 180 g and 100 - 150 g, respectively, were used in all experiments A solution of ethylene dibromide in soybean oil 1:50 v/v, or carbon tetrachloride per se, were administered by stomach tube. The amount of EDB given was 110 mg/kg body weight, for both species, and that of carbon tetrachloride was 4.4 g/kg body weight for rats and 6.6 g/kg body weight for chicks. Control animals were given soybean oil alone. All animals were kept without food from the time of treatment until sacrifice. Water was supplied ad libitum.

Biochemical Assays

The animals were killed by cervical dislocation at various intervals after treatment as designated in the tables. Determinations were carried out in individual animals. Blood for alkaline phosphatase determination was collected rapidly in heparinized tubes, and organs were removed quickly and weighed. Livers were homogenized in 20 parts of cold saline solution. Duplicate samples of 0.2 ml homogenate were taken for the determination of rat liver alkaline phosphatase [12]. For chick liver alkaline phosphatase, the homogenate needed further fivefold dilution and samples of 0.1 ml were taken. One of the duplicate tubes was used as a blank after addition of one drop of concentrated hydrochloric acid solution.

NAD(P) were determined by reading the $E_{260}m\mu$ of the centrifuged and filtered homogenate, obtained after precipitating the protein in 1 ml liver homogenate with 9 ml of 5% w/v trichloroacetic acid. The E_{260} readings were corrected to the amount of liver (in g) corresponding to 100 g body weight, per 1000 ml 5% w/v trichloroacetic acid solution.

Ascorbic acid was determined by the method of Roe and Kuether [13]. Free sulfhydryl groups were determined by the method of Ellman [14] with the reagent 5.5' dithio-bis 2.2' dinitro-benzoic acid and also by the same method as modified by Jocelyn [15] for determination of free and protein-bound SH-groups.

Results

The mean values of adrenal ascorbic acid, liver NAD (P), liver and blood alkaline phosphatase obtained after EDB and CCl_4 poisoning of rats are summarized in Table I.

TABLE I. Effect of administration of ethylene dibromide and carbon tetrachloride to rats on adrenal ascorbic acid, liver NAD (P), liver and blood alkaline phosphatase. (Values are expressed as mean ± standard error).

Treatment	Period of treatment (hr)	Sex (No. of rats)	Weight of organs g/100 g body weight		Ascorbic acid μg/adrenal /100 g body weight	Liver NAD(P) E_{260} †	Alkaline phosphatase μmole of nitrophenol /hr	
			liver	adrenals			liver /100g body weight	1 ml blood
EDB 120 mg/kg	4 - 5	(16) ♀	3.97 ± 0.11	0.030 ± 0.001	89 ± 4	0.825 ± 0.032	35 ± 9	5.1 ± 0.1
Control	"	(12) "	4.00 ± 0.10	0.026 ± 0.001	94[a] ± 5	0.881[a] ± 0.038	35 ± 8	4.8 ± 0.2
CCl_4 4.4g /kg	"	(9) "	3.83 ± 0.10	0.029 ± 0.001	90 ± 5	0.874 ± 0.040*	33 ± 6	4.7 ± 0.3
Control	"	(8) "	3.93 ± 0.09	0.029 ± 0.001	98[a] ± 5	1.028[a] ± 0.042	33 ± 5	4.4 ± 0.3
EDB 120 mg/kg	20	(16) "	4.19 ± 0.11**	0.033 ± 0.001*	110 ± 6*	0.733 ± 0.024	251 ± 46*	5.6 ± 0.2
CCl_4 4.4 g / kg	"	(15) "	4.35 ± 0.12**	0.036 ± 0.001**	97 ± 6*	0.584 ± 0.025**	242 ± 56*	5.1 ± 0.2
Control	"	(16) "	3.52 ± 0.11	0.029 ± 0.001	136[a] ± 5	0.786[a] ± 0.025	16 ± 2	4.0 ± 0.1
EDB 120 mg/kg	"	(10) ♂	4.80 ± 0.13**	0.027 ± 0.001*	87 ± 4	0.900 ± 0.031	177 ± 15*	10.5 ± 0.9*
CCl_4 4.4 g/kg	"	(11) "	5.60 ± 0.13**	0.028 ± 0.001*	74 ± 3	0.950 ± 0.031	105 ± 15	11.4 ± 0.7*
Control	"	(12) "	4.00 ± 0.12	0.024 ± 0.001	78[a] ± 4	0.960[a] ± 0.031	66 ± 13	7.0 ± 0.7

In Tables I and II, analysis of variance used to evaluate the differences between the control and treated groups of each case.

* $P < 0.05$

** $P < 0.01$

† The E_{260} corrected to the amount of liver (in g) corresponding to 100g body weight, per 1000 ml 5% w/v TCA solution

a) considerable fluctuations in liver NAD(P) and in adrenal ascorbic acid content of rats were also observed by other authors [41, 42].

A significant increase of liver weight in both sexes and from both poisons was observed after 20 hr of intoxication. An analogous change occurred in adrenal weight. No liver enlargement was noticed a few hours after treatment. The increase in liver weight may be the result of triglyceride accumulation [16, 17] or an increase in water content [18].

Adrenal ascorbic acid levels in the animals were within normal limits a short time after poisoning (up to 4 hr). Female rats, after 20 hr of intoxication, suffer a significant depression in adrenal ascorbic acid, whereas adrenals of male rats showed no significant changes in ascorbic acid level at this time. The adrenal enlargement in females coincided with significant ascorbic acid depletion.

The liver NAD (P) concentration in female rats decreased at 4 - 5 hr after EDB and CCl_4 and 20 hr after CCl_4 administration. Male liver NAD (P) showed no changes from the control. A similar extent of NAD (P) depression has been observed by Gallagher and Rees [6] and Rees and Sinha [19] in carbon tetrachloride poisoning in rats.

Alkaline phosphatase activity in liver and blood was increased significantly by both poisons 20 hr after treatment, in spite of individual variations.

Corresponding data obtained with chicks are presented in Table II. EDB poisoning caused more drastic effects in chicks than did carbon tetrachloride. The liver weight increase caused by the two poisons after 20 hr of intoxication was in accordance with the effect found in rat liver. Adrenal weight and its ascorbic acid content are not given in the table because they were not affected by the poisons.

An early liver NAD (P) levels depression was observed with the two fumigants, in chicks, followed by an elevation after 20 hr.

Alkaline phosphatase activity in chicks suffered changes different from that noticed in rats. There was a depression in activity in liver alkaline phosphatase by EDB poisoning, whereas carbon tetrachloride did not cause any changes.

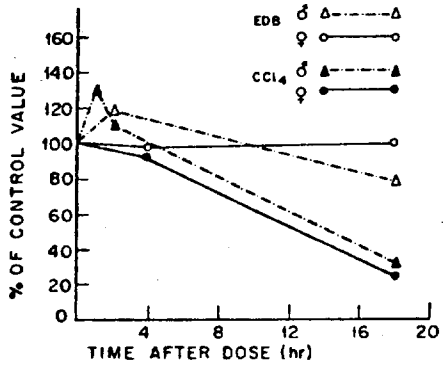

FIGURE 1. Rats

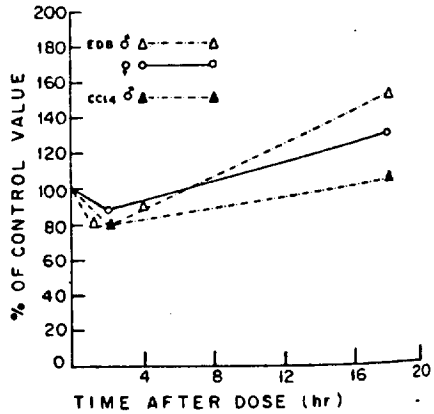

FIGURE 2. Chicks

Ascorbic acid concentration in liver homogenates. Results are expressed as percentage of the respective control values.

TABLE II. Effect of administration of ethylene dibromide and carbon tetrachloride to chicks on adrenal ascorbic acid, liver NAD (P), liver and blood alkaline phosphatase.
(Values are expressed as mean ± standard error).

Treatment	Period of treatment (hr)	Sex (No. of chicks)	Weight of liver g/100 g body weight	Liver NAD(P) E_{260} †	Alkaline phosphatase µmole of nitrophenol/hr	
					liver/100g body weight	1 ml blood
EDB 120 mg/kg	1	(8) ♂	2.41 ± 0.10	0.510 ± 0.04*	1126 ± 62	88 ± 32
Control	"	(7) "	2.58 ± 0.11	0.650 ± 0.04	1369 ± 70	99 ± 30
CCl_4 6.6 g/kg	"	(9) "	2.74 ± 0.09	0.515 ± 0.03*	1080 ± 80	-
Control	"	(9) "	2.87 ± 0.10	0.669 ± 0.03	1305 ± 85	-
EDB	4-5	(9) "	2.08 ± 0.11	0.500 ± 0.03	842 ± 50	125 ± 50
Control	"	(9) "	2.18 ± 0.12	0.610 ± 0.03	893 ± 52	185 ± 45
EDB	20	(8) ♀	3.41 ± 0.22	0.636 ± 0.03*	806 ± 84*	28 ± 7*
CCl_4	"	(6) "	2.93 ± 0.28	0.630 ± 0.03*	1450 ± 94	31 ± 7*
Control	"	(8) "	2.55 ± 0.24	0.505 ± 0.03	1287 ± 89	65 ± 6
EDB	"	(16) ♂	3.28 ± 0.12**	0.770 ± 0.04*	850 ± 110*	84 ± 8
CCl_4	"	(16) "	2.88 ± 0.12	0.738 ± 0.08*	1282 ± 110	105 ± 8
Control	"	(16) "	2.57 ± 0.12	0.608 ± 0.04	1130 ± 110	81 ± 8

* $P < 0.05$.
** $P < 0.01$
† The E_{260} corrected to the amount of liver (in g) corresponding to 100 g body weight per 1000 ml 5% w/v TCA solution.

The effects of ethylene dibromide and carbon tetrachloride injury on the mean level of liver ascorbic acid in rats and chicks are shown in Figures 1 and 2, respectively. The results are calculated as percentages of the respective control values. The mean level of 57 control rats expressed in μg ascorbic acid/liver/100 g body weight was 1247 ± 300, and that of 49 chicks was 692 ± 144. A large individual variation was noticed, but the differences between treatments were clear in each experiment.

Figure 1 clearly shows the different effects of EDB and CCl_4 on rat liver ascorbic acid after 20 hr: a slight or no depression in EDB poisoning as opposed to a marked one in CCl_4 intoxication. Depression in rat liver ascorbic acid was found by Burlina and Lippi [20] and by Lindan and Work [18] after administration of diets producing necrosis.

During the first two hr after administrating the two fumigants to chicks, a significant decrease in the level of liver ascorbic acid was observed (Figure 2). The subsequent rise in ascorbic acid level was significant only in EDB poisoning. In CCl_4 poisoning normal levels were observed in chicks of both sexes. Therefore, only results obtained with male chicks are presented. The ascorbic acid synthesis in chicks is localized in the kidney [21]. This may explain the relative lack of effect of the two poisons on liver ascorbic acid in chicks, differently from rats.

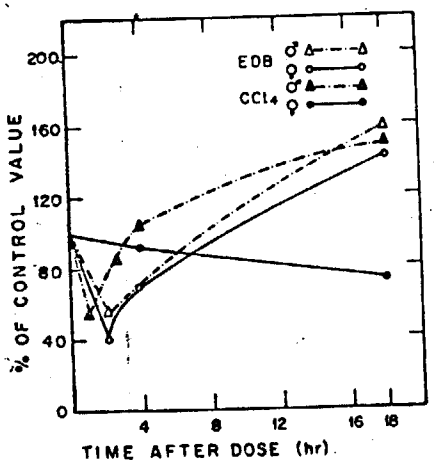

FIGURE 3. Rats

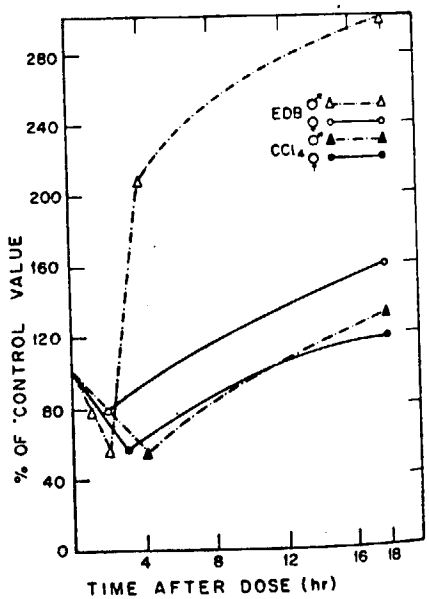

FIGURE 4. Chicks

Free sulfhydryl concentration in liver homogenates. Results are expressed as percentage of respective control values.

The results of the determination of free SH groups, calculated as percentage of the corresponding controls, are summarized in Figures 3 and 4. The mean levels of controls expressed in μmole SH/liver/100 g body weight for 34 female rats were 16.73 ± 2.2; for 21 male rats, 30.64 ± 3.75; for 31 female chicks, 9.98 ± 2.77; and for 34 male chicks, 6.01 ± 0.68. There was a pronounced sex difference in control levels of free SH groups in livers of the two species.

From the figures it is clear that the initial drop of free sulfhydryl groups was a common effect of the two poisons in the two sexes and species of animals. This drop was followed by an elevation in the level at different periods of time, except for female rats poisoned with CCl_4. Male chicks poisoned with EDB showed quicker adaptation. A fall in free sulfhydryl groups in liver has been noticed by many investigators studying halogenated hydrocarbon poisoning [9, 22, 23].

The striking fall of liver sulfhydryl level makes the differentiation between free and protein-bound SH groups of interest. In experimental animals killed 2 hr after EDB intoxification, no effect on bound sulfhydryl groups was observed. The values in micromoles bound SH per g liver were 16.3 and 15.4 for rats; 10.8 and 11.9 for chicks in experimental and control animals respectively.

Discussion

The resemblance between the biological disturbances caused by EDB and carbon tetrachloride in both sexes and in both species was particularly striking with regard to the drop in the level of sulfhydryl groups in the liver during the first hours of intoxication. This drop may be the results of alkylation in the detoxification process or oxidation to disulfide as a result of the pro-oxidant action of the two poisons.

Alkyl halides are known to alkylate sulfhydryl groups in a detoxification process and to form mercapturic acid derivatives which are excreted in the urine of the treated animals [24, 25]. EDB was found to be metabolized in the rat, and as a result S - (β-hydroxy-ethyl) mercapturic acid was excreted in the urine [26].

It was previously shown that liver contains enzymes involved in the conjugation of EDB with glutathione [27]. No sulfur containing detoxification compounds were detected in the urine of carbon tetrachloride-treated animals, but decrease in content of thiol groups in the liver was found also by other authors [28]. In case of CCl_4 the prooxidant effect seems to be the main cause of SH decrease.

The elevation of sulfhydryl groups level 20 hr after EDB intoxication may be explained by the enhanced synthesis of glutathione. It is known that the bulk of the free sulfhydryl groups are derived from glutathione [29, 30], whose turnover in liver is very rapid ($t\frac{1}{2}$ = 4 hr) [31, 32]. A rise in glutathione level which points to liver cell regeneration, was found after subtotal hepatectomy [33]. Hsu and colleagues recently found a similar depression and subsequent rise of liver glutathione in ethionine poisoned rats [34].

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The early depletion of sulfhydryl groups in both EDB and CCl_4 poisoning supports the hypothesis that the initial reaction in the *in vivo* degradation of these halogenated hydrocarbons is the homolytic fission of these compounds [35, 36]. Both the alkyl radical and the nascent halogen formed enhance the processes of alkylation and of oxidation of sulfhydryl groups [35] and at the same time may start chain reactions leading to oxidation of unsaturated fatty acids [37, 38].

Increased levels of peroxide in liver unsaturated fatty acids were found to be accompanied by inhibition of enzymatic synthesis of ascorbic acid [39]. This may explain the depletion of liver ascorbic acid in rats after CCl_4 poisoning. No appreciable loss of ascorbic acid was found after 18 hr intoxication by EDB. On the other hand peroxide formation was supposed to cause fatty liver, a disturbance which is pronounced in carbon tetrachloride poisoning [16, 17]. In EDB poisoning, fat accumulation was less extensive than in CCl_4 intoxication, but necrosis of liver was more apparent [40]. The toxic effect of EDB may result mainly from the impairment of -SH groups metabolism.

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EFFECTS OF STILBESTROL ON WEIGHT AND ASCORBIC ACID LEVEL OF GUINEA PIG ADRENALS

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INTRODUCTION

DURING the course of a study of the histogenesis of the estrogen-induced tumors of guinea pigs (Nadel, 1949), the influence of ascorbic acid upon the development of this type of tumor was investigated. An unexpected finding, compatible with acute scurvy (Meyer and McCormick, 1928), developed when stilbestrol pellets were implanted into guinea pigs kept on a diet partially deficient in ascorbic acid.

A study of the possible interrelationship between ascorbic acid and stilbestrol metabolism was undertaken. This paper deals with effects of stilbestrol implants on adrenal ascorbic acid concentration and adrenal weights of female guinea pigs maintained at different levels of ascorbic acid deficiency.

METHODS

Stock virgin female guinea pigs raised at the National Institutes of Health Breeding Farm were used in all the experiments. A stock diet of commercial Purina rabbit chow and fresh cabbage was supplemented with carrots once a week. Animals were transferred from this stock diet to the scorbutogenic diet described below. The day of transfer is indicated as Day O.

Scorbutogenic diet: A special diet, prepared in pellet form from a formula by Morris (1947) is given below. When guinea pigs weighing between 275–325 grams were transferred to this diet, they died with clinical signs and histological evidence of scurvy within 35 days.

Morris Scorbutogenic Diet

	Pounds
Alfalfa (ground)	900
Whole wheat (ground)	336
Molasses	150
Ground oats	100
Soy bean oil meal	100
Cerogras	100
Peanut oil meal	100

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Wheat bran	80
Rice offal	50
Salt (NaCl)	40
Apple pomace	30
Wheat germ	20
Brewer's dehydrated yeast	10
Shark oil	2
Liver meal	2
Mannamar	2
Irradiated yeast	1

Pellets, thus prepared, were mixed with shark and wheat germ oils just before use to insure against the loss of vitamins through storage. Twenty grams of each of the oils were added dropwise to 10 pounds of diet and the feed stirred slowly by hand over a period of 20–30 minutes to insure a good distribution of the added factors. Twenty pounds of feed were prepared in this manner twice weekly and kept at 4° C. Directly before each feeding, an additional supplement in the form of 10 grams of powdered Brewer's yeast was dusted onto each 1000 grams of diet after the method of Reid (1947, a).

Ascorbic acid solutions were prepared by dissolving crystalline ascorbic acid in distilled water and adjusting the resultant solution to pH 6.9 with 0.5% sodium bicarbonate. From a stock solution of 100 mg. of ascorbic acid per ml., dilutions were prepared for intraperitoneal injection immediately before use. Aliquots of these solutions analyzed immediately after injections showed no loss in ascorbic acid.

Hormone pellets: Pellets of 40% stilbestrol in cholesterol and of 100% cholesterol were prepared by fusion after established methods (Shimkin and White, 1941). The weights of the pellets thus prepared ranged from 35–45 mg. They were inserted intramuscularly in the dorsal cervical midline through a small surgical incision which was closed by a single Michel clip. All pellets were removed and weighed at the time of autopsy. Losses in the weight of these pellets, presumably because of absorption by the tissues of the guinea pigs, averaged less than 2 mg.

Post-mortem examinations: Animals were sacrificed by a blow on the head. The adrenal glands were removed quickly, placed in a Petri-dish surrounded by dry ice until partly frozen and then weighed to the nearest mg. on a Roller-Smith micro torsion balance. Individual glands were kept in dry ice until analyzed for ascorbic acid. The analyses were made usually within 15 minutes after the removal of the gland.

Ascorbic acid analysis: The adrenals, frozen in solid carbon dioxide, were homogenized for 1 minute in a Semimicro Waring Blendor with 50 ml. of ice-cold metaphosphoric-acetic acid mixture. Homogenate thus prepared was mixed with celite and filtered. The ascorbic acid was determined on aliquots of filtrates by the titrimetric method of Bessey (1944).

Experimental procedures: All animals were maintained on the stock diet prior to transfer to the special experimental diet. Twenty-seven animals on the stock diet (Series 1) were sacrificed at intervals to determine the normal relation of adrenal weight to body weight and adrenal ascorbic acid concentration (Figure 1).

In Series 2, twenty-six animals were placed on the scorbutogenic diet. These were serially sacrificed at regular intervals to study the effects of ascorbic acid deficiency on the adrenal ascorbic acid concentration (Figure 2).

TABLE 1. EFFECT OF STILBESTROL ON THE WEIGHT AND ASCORBIC ACID CONCENTRATION OF THE ADRENALS OF GUINEA PIGS ON THE SCORBUTOGENIC DIET

Group	Treatment		Adrenal wt. (mg.)	AD/W	Adrenal ascorbic acid (mg. %)
	Stilbestrol	Ascorbic acid[1] (mg./day)			
W	+	0	275	101	11
			294	110	10
			253	107	13
Average ± S.D.			274	106 ± 2.65	11.3 ± 0.9
X	0	0	159	73	16
			160	74	25
			204	82	18
Average ± S.D.			174	76.3 ± 2.86	19.7 ± 2.7
Y	0	25	155	67	95
			156	58	108
			154	63	100
			155	52	94
Average ± S.D.			155	60 ± 3.24	99.2 ± 3.2
Z	+	25	311	107	31
			208	76	61
			211	84	40
			210	77	25
Average ± S.D.			235	86 ± 7.23	39.2 ± 7.9

[1] Groups W and X were sacrificed on Day 23. Groups Y and Z were injected intraperitoneally with 25 mg. ascorbic acid on Days 24 and 25 only and were sacrificed 18 hours after the second dose.

In Series 3, a study was made of the effect of stilbestrol on the weight and ascorbic acid content of the adrenal glands of guinea pigs on a scorbutogenic diet as well as during recovery subsequent to ascorbic acid therapy (Table 1). Fourteen animals (250–300 gm.) were divided into groups W, X, Y and Z. Groups W and Z received stilbestrol implants on Day O while X and Y received control cholesterol blanks. All groups were kept on the scorbutogenic diet for 23 days when groups W and X were sacrificed. The animals in groups Y and Z received a daily intraperitoneal injection of 25 mg. of ascorbic acid on Days 24 and 25 only, and were sacrificed 18 hours after the second ascorbic acid injection.

In Series 4, a study was made of the effects of stilbestrol and varying amounts of ascorbic acid on the adrenal weight and on the adrenal ascorbic acid concentration of guinea pigs on the scorbutogenic diet (Table 2). Twenty-eight animals placed on the scorbutogenic diet were divided into groups A, B, C, D, and E. On Day O groups A and B were treated with stilbestrol implants while groups C, D, and E received cholesterol pellets. Daily intraperitoneal ascorbic acid injections of 4 mg. (a scurvy preventive dose) each were given for 14 days to animals in groups A and C, and 100 mg. (a high dose) to animals in groups B and D. Thus animals in groups A and B received stilbestrol plus 4 and 100 mg. of ascorbic acid daily, respectively; those in groups C, D, and E had 4, 100 and 0 mg. of ascorbic acid daily,

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TABLE 2. EFFECTS OF STILBESTROL AND ASCORBIC ACID ON ADRENAL ASCORBIC ACID AND AD/W[1] OF GUINEA PIGS ON SCORBUTOGENIC DIET

Group	Treatment		Adrenal wt. (mg.)	AD/W	Adrenal ascorbic acid (mg. %)
	Stilbestrol	Ascorbic Acid[2] (mg./day)			
A	+	4	206	62	88
			228	74	38
			268	90	24
			270	111	16
			182	59	55
Average ± S.D.			231	79.3 ± 9.64	44.2 ± 13.
B	+	100	208	68	121
			165	52	106
			224	75	112
			212	76	118
			216	79	156
			136	50	114
			152	57	129
Average ± S.D.			188	65.3 ± 4.58	122.3 ± 6.
C	0	4	94	38	91
			106	40	122
			150	56	89
			120	44	96
			102	36	57
Average ± S.D.			114	42.8 ± 3.59	91 ± 10.
D	0	100	128	42	140
			90	31	156
			91	31	173
			111	40	158
			130	49	121
Average ± S.D.			110	38.6 ± 3.45	149.6 ± 9.
E	0	0	102	43	35
			116	53	31
			114	57	19
			132	46	28
			106	45	34
			117	52	29
Average ± S.D.			115	49.3 ± 2.4	29.3 ± 2.

[1] AD/W = mg. adrenal weight per 100 gm. body weight.
[2] Ascorbic acid was injected intraperitoneally once daily for 14 days.

respectively, and no stilbestrol. All groups were sacrificed at the end of the experimental period which was 18 hours after groups A through D had received their 14th (last) daily injection of ascorbic acid.

RESULTS AND DISCUSSION

Relation of adrenal weight to body weight is graphically shown in Figure 1 where adrenal weight in milligrams per 100 grams of body weight (AD/W) is plotted against body weight. Adrenal weight increased at a faster rate than body weight. As a result AD/W for

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growing animals was greater with increase in age and weight. Animals weighing 150–350, 400–500 and 501–800 gm. had mean AD/W of 42.9, 52.4 and 68.8, respectively. Mixner, Bergman and Turner (1943) reported similar findings.

Adrenal ascorbic acid concentrations determined on 27 growing animals on regular stock diet averaged 144±5 mg. %. Individual ascorbic acid values ranged from 103–241 mg. %. The adrenal ascorbic acid concentration was 110–120 mg. % for five animals, 121–140 for eight, 141–160 for seven, 161–180 for five, 103 for one, and 241 for one. These adrenal ascorbic acid values do not show any correlation

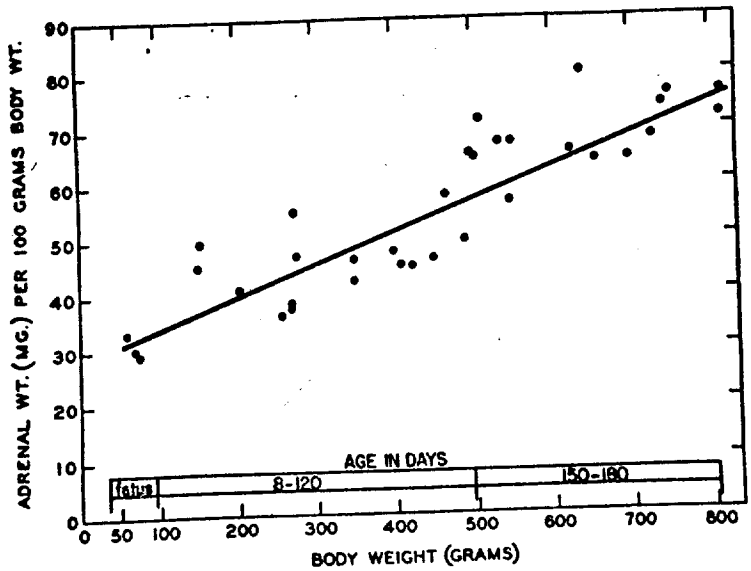

FIG. 1. Relationship between adrenal weight (in mg. per 100 gm. of body weight) and body weight.

with the age of the animal, its body weight or the weight of the adrenal glands. Reid (1947, b) has observed a wide range in the adrenal ascorbic acid values of male guinea pigs. Normal values for individual animals as reported in the literature range from 75 mg. % (Follis, 1947) to 336 mg. % (Roe, Hall, and Dyer, 1941). The average value reported by Kuether, Telford, and Roe (1944) was 118 mg. % and that reported by Long (1947, a) was 137 mg. %.

The effect of the scorbutogenic diet on the adrenal ascorbic acid concentration is graphically illustrated in Figure 2. The mean adrenal ascorbic acid values with standard errors obtained from guinea pigs serially sacrificed are plotted against the time during which the animals were on the scorbutogenic diet. A free hand curve was drawn through the mean values for Day 0 and for each day on the scorbutogenic diet. With the Day 0 mean adrenal ascorbic acid value of

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144 mg. %, those animals on the scorbutogenic diet for 1, 4, 14, 23, and 33 days show a drop of 32, 70, 80, 88, and 95%, respectively, in adrenal ascorbic acid concentrations.

Our animals lost about 32% of their adrenal ascorbic acid concentration when kept on the scorbutogenic diet for 24 hours, while Venning, Kazmin and Bell (1946) and Long (1947, b) found that their animals, which had been getting adrenocorticotropic hormone injections, lost only 10–15% under otherwise similar conditions of ascorbic acid starvation. Has adrenocorticotropic hormone a sparing effect on adrenal ascorbic acid depletion? The mechanism of adrenal ascorbic

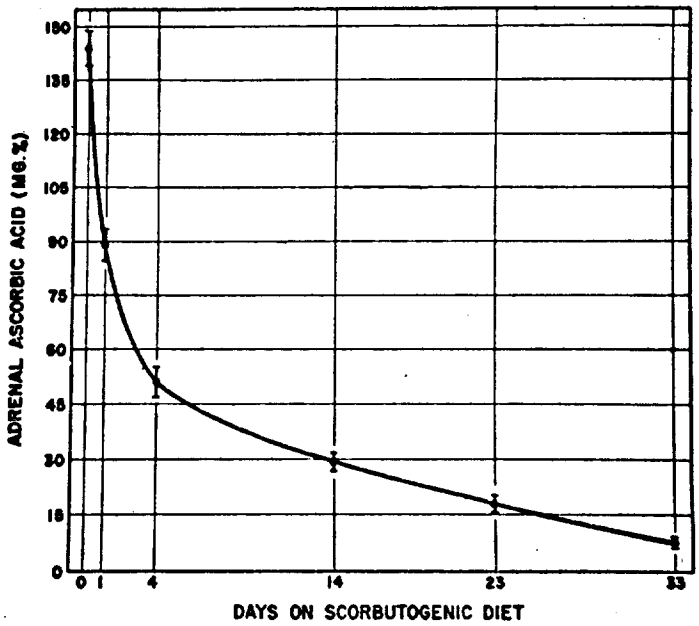

FIG. 2. Depletion of adrenal ascorbic acid in guinea pigs on scorbutogenic diet.

acid depletion and its replenishment needs further study for its proper understanding.

Changes in AD/W and adrenal ascorbic acid concentration caused by the scorbutogenic diet, stilbestrol therapy and the subsequent degree of recovery after ascorbic acid injections are shown in Table 1. Scorbutogenic diet alone (group X) increased the AD/W from about 40, normal for 200–300 gm. animals, to about 75. This value was further increased to about 105 in stilbestrol-treated animals (group W). Adrenal ascorbic acid concentrations in stilbestrol-treated animals were significantly lower than those in non-treated cases. When ascorbic acid was administered to animals of groups Y and Z, those without stilbestrol (group Y) showed a remarkable rise in their adrenal ascor-

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bic acid values to low normal, while in the stilbestrol-treated group Z, the rise in this value was comparatively small. The degree of recovery of AD/W toward normal was similar both in stilbestrol-treated (groups W vs. Z, 19%) and non-stilbestrol treated animals (groups X vs. Y, 21%). Thus treatment with stilbestrol decreased the adrenal ascorbic acid concentration over and above its depletion due to the scorbutogenic diet and these treated animals did not respond to ascorbic acid therapy as readily as ones without stilbestrol. The presence of stilbestrol in animals appeared to prevent the return of the adrenal ascorbic acid concentration to normal levels within the time studied.

Effects of stilbestrol and known ascorbic acid therapy on animals given the scorbutogenic diet are recorded in Table 2. In this series, 200–300 gm. animals were placed on the scorbutogenic diet for 2 weeks to avoid the weight losses reported when guinea pigs are kept on scurvy-producing diets longer than 15 days (Dunker, Fellers and Esslen, 1942). All but 3 of the animals gained weight.

Stilbestrol-treated animals, groups A and B, had the largest AD/W. Animals receiving 100 mg. of ascorbic acid daily (group B) were in the low normal range for adrenal ascorbic acid (122 mg.%), while those receiving 4 mg. daily (group A) had a value of 48 mg.%.

The daily administration of 4 mg. of ascorbic acid to groups A and C was calculated to provide the dose (1.23 mg.% of body weight) which Kuether, Telford and Roe, (1944) reported as sufficient to prevent changes associated with scurvy in the teeth of guinea pigs. Comparison of animals receiving the scurvy preventive dose of 4 mg. of ascorbic acid, group C, with those receiving 100 mg., group D, shows that the adrenal ascorbic acid concentration is much higher and the AD/W slightly lower in the latter group.

Animals receiving 4, 100, and 0 mg. of ascorbic acid and no stilbestrol, groups C, D, and E, respectively, had lower values for AD/W than groups A and B which received stilbestrol. The AD/W for control group E was slightly higher than that for groups C and D. Adrenal ascorbic acid concentration in these groups increased with increase in ascorbic acid intake.

SUMMARY AND CONCLUSIONS

Adrenal ascorbic acid concentration of 144 ± 5 mg.% has been established as the mean normal value for growing female guinea pigs used in these experiments.

Scorbutogenic diet reduced this mean normal value of 144 mg.% to 90 mg.% in 24 hours, a 32% reduction, and to 7.6 mg.% in 33 days, a 95% reduction.

The adrenal weight of normal growing animals increased at a much faster rate than body weight.

Ascorbic acid deficiency was associated with an increase in adrenal

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weight and AD/W (mg. adrenal weight per 100 gm. body weight) in guinea pigs.

Estrogen-treated animals always had higher AD/W and lower ascorbic acid concentration than their non-treated counterparts, regardless of ascorbic acid intake of the animals. Increase in ascorbic acid intake increased the adrenal ascorbic acid concentration.

Estrogens induced enlargement of the adrenal gland and caused an increase in AD/W. Increase in AD/W in growing animals might be a reflection of the normal increase in estrogens as the animals grow older.

Ascorbic acid concentration in the adrenal gland of normal growing guinea pigs seemed to be a resultant of ascorbic acid intake and rate of weight increase of the adrenal gland with age. There might be dietary and other factors involved in determining the ability of the adrenal gland to concentrate available ascorbic acid.

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Effect of Ascorbic Acid on Hydroxylase Activity

I. Stimulation of Tyrosine Hydroxylase and Tryptophan-5-Hydroxylase Activities by Ascorbic Acid

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The effect of ascorbic acid on the hydroxylase and dehydrogenase activities was studied *in vivo* to clarify its role in the biological oxidation. Tyrosine hydroxylase and tryptophan-5-hydroxylase which require 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine ($DMPH_4$), NADP and molecular oxygen were investigated with the non-scorbutic and scorbutic guinea pigs. Several dehydrogenases such as glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase and malic enzyme, which require no molecular oxygen but NADP, were also studied by comparing the activities in the non-scorbutic guinea pig with those in the scorbutic guinea pig. The results indicated that the activities of tyrosine hydroxylase and tryptophan-5-hydroxylase of the adrenal gland, liver and brain of the non-scorbutic guinea pig were higher than the values found in the scorbutic guinea pig, although no significant change in the activity of dehydrogenase was observed in the tissues of the non-scorbutic and scorbutic guinea pigs.

It has been known that the most prominent property of ascorbic acid is to be easily oxidized and reversibly reduced, and that the essential function of ascorbic acid might be related with the biological oxidation. Studies were undertaken to explore the effect of ascorbic acid on the levels of hydroxylase and dehydrogenase as typical enzymes concerned with the biological oxidation.

There are some reports on the changes of activities of hydroxylase and dehydrogenase by ascorbic acid. Robertson (1) has indicated that during the formation of collagen ascorbic acid may stimulate the hydroxylation of proline. Hofmann *et al.* (2) and Staudinger (3) have suggested that ascorbic acid is involved in the hydroxylation of corticosteroid in adrenal homogenate. Despite of these investigations, however, the effect of ascorbic acid on the activities of tyrosine hydroxylase and tryptophan-5-hydroxylase has not been clarified yet.

The present paper reports the effect of ascorbic acid on the activities of tyrosine hydroxylase and tryptophan-5-hydroxylase (1.99.1.4) as representative hydroxylases, and on the activities of glucose-6-phosphate dehydrogenase (1.1.1.49), isocitrate dehydrogenase (1.1.1.42) and malic enzyme (1.1.1.40) as representative dehydrogenases. The two hydroxylases require molecular oxygen, $DMPH_4$ and NADP. On the other hand, these dehydrogenases require only NADP as coenzyme. The results obtained here indicate that ascorbic acid does not stimulate the activities of dehydrogenases *in vivo* but does the activities of hydroxylases.

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EXPERIMENTAL

1. Materials

L-Tyrosine-3, 5-^{3}H (35.3 Ci per mmole) was obtained from Radiochemical Centre, Amersham, England. It was purified as follows: to remove labile ^{3}H, methanol (approximately 5 ml) was mixed with 1 mCi of L-tyrosine-3, 5-^{3}H and the mixture was evaporated *in vacuo* to dryness. This treatment was repeated several times, and the final residue was dissolved in 1 ml of distilled water and stored at −20°. $DMPH_4$ was purchased from Calbiochem. Glucose-6-phosphate and NADP were purchased from Sigma. All other chemicals were obtained from commercial sources.

2. Ascorbic Acid Deficient Diet

The ascorbic acid deficient diet consisted of the following composition: protein, 24 %; carbohydrate, 56 %; fat, 3.5 %; crude fiber, 4.5 %; ash, 6.0 % and water, 6.0 %. Vitamin mixture was prepared as follows (per kilogram of diet), vitamin A, 10,000 IU; vitamin D_3, 2,000 IU; vitamin E, 10 mg; thiamine, 5.6 mg; riboflavin, 10 mg; pyridoxine, 4 mg; vitamin B_{12}, 0.002 mg; calcium pantothenate, 26 mg; niacin, 44 mg; folic acid 0.8 mg; and choline chloride, 400 mg.

3. Animals and Preparation of Homogenate

Male guinea pigs, weighing about 300 g. were fed ascorbic acid deficient diet for 18 days. The animals were separated into two groups: one group was administered 200 mg of ascorbic acid per day during the last three days by intraperitoneal injection in a volume of 2 ml (the non-scorbutic group); and the other was given the same volume of 0.9 % NaCl solution (the scorbutic group). Thses groups were pair fed. Twenty-four hours after the last administration, they were sacrificed by decapitation and the adrenal gland, liver and brain were removed immediately. The tissues were weighed and then homogenized with 2 volumes of ice cold 0.32 M sucrose using a Potter-Elvehjem homogenizer.

4. Assay of Tyrosine Hydroxylase

The activity of tyrosine hydroxylase was assayed by measuring the rate of release of tritium from L-tyrosine 3, 5-^{3}H into water as described by Nagatsu *et al.* (4). The incubation mixture contained sodium acetate (pH 6.0), 200 μmoles; potassium phosphate (pH 6.0), 10 μmoles; $DMPH_4$, 0.5 μmole; 2-mercaptoethanol, 50 μmoles; $FeSO_4$, 0.5 μmole; 3,5-^{3}H-tyrosine, 0.2 μmole (150,000 cpm) and enzyme (5 to 10 mg of protein), in a final volume of 1.0 ml. Incubation was carried out for 30 min at 38°. The reaction was terminated by the addition of 0.05 ml of glacial acetic acid. The tube was centrifuged, and the entire supernatant solution was transferred to a column (0.5×3.0 cm) of Dowex 50-H$^+$ rinsed with 5.0 ml of water. The effluent was collected, an 1.0 ml aliquot of which was transferred to a scintillation vial containing 10 ml of Bray's solution (5). Radioactivity was measured in a liquid scintillation spectrometer.

5. Assay of Tryptophan-5-Hydroxylase

The activity of tryptophan-5-hydroxylase was assayed by the modification of the procedure described by Ichiyama *et al.* (6). The enzyme assay was performed in a Warburg flask containing Tris-acetate buffer (pH 8.1), 50 μmoles; L-tryptophan-1-^{14}C, 11.3 mμmoles (80,000 cpm) and 0.2 ml of homogenate in a final volume of 0.5 ml. Each vessel was incubated for 60 min at 37°. The reaction was terminated by the addition of 0.5 ml of 6 % perchloric acid. $^{14}CO_2$ evolved from L-tryptophan-1-^{14}C was trapped with hyamine and its radioactivity was measured by a liquid scintillation spectrometer.

6. Assay of Dehydrogenases

The activity of glucose-6-phosphate dehydrogenase was calculated from the initial rate of NADPH increase measured at 340 mμ in the test system described by Warburg and Christian (7). Malic enzyme was assayed essentially according to the method of Ochoa (8). The activity of isocitrate dehydrogenase was measured by the appearance of NADPH, determined by the increase in optical density at 340 mμ as described by Ochoa (9). The assays of these dehydrogenases were made in a cuvett of 1-cm path length; all components except enzyme were brought to temperature equilibrium at 25°, and then the reaction was started by the addition of enzyme.

Protein was determined according to Lowry *et al.* (10). The concentration of ascorbic acid in the tissues was estimated by phenylhydrazine method (11).

RESULTS

1. Concentration of Ascorbic Acid in the Tissues of Scorbutic and Non-scorbutic Guinea Pigs

The concentration of ascorbic acid was measured in the liver, adrenal gland and brain of the non-scorbutic and scorbutic guinea pigs. In the non-scorbutic guinea pig, the ascorbic acid concentration in adrenal gland was higher than that of other tissues, whereas in scurvy the concentration of ascorbic acid was highest

TABLE 1
Ascorbic acid content in the tissues of scorbutic and non-scorbutic guinea pigs

	Non-scorbutic	Scorbutic
Liver	0.255	0.006
Adrenal gland	1.105	0.015
Brain	0.266	0.079

All values are expressed as mg/g tissue.
The scorbutic guinea pigs were fed ascorbic acid deficient diet for two weeks.

in the brain, as indicated in Table 1. The concentration of ascorbic acid in liver, adrenal gland and brain of the scorbutic guinea pig was lower with high significance than that of the non-scorbutic guinea pig.

2. Effect of Ascorbic Acid on Dehydrogenase Activities

Table 2 represents the activities of glucose-6-P dehydrogenase, isocitrate dehydrogenase and malic enzyme of the non-scorbutic and scorbutic guinea pigs. The results indicated that there was no significant difference between the non-scorbutic and scorbutic guinea pigs in the specific activity as well as the total activity of the glucose-6-P dehydrogenase in the adrenal gland, liver and brain. No significant difference of the malic enzyme activity in adrenal gland, liver and brain was observed between the non-scorbutic and scorbutic guinea pigs. Table 2 also shows the specific activities and total activities of isocitrate dehydrogenase in adrenal gland, heart and brain of the non-scorbutic and scorbutic guinea pigs. Again, no difference in the isocitrate dehydrogenase activity was observed in the non-scorbutic and scorbutic guinea pigs. These results suggest that ascorbic acid does not affect the activities of dehydrogenases that require NADP but not molecular oxygen.

TABLE 2
Effect of ascorbic acid on dehydrogenase activity

			Non-scorbutic	Scorbutic
Glucose-6-phosphate dehydrogenase	Adrenal gland	S.A.	22.1 ± 1.8	22.3 ± 3.1
		T.A.	524 ± 101	503 ± 48
	Liver	S.A.	1.3 ± 0.1	1.1 ± 0.2
		T.A.	2,577 ± 533	2,610 ± 289
	Brain	S.A.	6.9 ± 0.1	7.4 ± 0.2
		T.A.	881 ± 46	866 ± 23
Isocitrate dehydrogenase	Adrenal gland	S.A.	31.3 ± 3.1	31.0 ± 0.7
		T.A.	738 ± 61	779 ± 46
	Heart	S.A.	115.2 ± 2.6	119.3 ± 3.8
		T.A.	36,000 ± 3,700	39,000 ± 3,300
	Brain	S.A.	5.4 ± 0.1	5.4 ± 0.7
		T.A.	660 ± 41	644 ± 78
Malic enzyme	Adrenal	S.A.	7.2 ± 0.2	7.8 ± 0.4
		T.A.	176 ± 44	172 ± 35
	Liver	S.A.	0.48 ± 0.06	0.36 ± 0.01
		T.A.	918 ± 109	915 ± 12
	Brain	S.A.	2.3 ± 0.2	2.3 ± 0.1
		T.A.	136 ± 27	130 ± 10

S.A. (specific activity) was defined as the amount catalyzing the production of NADPH (mμmoles) in 1 min per mg of protein. T.A. (total activity) was defined as the amount catalyzing the production of NADPH (mμmoles) in 1 min per whole tissue. Values represent the mean of 3 guinea pigs ± S.E.M.

3. Effect of Ascorbic Acid on Hydroxylase Activity

The data given in Table 3 show the effect of ascorbic acid on the activity of tyrosine hydroxylase in adrenal gland, liver and brain. The tyrosine hydroxylase activity of the non-scorbutic guinea pig was 2 fold higher than that of the scorbutic guinea pig in adrenal gland, 3 fold higher in liver and 4 fold higher in brain.

Table 4 represents the effect of ascorbic acid on the activity of brain tryptophan-5-hydroxylase. The rate of serotonin formation in the non-scorbutic guinea pig was 0.269 mμmole per hour per gram brain, whereas the

TABLE 3
Effect of ascorbic acid on tyrosine hydroxylase activity

		Non-scorbutic	Scorbutic
Adrenal gland	S.A.	3.5 ± 0.2	1.8 ± 0.3
	T.A.	133.9 ± 26	77.0 ± 14.0
Liver	S.A.	0.14 ± 0.01	0.05 ± 0.009
	T.A.	364.2 ± 73.0	109.8 ± 52.0
Brain	S.A.	0.82 ± 0.10	0.21 ± 0.05
	T.A.	246.4 ± 92.0	60.4 ± 21.0

S.A. (specific activity) was defined as the activity of enzyme which catalyzes the release of ^{3}H from ^{3}H-tyrosine equivalent to the formation of 1 mμmole of dopa in 30 min per mg of protein. T.A. (total activity) was defined as the activity of the enzyme which catalyzes the release of ^{3}H from ^{3}H-tyrosine in 30 min per whole tissue. Values represent the mean of 9 guinea pigs ± S.E.M.

TABLE 4

Effect of ascorbic acid on brain tryptophan-5-hydroxylase activity

Non-scorbutic	Scorbutic
0.269±0.035	0.169±0.013
159 (%)	100 (%)

Enzyme activity was defined as the amount which synthesized 1 mμmole of serotonin in hour per gram of brain. The values were calculated on gram of brain and were expressed relative increase to the scorbutic group.

rate in the scorbutic guinea pig was 0.169 mμmole. There was 59% increase in the brain tryptophan-5-hydroxylase activity of the non-scorbutic guinea pig compared with the scorbutic guinea pig.

Fig. 1 summarizes the effect of ascorbic acid on hydroxylase and dehydrogenase activities in brain. These results suggest that ascorbic acid is involved in some biological oxidation reactions, and that the administration of ascorbic acid does not affect the activity of dehydrogenase which requires only NADP as coenzyme, but stimulates the activity of hydroxylase which requires molecular oxygen, $DMPH_4$ and NADP.

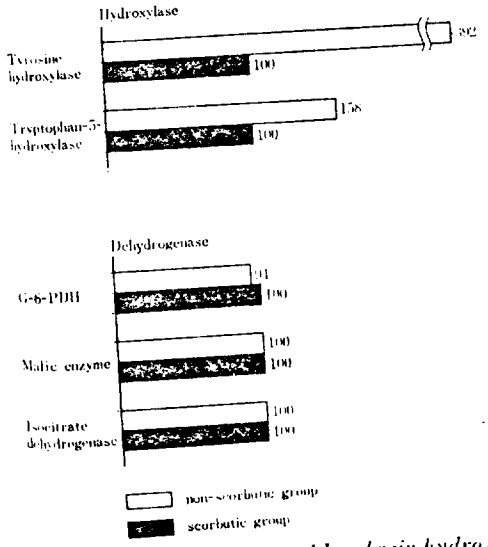

FIG. 1 *Effect of ascorbic acid on brain hydroxylase and dehydrogenase activity*

The values were caluculated on specific activity and were expressed relative increase to the scorbutic group.

In order to investigate whether this effect of ascorbic acid on tyrosind hydroxylase and tryptophan-5-hydroxylase activity is direct or not, we performed the following experiment *in vitro*. When ascorbic acid, 1×10^{-6}M to 1×10^{-4}M was added to the homogenate of adrenal gland of the scorbutic guinea pig, no remarkable stimulation of tyrosine hydroxylase and of tryptophan-5-hydroxylase activities were observed.

DISCUSSION

There were some reports on the changes of the enzyme activity involved in the biological oxidation by administration of ascorbic acid. Banerjee (12) reported that the activities of succinate, malate and lactate dehydrogenases moderately decreased in scurvy, whereas no change in the activity of isocitrate dehydrogenase which requires NADP as coenzyme was observed in normal and scorbutic guinea pigs.

Gould and Woessner (13) suggested that in ascorbic acid deficiency, the hydroxylation of proline was blocked and a protein that resembled collagen but contained no hydroxyproline accumulated. Kresten *et al.* (14) indicated that the rate of hydroxylation of deoxycorticosterone was increased by ascorbic acid. Staudinger (15) showed that the hydroxylation of compounds such as acetoanilide, was stimulated by ascorbic acid in the presence of NADH and rat liver microsomal fraction.

The experiment reported here showed that the activity of hydroxylase such as tyrosine hydroxylase and tryptophan-5-hydroxylase was found to decrease in scurvy. Thus, this change might be due to the following causes. 1) Directly ascorbic acid activates the hydroxylase activity. 2) Ascorbic acid affects the activation of low molecular coenzyme or metal ion such as Fe^{2+}. 3) Ascorbic acid reflects on increase in the amount of protein moiety of the enzyme, or 4) other protein such as cytochrome P-450 (16, 17) is involved in enzyme activation.

When ascorbic acid, 1×10^{-6}M to 1×10^{-4}M was added to the adrenal homogenate of the scorbutic guinea pig, the increase in tyrosine hydroxylase activity was not observed. This result showed that the effect of ascorbic acid on this enzyme system was not direct. Petrack *et al.* (18) also indicated that partially purified tyrosine hydroxylase did not require ascorbic acid.

Nagatsu *et al.* (19) and Petrack *et al.* (18) described that Fe^{2+} stimulated the tyrosine hydroxylase activity *in vitro* and that the higher activity and the greater stability were obtained when Fe^{2+}, $DMPH_4$ and mercaptoethanol were added. Therefore, in all experiments, sufficient amount of Fe^{2+}, $DMPH_4$ and mercaptoethanol were added to the reaction mixtures. It is clear that the stimulation of enzyme activity after administration of ascorbic acid is not due to Fe^{2+} or coenzyme such as $DMPH_4$.

And also it was considered that the stimulatory effect of ascorbic acid on the hydroxylase activity might be due to an increase in the amount of the protein moiety of the enzyme or other protein involved in enzyme activation. Further studies are necessary to clarify the role of ascorbic acid in the stimulation of hydroxylase activity *in vivo*.

In the next paper, we will investigate whether protein synthesis is increased by the administration of ascorbic acid in the scorbutic guinea pig.

Acknowledgement

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Determination of Ascorbic Acid

Application of the Indophenol-Xylene Extraction Method to Determination in Large Numbers of Tomato and Tomato Juice Samples

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The xylene method, as modified for rapid determination of ascorbic acid in tomatoes and tomato juice, is particularly adaptable to the determination of large numbers of samples and can be handled with accuracy by comparatively inexperienced technicians. The difficulties involved in the choice of extractant, clarification of extracts, choice of buffer, ascorbic acid losses following addition of buffer, and oxidizing substances in xylene are described, and methods of avoiding these difficulties are given.

IN PREVIOUS investigations of the ascorbic acid content of plant materials a titrimetric method (*6, 7*) was used in these laboratories. In the hands of an experienced operator this method was satisfactory and gave values which were in agreement with the photometric method of Bessey (*1, 15*). Critical evaluations of the titrimetric method have been presented by various workers (*8, 9, 11*). Since it requires experienced technicians, the authors have recently replaced it with the xylene method (*3, 4, 17, 18*) which is more objective, gives good results in the hands of comparatively inexperienced technicians, and hence is more suitable for large-scale routine analyses.

This paper gives a brief description of the xylene method as adapted for analysis of large numbers of tomato and tomato juice samples, the various difficulties encountered, and the precautions which are necessary to avoid them.

REAGENTS

ACETATE BUFFER. Dissolve 500 grams of C.P. sodium acetate trihydrate in enough distilled water to make 1 liter of solution; then mix with 1 liter of C.P. glacial acetic acid.

ASCORBIC ACID STANDARD. Prepare daily by dissolving a weighed amount of the vitamin in some of the acid which is used for extracting the samples. A solution which contains 20 micrograms per ml. is convenient, and 1- to 7-ml. aliquots of such a solution can be used. The ascorbic acid can be titrated iodometrically (*2*) to test its purity.

XYLENE. Use C.P. or reagent grade, providing it meets the test for oxidizing substances (see below). Redistill from glass if oxidizing substances are present.

2,6-DICHLOROPHENOLINDOPHENOL SOLUTION. Prepare by dissolving 40 mg. of the crystals in hot water, filter, cool, and dilute to 100 ml. Store this concentrated solution in the cold room at 3° to 5° C., and dilute about 15 ml. to 100 ml. before use. Use enough of the concentrated dye so that when a 5-ml. aliquot of the diluted dye is mixed with 2 ml. of the buffer and 5 ml. of the extracting acid and then is transferred to 15 ml. of xylene, the xylene solution gives 30% transmission in the Evelyn colorimeter with filter 520.

METHOD AND APPARATUS

The extract is prepared and filtered essentially as described by Morell (*16*). The first portion of the filtrate is poured back through the filter if it is not perfectly clear. An aliquot of from 1 to 10 ml. of the filtrate, depending on the ascorbic acid concentration, is pipetted into a large test tube, and about 2 ml. of the acetate buffer, followed immediately by 5 ml. of the dye, are added from automatic pipets. The solution is mixed thoroughly, but briefly, after each addition. After about 15 seconds, 15 ml. of xylene are added from another automatic pipet, and the tube is stoppered with a rubber stopper and shaken vigorously for 10 to 15 seconds. The xylene layer is drawn through a cotton plug into a colorimeter tube by means of the apparatus illustrated in Figure 1. This solution is read in an Evelyn colorimeter with filter 520 or 515.

The use of a rubber stopper has no harmful effect on the xylene solution. However, it is necessary to avoid rubber connections on the automatic pipets by using glass siphons for filling them. It is essential to allow about 15 seconds for the reaction with the dye, because even with moderate amounts of ascorbic acid the reaction is not completed in less time (*10*).

The xylene layer can be clarified by any one of three methods. It can be allowed to stand until it is clear, centrifuged, or drawn through a small wad of absorbent cotton (cf. Kirkpatrick, *12*). The authors prefer the latter. A fresh cotton plug, which need not be moistened, is inserted for each aliquot. The clarified xylene solutions are read in a photoelectric colorimeter, usually within an hour. Comparisons show that the methods of clarifying the xylene all give the same results. The intensity of the color of the dye after extraction with xylene is stable for at least 3 hours (*3*), but changes appreciably on standing overnight. The xylene can be used again after distillation in an all-glass still. It is preferable to dry the xylene with calcium chloride before redistilling.

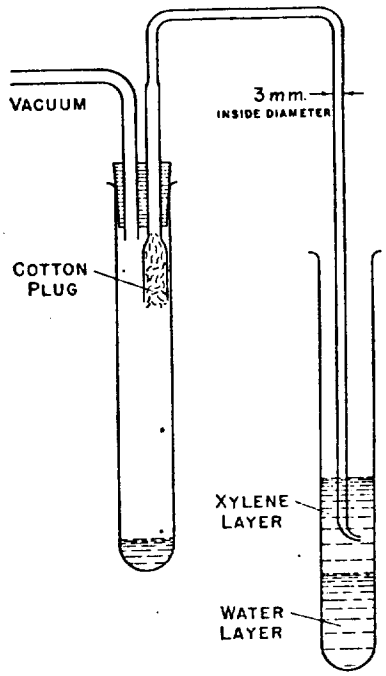

Figure 1. Apparatus for Clarifying Xylene Solutions of 2,6-Dichlorophenol-Indophenol

Xylene is drawn through absorbent cotton plug into colorimeter tube.

The ascorbic acid concentration of the aliquot is ascertained from a standard curve, which is constructed by plotting on semilogarithmic graph paper the per cent transmission of a dye-xylene solution against ascorbic acid concentration. The values obtained between 35 and 90% transmission show excellent agreement with Beer's law.

When this method is used in routine analyses of tomato samples duplicate determinations seldom give transmission values which differ by more than one scale division on the Evelyn colorimeter galvanometer—i.e., 1% transmission. Such a difference corresponds to from 2 to 4 micrograms of ascorbic acid.

This method gives results which agree satisfactorily with the Bessey photometric method (Table I).

Table I. Comparison of Xylene and Bessey Photometric Methods for Determination of Ascorbic Acid in Ripe Tomatoes

Sample No.	Mg. of Ascorbic Acid per 100 Grams of Fresh Fruit: Bessey photometric method	Xylene method
1	24.0	22.6
2	25.2	25.6
3	18.9	18.9
4	20.3	21.3
5	19.9	19.2
6	19.9	19.4
7	16.1	16.7
8	17.0	17.0
	Av. 20.16	20.09

DIFFICULTIES AND NECESSARY PRECAUTIONS

Choice of Extractant. The authors have compared 3% metaphosphoric acid with the 5% sulfuric–2% metaphosphoric acid mixture recommended by Mack and Tressler (*14*); with fresh acid solutions, identical results were obtained. The authors prefer to use 3% metaphosphoric acid for the following reasons (see also Fujita and Ebihara, *5*): It is more stable at room temperature, as is illustrated in Figure 2. The data in this figure were obtained by measuring the amount of metaphosphate and orthophosphate phosphorus (*19*) present at various times. Initially only 86% of the phosphorus was in the form of metaphosphate phosphorus. There is about the same percentage hydrolysis of the metaphosphoric acid in the sulfuric-metaphosphoric acid mixture in 6 hours as in the 3% metaphosphoric acid in a week. This necessitates more frequent preparation of the extracting acid if a mixture of the two acids is used.

Pure ascorbic acid is more stable in 3% metaphosphoric acid than in the metaphosphoric acid–sulfuric acid mixture. The 3% metaphosphoric acid is preferable, at least in the case of fresh tomatoes and canned tomato juices, because it is much easier to obtain perfectly clear filtrates using metaphosphoric acid alone than the acid mixture. With the former acid, the filtrates are nearly always perfectly clear from the start using Whatman No. 1 filter paper. With the mixture, it is usually necessary to pour the first portion of the filtrate back through the paper.

Other workers have used mixtures of several types of acids with metaphosphoric acid for extracting plant materials (*13, 14, 20*) because they considered a low pH essential to ensure stability of the ascorbic acid in the extracts. The authors have found that the ascorbic acid in 3% metaphosphoric acid extracts of tomatoes is stable on standing overnight at room temperature. Hence, they do not consider the use of a strong acid essential in ascorbic acid analyses of tomatoes.

Other stabilizing agents, such as oxalic acid, would presumably be satisfactory, provided they meet the tests required of a stabilizing agent; the authors have not tried this acid.

It is essential that approximately clear filtrates be obtained in the case of tomatoes and tomato juice because the suspended particles carry carotenoids which are extracted by the xylene and give an appreciable blank value. Centrifugation is not nearly so efficient in removing these carotenoid-carrying particles as is filtration. With dehydrated cabbage and sweet and Irish potatoes clear filtrates are not necessary, as no appreciable xylene-extractable pigments have been found in the filtrates. When

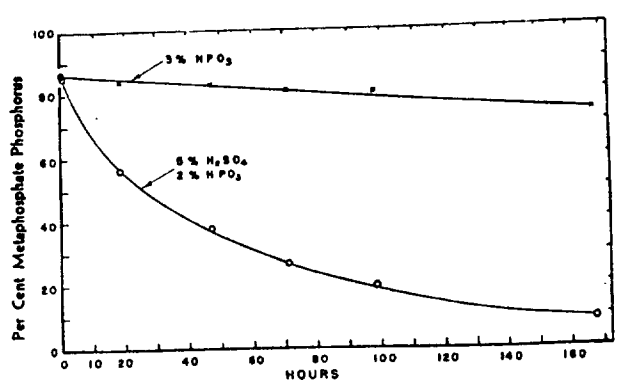

Figure 2. Relative Stability of a 3% Metaphosphoric Acid Solution and a Mixture of 5% Sulfuric and 2% Metaphosphoric Acids at Room Temperature

applying the method to other products it is advisable to extract the filtrate directly with xylene and compare the resulting extract with a sample of pure xylene. This would indicate the necessity of obtaining clear filtrates by refiltration.

Choice of Buffer. With 3% metaphosphoric acid as an extractant it makes very little difference whether an acetate buffer, such as that described above, or the citrate buffer of Bessey (*1*) is used. The final pH of the solution is not critical; values anywhere between pH 3 and 4 are satisfactory. However, if large amounts of a strong acid extractant, such as the 2% metaphosphoric-5% sulfuric acid mixture are used, the acetate buffer is preferable because of its stronger buffer capacity.

Ascorbic Acid Losses Following Addition of Buffer. It is essential that the dye and xylene be added immediately after the buffer has been added to the extract. Only enough time should elapse to permit a completion of reaction of the dye with the ascorbic acid. In some tomatoes the ascorbic acid is very rapidly destroyed after the buffer is added. This is illustrated in Figure 3, which shows the behavior of similar extracts from two tomatoes; in one case a considerable portion of the ascorbic acid is lost in a very short time after addition of the buffer. Similar results are obtained by the xylene and Bessey photometric methods (*1*). The rate of loss in extract A is about as rapid as the authors have ever observed. The rate in extract B is intermediate. In all extracts studied there was some loss if the aliquots were allowed to stand after the buffer was added. However, the amount of ascorbic acid lost during a determination is apparently not significant when determinations are made rapidly, as described above. The loss occurs as rapidly in extracts that have been boiled as it does in unboiled extracts, and hence is probably not enzymatic, even if any enzyme is extracted from the tomatoes by the acid. It is not accelerated by the addition of small quantities of copper and iron salts. The type of knife used to cut the fruits likewise makes no difference, as shown by comparing a new chromium-plated knife and an old chromium-plated knife, on which the brass plate was exposed, with grinding the tomatoes directly in the blender without slicing.

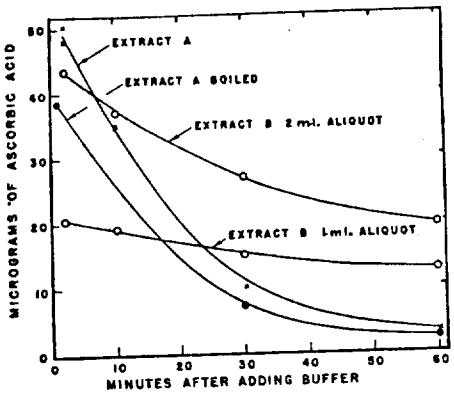

Figure 3. Decrease in Ascorbic Acid Content of Aliquots of Extracts of Tomatoes after Adding Acetate Buffer

The disappearance of ascorbic acid always seems to be more rapid in 2-ml. aliquots of the extracts than in 1-ml. aliquots. In some cases the reduced ascorbic acid content of a 2-ml. aliquot 1 hour after adding the buffer was less than that of a 1-ml. aliquot which had been similarly treated. As a consequence the authors had difficulty getting different sized aliquots to check in routine analyses in which several minutes elapsed between adding the buffer and the dye. This difficulty disappeared when the dye was added as rapidly as possible after the buffer. This loss in ascorbic acid occurs when either 3% metaphosphoric acid or 5% sulfuric-2% metaphosphoric acid is used for extracting the sample.

Table II. Effect of Xylene on Oxidation of Leuco-2,6-dichlorophenol-indophenol

Ascorbic Acid	Xylene 1 (Reagent) 7 min.	15 min.	30 min.	60 min.	Xylene 2 (Reagent) 7 min.	15 min.	30 min.	60 min.	Xylene 3 (Technical Grade) 7 min.	15 min.	30 min.	60 min.
Micrograms	*Per cent transmission*[a]											
0	29.8	30	30	30.5	29.8	30	30	30.3	30	30	30.3	30.5
20	33.5	33.8	34	34.3	32.5	32	31.8	30.5	33	33	32.8	31.8
100	53.3	53.8	53.8	54	52	51	47	42.3	51	50	46	33
160	99.5	99.5	99.5	99	92.5	81	60	39.3	94.5	91.5	86.5	79

[a] Per cent transmission as measured in an Evelyn colorimeter with filter 520. Time measured from point of addition of dye to acid solution.

Oxidizing Substances in Xylene. Most c.p. grades of xylene are suitable to use in the determination. However, the presence of oxidizing substances has been observed in technical grades of xylene and occasionally even in reagent grades. The effect of these substances on the leuco form of the dye is shown in Table II. These values were obtained by taking 5 ml. of 3% metaphosphoric acid containing an appropriate amount of ascorbic acid, and adding 2 ml. of citrate buffer, 5 ml. of dye solution (standardized for 30% transmission), and 15 ml. of xylene. The xylene was removed, and the per cent transmission was measured. It can be seen that at an ascorbic acid concentration of 160 micrograms the xylene-dye mixture should show approximately 100% transmission, and at 0 ascorbic acid concentration the transmission is approximately 30%. Only in the case of xylene 1 was the color relatively stable in the xylene solution; in the other xylene samples a fairly rapid development of color was indicated by the progressively lower transmission readings obtained when the mixture was allowed to stand for various lengths of time. For this reason it is always advisable to test the xylene for oxidizing substances before use. A simple test consists of completely decolorizing a small amount of dye solution with ascorbic acid and then shaking vigorously with the xylene to be tested. If no color develops in the xylene layer within 10 minutes it is considered suitable for use. If oxidizing substances are found the xylene should be distilled in an all-glass still.

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The Effect of Ascorbic Acid Supplementation on Tooth Mobility

by

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LOOSENING OF THE TEETH has long been regarded as a symptom of scurvy.[1,2] Westin's[3] studies led him to conclude that the increased tooth mobility seen in scorbutic men is caused primarily by destruction of the supporting structures. The clinical and histologic findings of Orban et al[4] in a case of scorbutic gingivitis are quite similar to those of Westin.

Waerhaug[5] reported that the teeth of young primates, raised on an ascorbic acid deficient diet, became markedly loose. He found that the early stage of increased mobility was facilitated by destruction of the periodontal fibers while later extreme mobility was aided by resorption of the alveolar wall. The histopathologic picture of the periodontal structures in C-avitaminosis did not resemble that of periodontitis in man.

Other investigators have reported on the effect of vitamin C supplementation alone,[6] and a combination of diet and nutritional supplementation,[7] on the tooth mobility of subjects without overt clinical symptoms of ascorbic acid deficiency. The results from both studies suggested some improvement from the systemic therapy.

Crandon[7] subsisted on a vitamin C free diet for six months. At the end of the experimental period, only a slight "bogginess" of the gingiva and interruptions in the lamina dura seen on roentgenograms were found. He made no reference to an increase in tooth mobility.

This paper reports on one part of a study designed to determine the effects of ascorbic acid supplementation on gingival health status, tooth mobility and levels of the vitamin in body fluids and tissue.

METHODS AND MATERIALS

Seventeen subjects, ranging in age from 21 to 55 years, participated in the study. The subjects were randomly placed in one of four groups. Group I (4 subjects) received a placebo for the 12-week experimental period. Group II (5 subjects) received a placebo for the first 6 weeks and received ascorbic acid supplementation for the second 6 weeks. Group III (4 subjects) received ascorbic acid supplementation for the first 6 weeks and placebo during the second 6 weeks. Group IV (4 subjects) received ascorbic acid supplementation for the entire 12-week period. The subjects received no dental treatment during the 30-day period prior to commencing the study and received no treatment during the experimental period.

The ascorbic acid supplement—300 mg—was contained in a sugar-coated, lemon-colored capsule. The placebo capsules were identical in all respects except they contained no ascorbic acid. The participants took one capsule daily with breakfast. The investigator (PPC) who assigned the subjects to the experimental groups dispensed the capsules. The investigators carrying out the clinical test procedures were unaware of the composition of the groups.

The mobility of six representative maxillary teeth (central and lateral incisor, cuspid, first and second premolar and first molar) was assessed on each subject on two pre-experimental days. It was also assessed at weeks 1, 2, 4, 6, 7, 8, 10 and 12, with assessments being made on two different days of weeks 6 and 12. The instrumentation and technic employed have been described in a previous report.[9] Their periodontal status ranged from a high degree of health to advanced stages of disease, while their pre-experimental tooth mobility ranged from low normal to highly elevated values.

The data were examined in a number of ways in order to investigate the effect of ascorbic acid on tooth mobility. In examining just the first 6 weeks of data the subjects in Groups I and II were combined into a single placebo group of 9 subjects, and the subjects in Groups III and IV into a single ascorbic acid group of 8 subjects. Groups I and IV provided a comparison of subjects on 12 weeks of placebo with subjects on 12 weeks of ascorbic acid. By looking at the last 6 weeks of data only, it could be determined whether the difference in tooth mobility between ascorbic acid and placebo subjects varied depending upon the treatment (placebo or ascorbic acid) for the first 6 weeks. Finally, since the subjects in Groups II and III were exposed to both placebo and ascorbic acid, it was possible to make a within-person evaluation of the effects of ascorbic acid on tooth mobility.

The research described in this paper was carried out at Wilford Hall, USAF Hospital, Lackland AFB, Texas, and the Dental Sciences Division, USAF School of Aerospace Medicine, Brooks AFB, Texas. It was published as a School of Aerospace Medicine Technical Report No. 68-112.

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Analysis of variance technics were used in the statistical analysis of these data. The 0.05 level of significance was used for all statistical tests.

RESULTS

Table presents the tooth mobility means of subjects receiving ascorbic acid supplementation (Groups III and IV) or placebo (Groups I and II) for the first 6 weeks of the study. There was no statistically significant change in tooth mobility for the subjects on either regimen.

TABLE 1
Tooth Mobility Means of Subjects Receiving Ascorbic Acid Supplementation or Placebo for First Six Weeks*

Week	Ascorbic Acid Supplementation (9)	Placebo (8)
Pre-experimental†	.00345	.00313
1	.00351	.00307
2	.00347	.00313
4	.00350	.00327
6†	.00352	.00319
S. D.**	.000180	.000154

Figures in parentheses represent number of subjects.
*Tooth mobility measured in 0.0001 inch (10^{-4}).
†Mean of two assessments.
**Standard deviation to be used in comparing means in the same column, for example, to compare week #1 with week #6 in column one use (S. D.) $\sqrt{\frac{1}{9}+\frac{1}{18}}$

The tooth mobility means of subjects receiving ascorbic acid supplementation (Group IV) or placebo (Group III) for the 12-week period are given in Table 2. For the four subjects receiving ascorbic acid supplementation daily, the means ranged from a high of .00370 at the first experimental assessment to a low of .00344 at the eighth week. The decrease in mean mobility from the first assessment to the mean of the two assessments, carried out during the twelfth experimental week, was -.00010 inch and had no statistical significance. There was slightly more variability between weeks for the 4 subjects receiving the placebo. The change from the first experimental assessment to the mean of the two assessments carried out at week 12 (+.00013 inch) was not, however, statistically significant.

Table presents the tooth mobility means of subjects receiving ascorbic acid supplementation (Groups II and IV) and placebo (Groups I and III) over the second 6 weeks of the study. The statistical analysis on these data indicated that the difference in tooth mobility between placebo and ascorbic acid subjects was not significant, either when the subjects had been on placebo for the previous 6 weeks or when they had been on the ascorbic acid supplement for the same time period.

Another way of looking at the data is presented in Table 4, which gives the time means for the two groups (II and III) that received both treatments. The analysis of these data which provided a within-person comparison showed no statistically significant differences in tooth mobility which could be related to the effect of ascorbic acid.

TABLE 2
Tooth Mobility Means of Subjects Receiving Ascorbic Acid Supplementation or Placebo for 12 Weeks

Week	Ascorbic Acid Supplementation (4)	Placebo (4)
Pre-experimental*	.00365	.00291
1	.00370	.00280
2	.00368	.00294
4	.00365	.00302
6*	.00363	.00298
7	.00358	.00286
8	.00344	.00288
10	.00350	.00275
12*	.00356	.00293
S.D.	.000166	.000117

*Mean of two assessments.

TABLE 3
Tooth Mobility Means of Subjects Receiving Ascorbic Acid Supplementation or Placebo for Second Six Weeks

	Placebo*		Ascorbic Acid†	
Week	Group I Placebo (4)	Group II Ascorbic Acid Supplementation (5)	Group III Placebo (4)	Group IV Ascorbic Acid Supplementation (4)
7	.00286	.00320	.00335	.00358
8	.00288	.00313	.00326	.00344
10	.00275	.00340	.00336	.00350
12**	.00294	.00334	.00331	.00356
S.D.	.000117	.000166	.000168	.000166

*Subjects on placebo for first six weeks.
†Subjects on ascorbic acid supplementation for first six weeks.
**Mean of two assessments.

TABLE 4
Tooth Mobility Means During Ascorbic Acid and Placebo Treatment (for Two Groups Receiving Both Treatments)

Week	Group II Placebo (5)	Group III Ascorbic Acid (4)
Pre-experimental*	.00331	.00325
1	.00329	.00331
2	.00329	.00326
4	.00346	.00334
6*	.00335	.00340
	Ascorbic Acid	Placebo
7	.00320	.00335
8	.00313	.00326
10	.00340	.00336
12*	.00334	.00331
S.D.	.000166	.000168

*Mean of two assessments.

DISCUSSION

The lack of response to the ascorbic acid supplementation could be due to a number of factors. Were the

participants in the groups receiving ascorbic acid faithful in taking the supplement? Data secured on whole blood, plasma, white blood cell and urine levels indicate the supplement was being taken.

A second point in question is the pre-experimental tooth mobility values of the participants. Each group contained subjects with elevated tooth mobility values. There was no decrease in the tooth mobility values of these individuals during the period of ascorbic acid supplementation.

One may ask if the supplement given was adequate. Would the results have been different if the supplement was increased to 500 or 1000 mg per day? An absolute answer cannot be given. However, the whole blood levels and urinary excretion rates indicate the subjects had achieved a satisfactory state of saturation.

The effectiveness of ascorbic acid supplementation in decreasing tooth mobility could be influenced by the pre-experimental ascorbic acid status of the subjects. If, prior to beginning the study, participants had subsisted on a vitamin C deficient diet long enough to induce tissue changes it is possible that tooth mobility values might decrease markedly with ascorbic acid supplementation. It is, however, difficult for an individual living on a normal dietary intake to avoid ingesting some ascorbic acid. Even if one abstains entirely from citrus and all other fruits he will secure a certain amount of ascorbic acid from vegetables and some animal products. The National Research Council has suggested a vitamin C intake of 75-100 mg per day for optimum human nutrition. This amount maintains tissue saturation and plasma vitamin C levels of 1 mg % without excessive loss in urine. However, it is probable that an intake of only 18-25 mg per day will prevent scurvy and maintain satisfactory health.[10]

SUMMARY

Seventeen subjects with wide variations in periodontal health and tooth mobility values were randomly placed in one of four experimental groups.

Group I (4 subjects) received a placebo for the 12-week experimental period. Group II (5 subjects) received a placebo for the first 6 weeks and ascorbic acid supplementation for the second 6 weeks. Group III (4 subjects) received ascorbic acid supplementation for the first 6 weeks and placebo during the second weeks. Group IV (4 subjects) received ascorbic acid supplementation for the entire 12-week period. The ascorbic acid supplement (300 mg.) was taken daily with breakfast. Mobility of six teeth was assessed on each subject on two pre-experimental days and at week 1, 2, 4, 6, 7, 8, 10 and 12 with assessments being made on two different days of weeks 6 and 12. The ascorbic acid supplementation had no statistically significant (P>) effect on tooth mobility.

Under the conditions of this study in which the subjects did not have overt symptoms of ascorbic acid deficiency ascorbic acid supplementation did not decrease tooth mobility.

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Metabolic availability of ascorbic acid in female guinea-pigs

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Stone (1965) stated that the presence of the gene controlling the ability to synthesize ascorbic acid (AA) should be demonstrable by placing guinea-pigs on an AA-free diet and following the decrease in tissue AA content. Failure to show a uniform fall in AA before death would show that AA was being synthesized. Williams & Deason (1967) reported great individual variation in AA requirements in guinea-pigs, and suggested the possibility of endogenous AA production. Odumosu & Wilson (1970a) showed that plasma and leucocyte AA concentrations decreased after 9 days of dieting, and weight loss began after 12 days in a group of guinea-pigs of both sexes. They died between days 28 and 36. Male guinea-pigs showed severe symptoms and all died after 27–30 days (Odumosu & Wilson, 1970b). Between days 24–27, buffy coat AA values changed from 38% to 44%, and liver values from 1·3 to 1·0% of the control values. Final plasma values were 0·093±0·032 mg per 10·6 ml.

Table 1 shows the survival times and mean tissue AA values for female (Duncan-Hartley) guinea-pigs on a scorbutic diet. These animals lost weight until day 18, but maintained their food intake until they died. They were divided into two groups on day 24 on the basis of their weight loss. Those that were likely to die (D guinea-pigs) were still losing weight, but the loss of weight had ceased in the potential survivors (S guinea-pigs).

The S guinea-pigs killed on day 32 had liver AA concentrations which were 3·9 times greater than those of the D guinea-pigs which were killed on day 30.

The liver AA concentrations of S guinea-pigs were also higher than D guinea-pig values from day 36. Liver AA values were 40% of the control values in 1 S guinea-pig killed on day 72. Two guinea-pigs survived 90 days. Plasma AA increased in parallel with, but more slowly than, liver AA in the S guinea-pigs. Adrenal AA in S guinea-pigs increased from zero on day 30 in parallel with, but more slowly than,

TABLE 1. *Survival times and tissue ascorbic acid values in guinea-pigs on a scorbutic diet*

Day of diet	Number surviving S	Number surviving D	Number dead S d	Number dead S k	Number dead D d	Number dead D k	Animals analysed Group	Animals analysed No.	Tissue weight g	Analysis AA mg% Plasma	Analysis AA mg% Liver
0	54	—									
1	48	—	—	6	—	—	S	6	426	0·884	20·42
1–23	46	—	—	—	—	2	D	2	306		
24	40	—	—	—	—	6	D	6	331	0·246	2·08
	17	23					—	—			
28	17	22	—	—	1	—	D	1	340		
30	16	22	1	—			S	1	526		
					—	11	D	11	314	0·122	2·16
32	15	11	—	2	—	—	S	2	524	0·171	3·90
33	13	11	—	—	3	—	D	3	307		
35	13	8	—	1	—	—	S	1	508	0·204	4·27
36	9	0	—	4			S	3	407	0·222	5·25
					2	6	D	8	303	0·084	2·97
39	8	0	1	—	—	—	S	1	448		
48	7	0	1	—	—	—	S	1	346		
54	5	0	—	—	—	—	W	2	482		
60	4	0	1	—	—	—	S	1	315		
62	3	0	—	1	—	—	S	1	465	0·571	4·69
72	2	0	—	1	—	—	S	1	380	0·178	8·17
98	1	0	—	1	—	—	S	1	504	1·400	11·04

S, Potential survivors. D, Guinea-pigs expected to die. d, Died. k, Killed. W, Withdrawn for treatment with PCMB. Tissue analysis figures are the means from the numbers of animals analysed.

plasma values. As adrenal AA increased, plasma cortisol concentrations diminished. Para-chloromercuribenzoate administration, inhibiting L-gulonolactone oxidase (Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1960), administered to 2 S guinea-pigs (W) on day 54 caused weight loss, followed by death in 7 days when liver AA was 20% of normal, and plasma AA was zero. It is concluded that in times of deficiency, some female guinea-pigs are able to readjust their ascorbic acid metabolism and so compensate for the influence of the defective gene responsible for hypoascorbemia (Stone, 1966).

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The relationship between ascorbic acid concentrations and cortisol production during the development of scurvy in the guinea-pig

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Male guinea-pigs weighing 400 g were fed on an ascorbic acid deficient diet for two weeks with a daily oral supplement of 50 mg of vitamin C, before being given the diet without the supplement for a period of 27 days. Ascorbic acid and cortisol estimations were carried out on the blood and tissues of six animals at intervals while the scorbutic diet was being administered, and changes in tissue and body weight were recorded. On the 27th day animals were spontaneously dying and showed morphological evidence of scurvy. The body weight increased until the twelfth day on the scorbutic diet in the same way as in control and hypervitaminotic animals (Odumosu & Wilson, 1970). Thereafter it decreased, and was 8·5% less than the initial weight on the 27th day ($P<0{\cdot}05$). The adrenal weights increased consistently from the sixth day and finally were 65% above the initial weights. The adrenal cortisol concentration remained constant until the sixth day. It had fallen to 75% of its initial level by the eighteenth day, but the decrease then stopped until the end of the experiment. From the first day of vitamin C deprivation the plasma cortisol increased, and on the 27th day it was 27 times its initial value. Values for the biliary cortisol increased in a comparable fashion. Like the adrenal cortisol, the adrenal ascorbic acid concentration did not alter during the first 6 days of the scorbutic diet. Thereafter adrenal ascorbic acid diminished rapidly. 87% of the ascorbic acid had disappeared by the eighteenth day, and ultimately 99·5% had been lost. Plasma ascorbic acid increased during the first 6 days and then fell consistently to almost zero on the 27th day. Leucocyte ascorbic acid did not decrease until after the sixth day. By the eighteenth day 40% had been lost, but thereafter leucocyte ascorbic acid fell significantly more slowly than plasma ascorbic acid as shown by the change in angle of the regression lines relating plasma and leucocyte values. During the development of scurvy the corticosteroid secretion increases on the sixth day at the same time as a change appears in the metabolic use of ascorbic acid. On the eighteenth day adrenal production of cortisol levelled out at the same time as the leucocytes began to conserve their stores of ascorbic acid. Throughout the experiment plasma ascorbic acid showed a more extensive range of variation than leucocyte ascorbic acid. We suggest that this indicates that plasma values give a measure of metabolic demands, and leucocyte values indicate storage capacity for ascorbic acid.

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The growth maintaining activity and intestinal absorption of ascorbic acid in guinea-pigs

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It has been shown that guinea-pigs develop scurvy on a scorbutic diet in 20–28 days (Evans & Hughes, 1963; Hodges & Hotston, 1970). However, the relationship between the development of scurvy, leucocyte ascorbic acid levels and intestinal absorption of vitamin C has not been accurately established. After maintenance on a normal diet supplemented with vitamin C (50 mg in 100 ml water), 24 guinea-pigs were given a scorbutic diet for two weeks while receiving 20 mg of L-ascorbic daily by stomach tube. They were weighed regularly, food intake was recorded daily, and blood samples were removed at 6 day intervals for estimation of ascorbic acid concentrations. They were then divided into three equal groups receiving 20 mg (control C), 100 mg (hypervitaminotic: HV) and zero (scorbutic: S) ascorbic acid daily. After 7 days the plasma ascorbic acid of the S group began to fall and the group lost weight after 10 days. Plasma ascorbic acid and weight increased after 7 days in the HV group. Leucocyte ascorbic acid levels fell after 10 days in the scorbutic group and increased slightly in the other two groups. On the 28th day two of the S group died. The mean ($\pm$S.E.M.) leucocyte ascorbic acid concentration of the group was $9{\cdot}3 \pm 1{\cdot}3/10^8$ cells and the plasma ascorbic acid was $0{\cdot}103 \pm 0{\cdot}016$ mg%. In the HV group the corresponding levels were $32{\cdot}8 \pm 1{\cdot}7$ μg/10^8 cells and $1{\cdot}097 \pm 0{\cdot}145$ mg%. Weight changes for the groups at the end of the period were: C group +23%, HV group +32%, and S group −27% of the initial weights.

On the 28th day the intestinal absorption of ascorbic acid was measured *in vivo* from a loop of the ileum during a 2 h period. The percentage disappearance of ascorbic acid from the loops, the intestinal uptake, and alteration in blood concentrations of ascorbic acid were measured in the different groups. Ascorbic acid uptake from the lumen was least in the scorbutic animals. About three times as much ascorbic acid remained in the lumen of the S group as in the C or HV groups. A larger percentage of ascorbic acid remained in the mucosal cells of the control and scorbutic animals than in the cells of the hypervitaminotic animals. The increase in leucocyte ascorbic acid after the period of intestinal absorption was greatest in the C group and least in the S group, with the values in the HV group coming between. It is concluded that intestinal absorption of vitamin C is diminished in the terminal stages of scurvy. Concurrently leucocyte ascorbic acid concentrations in scorbutic animals fail to increase as easily as those in control or hyperscorbutic animals.

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A sex difference in scorbutic guinea-pig survival. By A. ODUMOSU and C. W. M. WILSON, *Department of Pharmacology, University of Dublin, Trinity College, Dublin 2, Republic of Ireland*

Odumosu & Wilson (1971) have shown that in times of ascorbic acid deficiency some female guinea-pigs can readjust their ascorbic acid metabolism and so compensate for the influence of the defective gene responsible for ascorbaemia (Stone, 1966), although males appear to be incapable of doing so. The extent to which the availability of gulono-lactone is the determining factor for this readjustment is unknown.

Twelve male and twelve female guinea-pigs, after receiving a normal supplemented diet for 6 weeks (Odumosu & Wilson, 1970*a*, *b*), were transferred to scorbutogenic diets with saline, intraperintoneal injection, and water *ad lib.* for 12 d (♂), and 20 d (♀), when the maximum points in the scorbutic weight curves are attained. Both groups were then subdivided into one subgroup receiving gulono-lactone supplement (30 mg/kg daily intraperitoneal injection) (GS), whereas the other

Table 1. (1) *Mean weights and alterations from mean* (*g*) *at days* 12 (♂) *and* 20 (♀), (2) *numbers losing weight and* (3) *nos remaining alive in groups of six* ♂ *and* ♀ *guinea-pigs fed on scorbutogenic diets and receiving daily supplements of saline* (*SS*) *or gulono-lactone* (*GS*)

	Males						Females					
Time on diet (d)	SS			GS			SS			GS		
	1	2	3	1	2	3	1	2	3	1	2	3
0	484·8±11·5	0	6	467·0±15·6	0	6	460·8±21·7	0	6	459·5±11·5	0	6
6	+29·2	0	6	+25·8	0	6	+7·2	0	6	+3·0	0	6
12	+31·4	3	6	+31·7	2	6	+9·7	1	6	+14·0	0	6
18	−5·6	6	3	−12·8	5	4	+11·0	2	6	+24·0	1	6
20	−64·0	3	3	−13·7	2	4	−7·3	5	6	+37·0	1	6
24	−107·0	3	3	−15·0	2	4	−13·5	4	6	−9·8	3	6
30	—	−	0	+30·5	1	2	−62·6	4	6	−21·3	4	6
36	—	−	0	+68·5	2	2	−12·0	2	2	−12·0	3	5
42	—	−	0	+108·0	2	2	−72·0	1	1	+93·0	3	3
52	—	−	0	+63·0	2	1	−25·0	1	1	+87·0	1	3
60	—	−	0	+35·0	1	0	—	−	0	+87·0	2	3
80	—	−	0	—	−	0	—	−	0	+39·0	3	2
84	—	−	0	—	−	0	—	−	0	+80·0	0	1
90	—	−	0	—	−	0	—	−	0	+86·0	0	1

continued to receive saline (SS). All the subgroups continued on the scorbutogenic diet, and were weighed at 3 d intervals. Table 1 shows the alterations in weight and survival times in the different subgroups. All the unsupplemented males lost weight and the last one died on day 29, as in previous experiments (Odumosu & Wilson, 1970*a*, *b*, 1971). The last unsupplemented female finally died on day 58, having survived for 29 d longer than her male counterpart. The last GS male was losing weight, and died on day 60. The second of the surviving GS females finally died on day 82 and the third one continued to gain weight and survive up to day 90 when she was returned to the normal supplemented diet.

It has been confirmed that male and female guinea-pigs react differently to the stress of ascorbic acid deficiency, females having a shorter initial weight gain, and surviving longer than males. This can be attributed to an increase in tissue ascorbic acid values after the initial fall in the surviving females (Odumosu & Wilson, 1971). Administration of gulono-lactone can more than double life expectancy in scorbutic male guinea-pigs. In female guinea-pigs gulono-lactone administration prolongs their life expectancy; the longer life of the female in comparison with the male is maintained.

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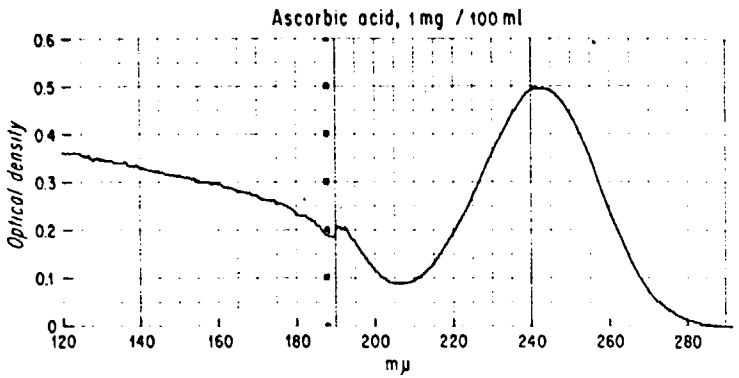

Fig. 1. Absorbance spectrum of ascorbic acid.

When dog blood was anticoagulated with one-tenth volume of oxalate (0.1M sodium oxalate) or citrate (3.8% sodium citrate), clotting could not be induced by the addition of any amount of ascorbic acid.

Ascorbic acid has a characteristic ultraviolet absorbance maximum near 243 mμ and a minimum near 206 mμ (Fig. 1) while heparin has an absorbance peak near 206 mμ with a sharp maximum near 191 mμ (Fig. 2). When both compounds are present in the same solution there is no shift in the absorbance maxima of either, but the absorbance peak for heparin at 206 mμ is no longer evident and the absorbance below 190 mμ is significantly depressed (Fig. 3).

DISCUSSION

Since by definition 1 unit of heparin is sufficient to prevent clotting of 1 ml of whole blood for 24 hours under specified conditions, the use of 5 units of heparin per milliliter represents generous anticoagulation. A number of experiments, primarily with dog blood, only a few of which are presented here, clearly show that 6 to 10 mg of ascorbic acid can virtually abolish the anticoagulant effect of 5 units of heparin. Furthermore, 10 mg of ascorbic acid distinctly prolongs the clotting time of 1 ml of normal dog blood, and this is reversed by adding heparin which, in this paradoxic circumstance, has coagulant properties.

We do not know the mechanism by which heparin and ascorbic acid interact. The main spectral absorbance peak of ascorbic acid, at about 243 mμ, was not altered by heparin. At 191 mμ, where heparin's main absorbance occurs, as well as at a secondary peak of ascorbic acid, there was a slight reduc-

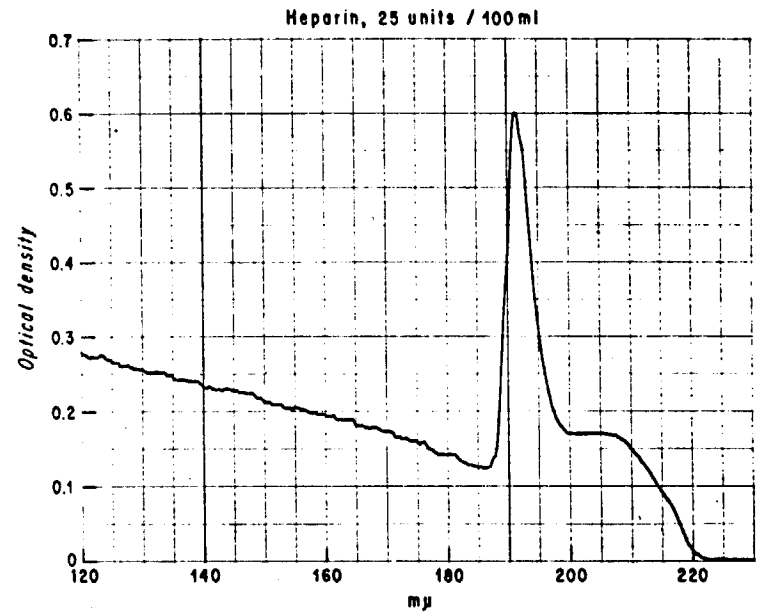

Fig. 2. Absorbance spectrum of heparin.

tion in light absorbance. The pronounced spectral change occurred below 190 mμ where absorbance of the mixture was invariably less than the sum of the absorbance of heparin

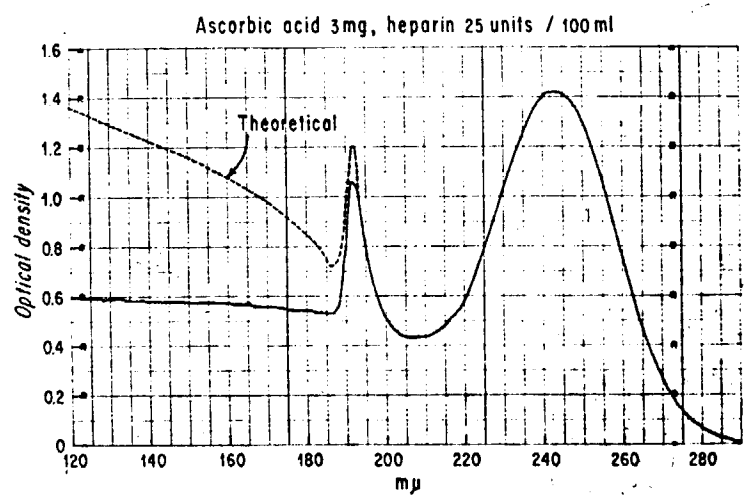

Fig. 3. Absorbance spectra of ascorbic acid and heparin.

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and ascorbic acid individually. The significance of this is uncertain, though, since thiodiglycol and cysteine also depressed light absorbance below 190 mμ but did not inhibit heparin's anticoagulant activity. These two compounds, like ascorbic acid, have strong reducing properties.

Since, for blood coagulation, heparin and ascorbic acid are mutually inhibitory, they may be inhibitory in other biologic systems as well. The adrenal cortex contains a high concentration of ascorbic acid which may be important in steroidal synthesis.[1-4] The intravenous administration of heparin[5,6] or heparinoids[7] interferes with adrenal synthesis of aldosterone, and protamine obstructs this action of heparin[6] as it does the anticoagulant property also.

Long-term administration of heparin also affects the osseous system, an effect shown clinically in the development of osteoporosis. Since osteoblasts are sensitive to ascorbic acid deprivation[8] and "the vitamin may be an important factor in stimulating skeletal aerobic metabolism,"[9] the heparin effect may be mediated through ascorbate metabolism.

Heparin is believed to form complexes with amines[10] and perhaps interferes with histaminic stimulation of gastric acid by this mechanism.[11,12] Or, heparin may induce the release of diamine oxidase which destroys histamine.[13] However, the intestinal tract has a fairly high content of ascorbic acid,[14-16] and reduced gastric acidity from histamine stimulation has been reported in scurvy.[17]

Since other tissues contain high concentrations of ascorbic acid—including the eye, anterior pituitary, corpus luteum, testicular interstitium, liver, and polymorphonuclear leukocytes—the effect of long-term administration of heparin on the function of these tissues might be interesting.

SUMMARY

In the proportion of about 2 mg of ascorbic acid to 1 unit (about 0.01 mg) of heparin, the anticoagulant effect of each substance is virtually eliminated. Depression of aldosterone synthesis and development of osteoporosis, both induced by heparin, may reflect this heparin-ascorbic acid competition.

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Effect of Benzanthrone on the Body Level of Ascorbic Acid in Guinea Pigs

By

K. P. Pandya, G. B. Singh and N. C. Joshi

(Received April 30, 1970)

Abstract: The effect of benzanthrone on the blood, adrenal and liver ascorbic acid levels in adult male guinea-pigs has been investigated. Benzanthrone and ascorbic acid were administered intraperitoneally and orally in doses of 25 mg and 50 mg/kg body weight respectively. Benzanthrone alone caused significant decrease of ascorbic acid levels in the blood, adrenal and liver. Supplementation of ascorbic acid appreciably restored the blood ascorbic acid. However, adrenal and liver ascorbic acid levels were restored only to some extent. Histochemical examination of ascorbic acid in the adrenal revealed almost similar changes. The mortality rate due to benzanthrone toxicity (500 mg/kg) was lowered by 40 % in non-scorbutic as compared to scorbutic guinea-pigs.

Key-words: Benzanthrone – ascorbic acid.

It has been observed in a dye producing factory that workers who come in contact with the dye benzanthrone (a derivative of anthraquinone) during its manufacture, pulverization and storage develop itching, burning sensation, erythema and pigmentation on exposed and even covered parts of the skin. In a few cases, pigmentation spreads rapidly. SINGH *et al.* (1967) studied the effect of local application of benzanthrone on the skin of mice and observed dermal changes when the animals were exposed to sunlight. In their preliminary report on benzanthrone toxicity SINGH & ZAIDI (1969) observed that oral administration of ascorbic acid partially inhibited the benzanthrone-induced dermal changes in experimental animals. Since ascorbic acid appears to protect the animals from the dermal toxicity of the dye, it was of interest to study the toxic action of the dye in scorbutic animals and the reversal of the toxic symptoms by ascorbic acid.

Material and Methods

Animals.

One hundred male guinea-pigs of I. T. R. C. colony, average weight 400 grams, were used.

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Plan of experiment.

1. *Body level ascorbic acid studies.* The animals which were kept on routine stock diet consisting of gram, bran and green leafy vegetables, were divided into four groups of ten each, as follows:

Group I. Normal control.

Group II. In this group 25 mg/kg body weight of benzanthrone, suspended in 1 ml normal saline, was injected intraperitoneally, once a day.

Group III. In this group 50 mg/kg body weight of ascorbic acid, dissolved in 1 ml distilled water, was fed orally, daily, through a No. 5 rubber catheter.

Group IV. The animals were treated as in group III, and 25 mg/kg body weight of benzanthrone in suspension was administered as in group II.

Animals of all the groups were sacrificed after 7 days by ether anaesthesia. Approximately 5 ml blood were drawn directly from the heart. The abdomen of each animal was opened and the liver and adrenals were dissected out. Histochemical examination of ascorbic acid in one of the adrenals (left) was done by the method of BACCHUS (1950). Estimation of the total ascorbic acid content of the blood, liver and the right adrenal was done by the method of SCHAFFERT & KINGSLEY (1955).

2. *Study of mortality rate.* Sixty male guinea-pigs were divided into three groups of 20 each, I) normal, II) scorbutic and III) non-scorbutic. Routine stock diet was fed to the animals of group I. Animals of groups II and III were fed a synthetic scorbutic diet (KAW *et al.* 1969) consisting of crushed barley, 64 parts, crushed gram (CICER ARIETINUM), 20 parts, casein, 12 parts, calcium carbonate, 3 parts, and sodium chloride, 1 part, vitamins A and D (2,400 and 400 i. u. respectively) were supplied twice weekly. 25 mg/kg body weight/day of ascorbic acid was fed to group III. After 15 days, 500 mg/kg body weight of benzanthrone in normal saline was injected intraperitoneally into each animal of all the groups. The mortality rate was recorded within twentyfour hours. The dose of benzanthrone was selected on the basis of our preliminary investigation which showed that 500 mg/kg body weight was sufficient to kill 50 per cent of stock diet fed animals in 24 hours.

Results

Histochemical studies.

Histochemical examination of ascorbic acid was done only in the adrenal because its concentration in the liver of various groups of animals was not sufficient to be visualised. The adrenal of animals belonging to groups I and III showed cells densely filled with compact ascorbic acid granules (figs. 1 & 2). In groups II and IV however, the number of ascorbic acid granules was scanty. They were discrete, smaller in size and irregularly scattered in the cytoplasm (figs. 3 & 4).

Estimation of ascorbic acid.

The results are given in table 1.

Ascorbic acid levels in the blood, adrenal and liver were significantly lowered ($P < 0.01$) in the benzanthrone treated animals of group II as com-

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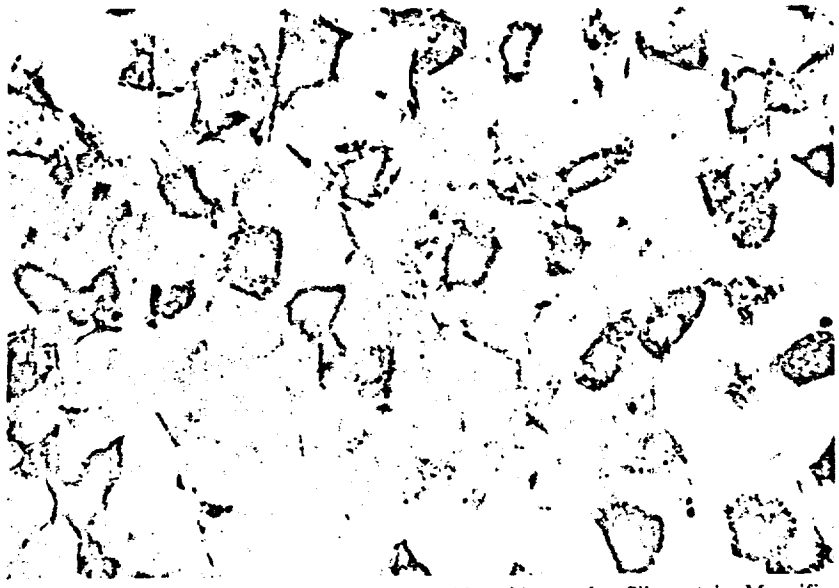

Fig. 1. Normal control. Densely compact ascorbic acid granules. Silver stain. Magnification × 120.

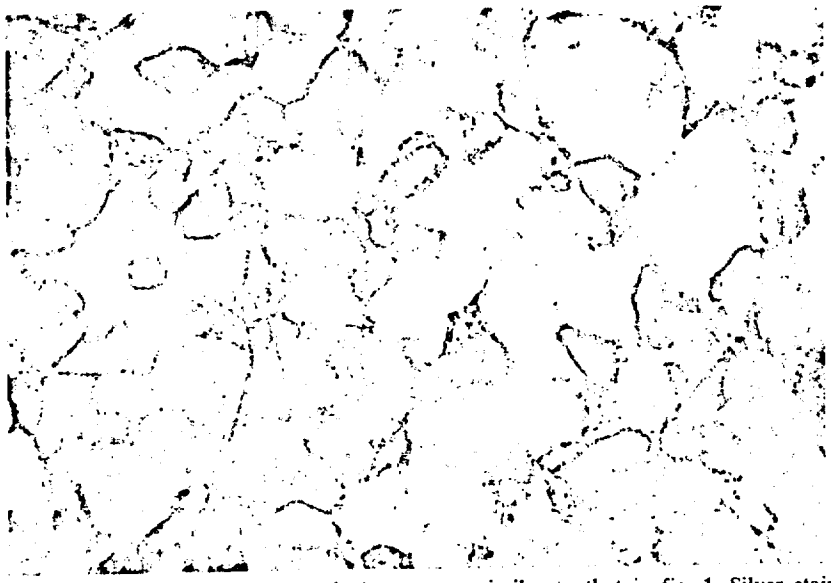

Fig. 2. Ascorbic acid administered. Appearance similar to that in fig. 1. Silver stain. Magnification × 120.

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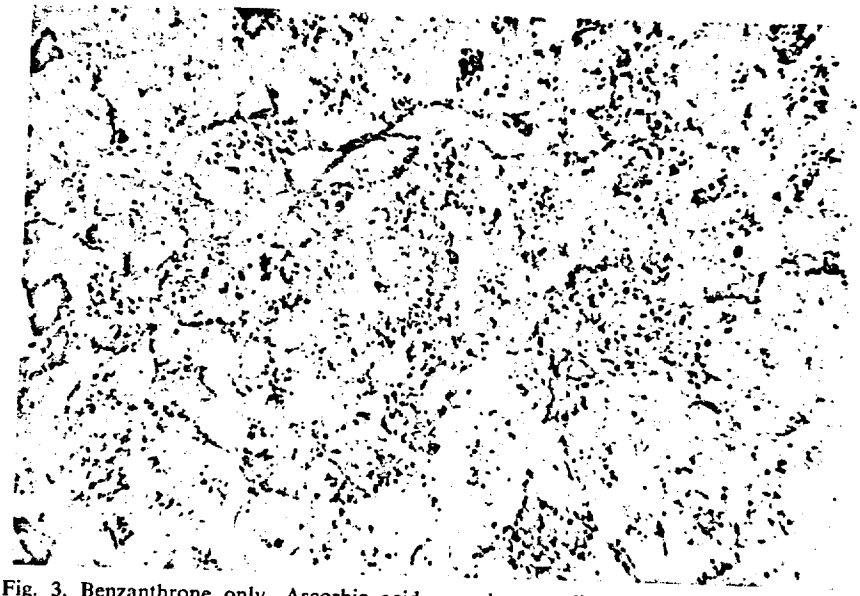

Fig. 3. Benzanthrone only. Ascorbic acid granules are discrete, smaller in size and irregularly scattered. Silver stain. Magnification × 120.

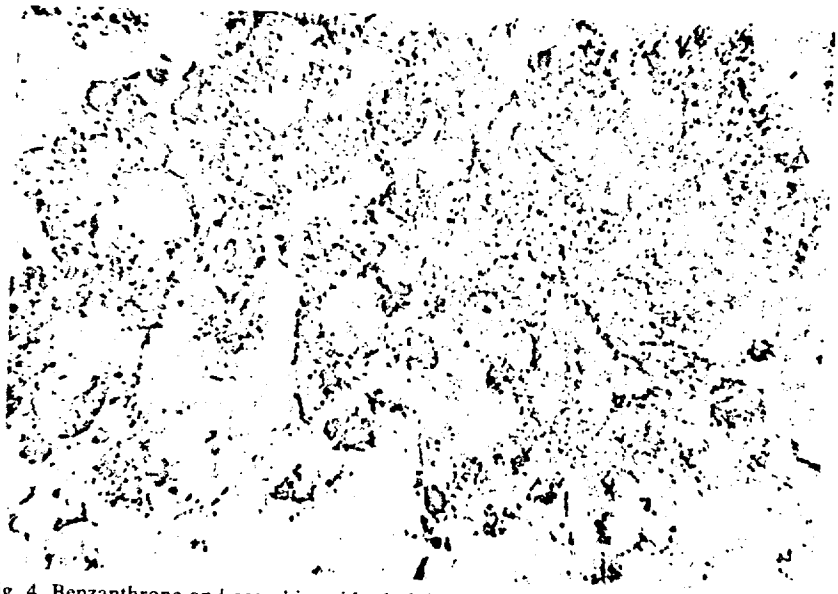

Fig. 4. Benzanthrone and ascorbic acid administered. Almost similar appearance to that observed in fig. 3. Silver stain. Magnification × 120.

Table 1.

Ascorbic acid content of the blood, adrenal and liver in control and experimental animals. Comparisons between groups have been made from a pooled estimate of the variance, for each tissue (after analysis of variance).

	Blood (mg/100 ml) Mean ± S. E. M.	Adrenal (mg/100 gram of wet wt.) Mean ± S. E. M.	Liver (mg/100 gram of wet wt.) Mean ± S. E. M.
Group I (Normal control)	1.161 ± 0.083	95.7 ± 6.4	21.81 ± 0.89
Group II (Benzanthrone administered)	0.430 ± 0.013	48.9 ± 4.2	17.88 ± 0.85
Group III (Ascorbic acid administered)	1.180 ± 0.090	100.4 ± 9.3	20.95 ± 0.94
Group IV (Benzanthrone & ascorbic acid administered)	0.982 ± 0.062	62.4 ± 2.3	19.53 ± 1.11

pared to the control group I. However, significant differences were not observed in the ascorbic acid contents of these tissues after administration of ascorbic acid in group III as compared to the controls. Ascorbic acid supplementation in benzanthrone treated animals (group IV) appreciably restored the blood ascorbic acid level ($P < 0.01$) as compared to animals of group II. However, in the case of the adrenal and liver, the ascorbic acid level was restored to some extent only.

Mortality rate.

The results are given in table 2. In animals supplemented with ascorbic acid (25 mg/kg/day) the mortality rate was lowered to the extent of 40 % as compared to animals of the scorbutic group. Scorbutic animals appeared to be completely unprotected due to the lack of ascorbic acid.

Discussion

MURAKAMI (1939) demonstrated that a diet deficient in ascorbic acid lowered the detoxication mechanism of guinea-pig liver, but this condition was reversed by supplementation with ascorbic acid. BUNDESEN *et al.* (1941) showed that the reaction of hypersensitive patients can be prevented if the

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Table 2.

Mortality rate in normal and synthetic diet fed scorbutic and non-scorbutic guinea-pigs. Mortality rate was lowered by 40 per cent in non-scorbutic as compared to scorbutic guinea-pigs.

	No. of animals	No. of animals died within 24 hours of benzanthrone administration (500 mg/kg)	Mortality rate
Group I Normal (Stock diet)	20	10	50 per cent
Group II Scorbutic (synthetic scorbutic diet)	20	20	100 per cent
Group III Non-scorbutic (Synthetic scorbutic diet with additional ascorbic acid)	20	12	60 per cent

ascorbic acid content of the blood is maintained at a sufficiently high level. BEYER (1943) found that the hepatic toxic effect of hydrazine was reduced by ascorbic acid and enhanced by ascorbic acid deficiency. The mechanism of action was not explained but it was suggested that ascorbic acid exerts its effect indirectly by allowing normal metabolism of carbohydrate and protein.

Ascorbic acid has also been reported to be effective in allergy, sensitivity and toxicity of the skin due to various synthetic dyes and its usefulness in the prevention of dermal lesions caused by the local application of benzanthrone was shown by SINGH & ZAIDI (1969). Ascorbic acid has been found useful in the prevention of chrome dermatitis (SAMITZ *et al.* 1968). WAPNICK *et al.* (1968) observed a decrease in white blood cell ascorbic acid levels in subjects suffering from iron toxicity.

FORSSMAN & FRYKHOLM (1947) observed that exposure to benzene produced an increased requirement of ascorbic acid. EDDY (1949) reported that ascorbic acid might exert a protective action against benzene poisoning. Recently, the effect of chronic benzene poisoning on ascorbic acid levels in the guinea-pig and rat was studied by IANCU *et al.* (1969). In the guinea-pig, benzene poisoning lowered ascorbic acid levels in the blood and organs (adrenals, liver) and intensified ascorburia. The toxic effect was attenuated by the administration of ascorbic acid at a dose of 6 mg/day and the mortality rate was lowered by 57 %. They concluded that benzene affected the metabolism of ascorbic acid by causing an increase in catabolism.

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In the present experiments, administration of benzanthrone caused a significant decrease of ascorbic acid level in the blood, adrenal and liver. Supplementation with ascorbic acid had only a slight effect on the levels in the adrenal and liver but restored the blood ascorbic acid. This would suggest an increased requirement of ascorbic acid in guinea-pigs after the administration of benzanthrone. Similar conclusions were also obtained from studies of mortality rates. A 40 % lowering of the mortality rate after supplementation of ascorbic acid (25 mg/kg/day) as compared to animals of the scorbutic group also emphasizes the protective role of the vitamin in cases of benzanthrone toxicity. These studies suggest the possible usefulness of ascorbic acid in clinical therapy, particularly of workers who are exposed to benzanthrone and develop various dermal symptoms.

Acknowledgements

The authors are grateful to Dr. S. H. Zaidi, Director, Industrial Toxicology Research Centre for his keen interest in the work. Technical assistance of Messrs. Mulkraj, Kanahiya Lal and V. G. Misra is highly appreciated. Thanks are due to Mr. Musleh Ahmad for the preparation of photomicrographs and Mr. P. A. George for the statistical analysis.

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18: 428-9, 1939.
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The Behavior of the Thrombocyte Count after Intravenous Injection of Large Amounts of Vitamin C

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(From the First Medical Clinic of Munich University - Director: Prof. Dr. W. Stepp)

In view of the different effects exerted by vitamin C in the human organism, special attention has been devoted to the behavior of the blood picture after oral and parenteral administration of l-ascorbic acid. In this case, it was found that both the red and white blood count can be affected by ascorbic acid under certain conditions (Kreitmair, Schnetz). Seyderhelm and Grebe found that reticulocytes are present in larger amounts in the blood stream after treatment with vitamin C, and assume that this is caused by an action on the bone marrow which results in the washing out (elimination) of young cells. Boger and Schroder, who described for the first time the hemostatic action of vitamin C, devoted very special attention, for obvious reasons, to the behavior of thrombocytes and found, in thrombopenic hemorrhages and hemophilia, a considerable increase in the number of thrombocytes after oral administration of vitamin C. Boger and Martin have confirmed these findings in many cases and have expressed for the first time the thought that this thrombocytopoiesis is due to an increased activity of the blood-forming spinal marrow. In the course of different check-ups of these findings, quite nonuniform results have been obtained. Thus, in thrombopenic hemorrhages, Koch and Walter were unable to find any effect of vitamin C administration on the thrombocyte count or on the tendency to bleed. According to Anstett, ascorbic acid is unable to halt the destruction of the increased number of thrombocytes present in blood after a blood transfusion. For this reason, we have again studied the effect of different large doses of l-ascorbic acid (in the form of Merck's cebion) on thrombocytes in a series of patients with various diseases, and have obtained quite remarkable (noteworthy) results from this study.

Method. Thrombocytes were counted by the known method of Fonio. The determination was always done before the beginning of treatment and after the daily intravenous administration at 2-day intervals. In a series of patients, the thrombocyte count was also determined after the intravenous injection of a larger vitamin C dose at 15-minute intervals. For control purposes, the thrombocyte count was also determined in some patients by the Flossner method. The results of our studies are summarized in Table 1.

Table 1 -

No.	Diagnosis	Vitamin C-dose	Thrombocytes in ccm, according to Fonio	Thrombocytes in ccm, according to Flossner	Time of Determination
1	Cachexia	3000 mg (daily 500 mg)	65000 150000 196000		a b h
2	Dry pleurisy	3500 mg (daily 500 mg)	260000 500000 455000		a c h
3	Liver cirrhosis	3000 mg (daily 500 mg)	88000 277000 396000		a b g
4	Hypophyseal cachexia	3500 mg (daily 500 mg)	100000 405000		a c
5	Diabetes mellitus	2000 mg (daily 500 mg)	290000 450000	602000 900000	a d
6	Diabetes mellitus	1500 mg (daily 500 mg)	170000 332000		a c
7	Croupous pneumonia	6000 mg (daily 1000 mg)	255000 530000 480000	650000 1300000 1355000	a b d
8	Influenzal pneumonia	1500 mg (daily 500 mg)	250000 370000	1000000	a b
9	Diabetes mellitus	100 mg	195000 200000		a b
10	Fracture of the neck of the femur	1500 mg (daily 500 mg)	272000 360000	674000 935000	a c
11	Chronic polyarthritis	900 mg (daily 300 mg)	290000 325000 300000		a b e
12	Heart failure	750 mg (daily 250 mg)	220000 215000 250000		a b e

Table 1 - cont.

No.	Diagnosis	Vitamin C-dose	Thrombocytes in ccm, according to Fonio	Thrombocytes in ccm, according to Flossner	Time of Determination
13	Lobar pneumonia	1000 mg (daily 500 mg)	260000 302000	700000 750000	a c
14	Pulmonary tuberculosis	1500 mg (daily 500 mg)	150000 270000		a b
15	Chronic polyarthritis	500 mg (daily 100 mg)	197000 184000 190000		a b e
16	Pleuritis carcinomatosa	100 mg	140000 132000		a b
17	Chronic bronchitis	400 mg (daily 100 mg)	168000 291000		a b
18	Werlhef's disease*	7000 mg (daily 500 mg)	8000 62000 70000 70000		a j f i
19	Lobar pneumonia	2500 mg (daily 500 mg)	220000 465000 200000		a b f
20	Gastric (peptic) ulcer	4000 mg (daily 500 mg)	150000 460000 390000		a b e

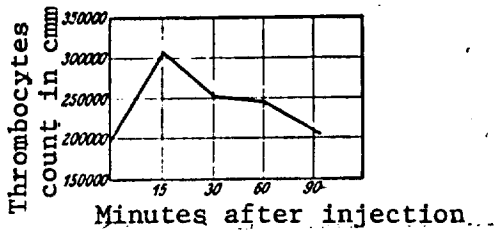
*(idiopathic thrombocytopenic purpuva)

a. Before start of treatment
b. one (1) day after treatment
c. two (2) days after treatment
d. three (3) days after treatment
e. four (4) days after treatment
f. five (5) days after treatment
g. six (6) days after treatment
h. seven (7) days after treatment
i. ten (10) days after treatment
j. fourteen (14) days after treatment

Thus, our studies have shown that in patients which received by the intravenous route for several days quite large amounts of vitamin C (a total of 3000 to 6000 mg) the number of thrombocytes exhibits rather regularly a definite tendency to increase, which always extends beyond the physiological variation. Values of 450,000-530,000 were observed by us as the greatest increase after such high doses of vitamin C. In some patients, in which a considerable thrombopenia was initially present, it was not possible to achieve an increase in the thrombocyte count above normal values

even with high doses of vitamin C. However, an increase in the thrombocyte count could be clearly noted also in these cases. Quite different were the results of our investigation in patients which did not receive such unphysiologically high doses of vitamin C. Daily intravenous injections of 100 and 300 mg for several days did not disclose any clearcut effect on the thrombocyte count. After a single injection of a large amount of Cebion (500 mg), a temporary increase of the thrombocyte count is noted after 15 minutes, which can no longer be detected after the lapse of a further 75 minutes.

Figure 1 - Effect of a single injection of vitamin C (500 mg) on the thrombocyte count.



In summary, we can thus say that unphysiologically large doses of the isolated vitamin C can induce a thrombocytosis, which very probably is the expression of stimulation of the blood-forming bone marrow. We believe that this effect of l-ascorbic acid has nothing to do with its vitamin nature, and that it rather represents one of the effects exerted by healing substances, which Stepp and Pfannenstiel have already pointed out quite early in isolated active principles.

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ÜBER DAS VERHALTEN DER THROMBOCYTENZAHL NACH INTRAVENÖSER INJEKTION GROSSER VITAMIN C-MENGEN.

Von

G. PAPAYANOPULOS und H. SCHROEDER.

Aus der I. Medizinischen Klinik der Universität München (Direktor: Prof. Dr. W. STEPP).

Bei den verschiedenartigen Wirkungen des C-Vitamins im menschlichen Organismus hat man dem Verhalten des Blutbildes nach peroraler und parenteraler Verabreichung der l-Ascorbinsäure ganz besondere Aufmerksamkeit geschenkt. Dabei hat man sowohl eine Beeinflussung des roten wie des weißen Blutbildes unter bestimmten Bedingungen feststellen können (KREITMAIR, SCHNETZ). SEYDERHELM und GREBE fanden ein vermehrtes Auftreten von Reticulocyten im strömenden Blut nach Vitamin C-Behandlung und nehmen als Ursache eine Einwirkung auf das Knochenmark an, die zu einer Ausschwemmung jugendlicher Zellen führt. Sie sprechen daher auch von einer „Blutverjüngung". BÖGER und SCHROEDER, die erstmalig die hämostyptische Wirkung des C-Vitamins beschrieben haben, widmeten naheliegenderweise dem Verhalten der Thrombocyten ganz besondere Aufmerksamkeit. Sie fanden bei thrombopenischen Blutungen und bei der Hämophilie einen erheblichen Anstieg der Thrombocyten nach peroraler Vitamin C-Darreichung. BÖGER und MARTIN haben diesen Befund in vielen Fällen bestätigt und zum erstenmal den Gedanken ausgesprochen, daß diese Thrombocytopoese durch eine Steigerung der Tätigkeit des blutbildenden Rückenmarkes bedingt ist. Bei verschiedenen Nachprüfungen dieser Befunde sind recht uneinheitliche Ergebnisse erhalten worden. So konnten KOCH und WALTER bei thrombopenischen Blutungen nach Vitamin C-Gaben weder eine Beeinflussung der Thrombocytenzahl noch der Blutungsneigung finden. Nach ANSTETT ist die Ascorbinsäure nicht in der Lage, die Zerstörung der nach einer Bluttransfusion im Blut vermehrt auftretenden Thrombocyten

aufzuhalten. Wir haben deshalb bei einer Reihe von Patienten mit den verschiedensten Krankheitszuständen noch einmal die Wirkung verschieden großer Dosen l-Ascorbinsäure in Form von Cebion (Merck) auf die Thrombocyten untersucht und sind dabei zu recht bemerkenswerten Ergebnissen gekommen.

Tabelle 1.

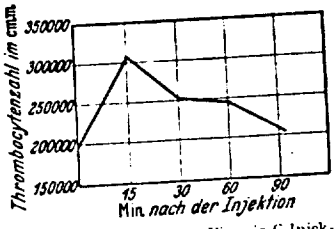
Nr.	Diagnose	Vitamin C-Dosis	Thrombocyten in cmm nach FONIO	Thrombocyten in cmm nach FLÖSSNER	Zeit der Bestimmung
1	Kachexie	3000 mg (täglich 500 mg)	65000 150000 196000		Vor Beginn 1 Tag nach 7 Tage nach
2	Pleuritis sicca	3500 mg (täglich 500 mg)	260000 500000 455000		Vor Beginn 2 Tage nach 7 Tage nach
3	Lebercirrhose	3000 mg (täglich 500 mg	88000 277000 396000		Vor Beginn 1 Tag nach 6 Tage nach
4	Hypophysäre Kachexie	3500 mg (täglich 500 mg)	100000 405000		Vor Beginn 2 Tage nach
5	Diabetes mellitus	2000 mg (täglich 500 mg)	290000 450000	602000 900000	Vor Beginn 3 Tage nach
6	Diabetes mellitus	1500 mg (täglich 500 mg)	170000 332000		Vor Beginn 2 Tage nach
7	Croupöse Pneumonie	6000 mg (täglich 1000 mg)	255000 530000 480000	650000 1300000 1355000	Vor Beginn 1 Tag nach 3 Tage nach
8	Grippe-Pneumonie	1500 mg (täglich 500 mg)	250000 370000	1000000	Vor Beginn 1 Tag nnch
9	Diabetes mellitus	100 mg	195000 200000		Vor Beginn 1 Tag nach
10	Schenkelhals-fraktur	1500 mg (täglich 500 mg)	272000 360000	674000 935000	Vor Beginn 2 Tage nach
11	Chronische Polyarthritis	900 mg (täglich 300 mg)	290000 325000 300000		Vor Beginn 1 Tag nach 4 Tage nach
12	Herz-insuffizienz	750 mg (täglich 250 mg)	220000 215000 250000		Vor Beginn 1 Tag nach 4 Tage nach
13	Lobäre Pneumonie	1000 mg (täglich 500 mg)	260000 302000	700000 750000	Vor Beginn 2 Tage nach
14	Lungen-tuberkulose	1500 mg (täglich 500 mg)	150000 270000		Vor Beginn 1 Tag nach
15	Chronische Polyarthritis	500 mg (täglich (100 mg)	197000 184000 190000		Vor Beginn 1 Tag nach 4 Tage nach
16	Pleuritis carcinomatosa	100 mg	140000 132000		Vor Beginn 1 Tag nach
17	Chronische Bronchitis	400 mg (täglich 100 mg)	168000 291000		Vor Beginn 1 Tag nach
18	Morbus Werlhof	7000 mg (täglich 500 mg)	8000 62000 70000 70000		Vor Beginn Am 14. Behandlungstage 5 Tage nach 10 Tage nach
19	Lobäre Pneumonie	2500 mg (täglich 500 mg)	220000 465000 200000		Vor Beginn 1 Tag nach 5 Tage nach
20	Magen-geschwür	4000 mg) (täglich 500 mg)	150000 460000 390000		Vor Beginn 1 Tag nach 4 Tage nach

der Behandlung

der Behandlung

Methodik: Die Thrombocyten wurden nach der bekannten Methode von FONIO ausgezählt. Die Bestimmung erfolgte immer vor dem Beginn der Behandlung und nach der täglichen intravenösen Verabreichung in Abständen von 2 Tagen. Bei einer Reihe von Kranken haben wir die Thrombocytenzahl auch nach der intravenösen Injektion einer größeren Dosis C-Vitamin in Abständen von 15 Minuten gezählt. Zur Kontrolle wurde bei einigen Kranken die Thrombocytenzahl auch mit der von FLÖSSNER angegebenen Methode bestimmt. Das Ergebnis unserer Untersuchungen gibt das Schaubild (Tabelle 1) wieder.

Bei unseren Untersuchungen hat sich also gezeigt, daß bei den Kranken, die mehrere Tage lang recht große Vitamin C-Mengen intravenös erhielten (im ganzen 3000 bis 6000 mg) ziemlich regelmäßig die Zahl der Thrombocyten eine unverkennbare Tendenz zu einer Steigerung zeigt, die stets über die physiologische Schwankungsbreite hinausgeht. Als höchste Steigerung nach derartig hohen C-Dosen beobachteten wir Werte von 450000—530000. Bei einigen Kranken, bei denen primär eine erhebliche Thrombopenie bestand, gelang es auch durch hohe Vitamin C-Dosen nicht, eine Steigerung der Thrombocytenzahl über die Norm zu erzielen. Jedoch war auch in diesen Fällen ein Anstieg in der Thrombocytenzahl deutlich zu erkennen. Ganz anders war das Untersuchungsergebnis bei den Kranken, die nicht derartig unphysiologisch hohe Vitamin C-Dosen erhielten. Intravenöse Gaben von täglich 100 bzw. 300 mg mehrere Tage lang gegeben, ließen keine deutliche Einwirkung auf die Thrombocytenzahl erkennen. Nach einer einmaligen Injektion einer größeren Cebionmenge (500 mg) kommt es nach 1/4 Stunde zu einem vorübergehenden Anstieg der Thrombocytenzahl, der nach dem Verlauf von weiteren 75 Minuten nicht mehr zu erkennen ist.



Wirkung einer einmaligen Vitamin C-Injektion (500 mg) auf die Thrombocytenzahl.

Zusammenfassend läßt sich also sagen, daß unphysiologisch große Dosen des isolierten C-Vitamins eine Thrombocytose hervorrufen können, die sehr wahrscheinlich Ausdruck einer Reizung des blutbildenden Knochenmarkes ist. Wir glauben, daß diese Wirkung der l-Ascorbinsäure nichts mit ihrem Vitamincharakter zu tun hat, sondern daß sie zu den Heilstoffwirkungen gehört, auf die STEPP und PFANNENSTIEL schon sehr früh bei den isolierten Wirkstoffen aufmerksam gemacht haben.

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UNTERSUCHUNGEN ÜBER HEMERALOPIE.

Ein Beitrag zu der Frage des Vorkommens von Vitamin A-Mangelzuständen.

Von

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Im Rahmen unserer Untersuchungen über die Häufigkeit des Vorkommens von allgemeinen Vitamin-Mangelzuständen bei unserer Bevölkerung haben wir uns auch mit der Frage des Vorkommens von Vitamin A-Mangelzuständen bei Gesunden und Kranken beschäftigt. Es galt hierbei nicht, die bei uns (besonders beim Erwachsenen) äußerst seltenen manifesten A-Avitaminosen, sondern vielmehr die große Gruppe derjenigen Vitamin A-Mangelzustände zu erfassen, die das weite Gebiet zwischen ausreichender Versorgung des Organismus mit Vitamin A und manifester Avitaminose ausmachen, und die als Hypovitaminosen bezeichnet werden. Für den *Nachweis eines Vitamin A-Mangelzustandes* standen *zwei*

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Role of nutrition in the prevention and treatment of periodontal disease

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Nutritional deficiency is not a significant factor in the type of periodontal disease commonly encountered in North America. Consequently, indiscriminate prescription of nutritional supplements, especially of vitamin C, has no practical value for the prevention or treatment of this disease.

La carence nutritive n'est pas un facteur important dans le type de maladie périodontique que l'on rencontre couramment en Amérique du Nord. Par conséquent, la prescription au hasard de suppléments nutritifs, surtout de vitamine C, n'a pas de valeur pratique pour prévenir ou traiter cette maladie.

All living tissues need an adequate supply of essential nutrients. They need even more during growth, repair and inflammation, or other cellular activity.

Cellular metabolism suffers and tissue function is impaired when nutrition is inadequate. As a consequence, the body is starved or shows signs of a specific nutritional deficiency.

These observations led to early speculation that nutritional deficiency might also play a role in chronic periodontal disease. By pinpointing the cause, scientists thought they might be able to prevent or cure the disease by stepping up the consumption of a deficient nutrient. The role of nutrition in periodontal disease has accordingly received considerable attention. This article reviews several previous studies and presents current information on the subject.

Local irritation

Since endocrine, dietary, functional and environmental changes affect cell metabolism, we would expect the periodontium to respond to metabolic alterations like other body tissues. But it is hard to determine how much of a particular nutrient is necessary to maintain periodontal health under various physiological and pathological conditions. The assessment of the nutrients and their interdependence in the diet is a problem in itself. Measurement of their absorption, metabolism and utilization is likewise difficult, and the presence of inflammation may add further complications.

Though marginal gingivitis and ulceration are caused by local irritation, they can be modified or accentuated by systemic influences that alter the body's resistance to injury or its capacity for repair. Nutrition is one of these systemic influences.

The diseases which the periodontal structures suffer are associated with their unique function of supporting the teeth. The periodontium is exposed to irritants that are peculiar to, and dependent on, the presence of teeth. These irritants are of three kinds (1) the bacterial masses which accumulate on the tooth surfaces in close proximity to the gingival margin, (2) chemical and thermal variation of foods tha

are bitten into and pressed against the gingival tissues, and (3) the forces of mastication transmitted directly to the soft tissues surrounding the necks of the teeth and the deeper periodontal tissues.

Though the nutritive aspect of the diet is of prime importance, its local effect cannot be ignored. Diet affects periodontal disease locally by encouraging or discouraging accumulations of bacteria on the tooth surface. In the common form of periodontal disease the local irritant is bacterial in origin.[1] Bacteria collect at the marginal gingiva as plaque or materia alba and produce irritant substances in direct contact with the gingival tissues. As the plaque spreads over the tooth surfaces its progress is hindered by mechanical removal. The latter is mediated by the friction of lips, cheek and tongue, by oral hygiene procedures, and by mastication. The amount of debris around the teeth at any given time thus reflects a balance between plaque formation and its mechancial removal. Since mastication is one of the cleansing factors, the consistency of the food is important but this quality must not be confused with the nutritional aspect of the diet after it has been absorbed.

Nutritional deficiency

The local irritant and the expected inflammatory response are evident in most cases of clinical periodontal disease. Sometimes, however, one or the other is conspicuous by its absence. Faulty nutrition is commonly blamed in such cases, especially when there is no evidence of systemic disease. However, the role of nutrition in the actiology and treatment of periodontal disease remains obscure. The literature on the subject is conflicting. Experiments which have been reported cannot readily be checked, and clinical reports are often at variance with clinical experience.

Gingivitis is more severe and more prevalent in malnourished groups; but their oral hygiene is also worse.[2,3] A hundred years ago Harris[4] observed that "persons who pay no attention to the cleanliness of the teeth are particularly subject to periodontal disease and that the disease seems to be the result of general debility or defective nutrition."

Daily nutritional requirements vary from person to person, one individual adapting to a low daily intake of a particular nutrient while another becomes ill. But, apart from such individual variations, the level of disease is linked with the degree of deficiency of the nutrient involved. Quantities of a nutrient that exceed the amount which a cell can use are simply wasted. Such an excess is usually asymptomatic except in those few instances where it hinders the absorption or utilization of another nutrient that is present in marginal amounts.

Kwashiorkor, a severe protein deficiency in African and Indian children, is characterized by loosening of the teeth which may fall out. Pindborg[5] has reported that 4 per cent of the victims suffered acute necrotizing gingivitis, noma, atrophy of the papillae of the tongue, coating of the tongue and angular cheilosis. Children free of the disease did not have these severe disorders. In kwashiorkor patients the periodontal index was 50 per cent higher. These children showed additional evidence of severe illness, such as diminution in body size, abdominal distension, oedema of the extremities, and sparse and depigmented hair which was ginger, yellow or whitish, rather than black.

Protein deficiency in its most severe form does not produce specific periodontal disease entities. Emslie,[6] who studied protein deficiencies in Africa, found that they were not a factor in the development of gingivitis or cancrum oris. Though terminal cases of noma were seen in German concentration camps, there are no reports that periodontal disease was a feature of starvation.

In animals, gingivitis occurs in the terminal stages of all vitamin deficiencies; but in Japanese prison camps, the only oral signs observed in American prisoners with pellagra were glossitis and buccal stomatitis.[7]

Several forms of malnutrition manifest periodontal lesions, but periodontal disease in the United States and in Canada is not of this type. Malnutrition is unusual on this continent and confined to the lower income groups. Yet the incidence of periodontal disease is high and the disease is prevalent in all strata of society.

Navajo Indians illustrate what happens to the oral mucosa when the diet falls short of accepted standards.[8] These semi-nomads live in a sandy, arid, desert region and subsist on what little they can produce for themselves or buy from a trading post. Their diet is almost completely devoid of fresh fruit, vegetables, dairy produce and fish. Yet Navajo children on the poorest diet were found to have the best gingival condition. Their gums, in fact, were better than those of the healthiest white children.

Darby[9] has described several examples of malnutrition among Navajos. In 60,000 patients he observed 10 cases of pellagra, two each of scurvy and beriberi, one of rickets and 73 of general malnutrition. Although the serum ascorbic acid was under 0.3 mg per cent in half the children he examined, Darby found no relationship between the serum level of this vitamin and gingival health.

Unpublished data from other surveys tend to corroborate these findings. For example, Papago Indians on the US-Mexican border also have healthy gums despite their very restricted diet. They subsist almost exclusively on tortilla and beans but get no fresh fruit or dairy produce. Hopi Indians, on the other hand, enjoy a more sophisticated diet but have higher gingival scores. Their gums resemble those of people living on a typical western diet.

Of all the groups examined, the healthiest were the Navajos living in the remote area of their reservation and the Papagos on the Mexican border. Both groups ate a very restricted diet, did not clean their teeth, and had a good deal of subgingival calculus despite the abrasive nature of their food. In various other surveys I have observed the poorest periodontal health in large groups of orphanage children and similar groups of institutionalized mentally sick patients. Both groups received adequate diets according to the accepted standards.

Vitamin C deficiency

Vitamin C deficiency deserves special consideration because of its specific effect on the periodontium. Fully developed cases of scurvy are now rare but were

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HEPARIN-ASCORBIC ACID ANTAGONISM

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We have noticed that when ascorbic acid is added to heparinized rat or dog blood to block destruction of serotonin during extraction and assay, the blood often clots despite adequate heparinization. This apparent antiheparin effect of ascorbic acid was studied.

METHODS

Normal mongrel dog blood was used for the most part, although normal human or rat blood reacted similarly. Ascorbic acid, in 0.9% sodium chloride, and heparin, also in physiologic saline, were added individually or together to freshly drawn blood in 3-ml glass tubes. The volume of blood was always 1 ml; its clotting time was determined, at room temperature, by tilting the tube at 1-minute intervals.

Absorbance spectra of heparin and ascorbic acid, alone or together, were measured in 10^{-3}N HCl.

RESULTS

Preliminary tests showed that 10 mg of ascorbic acid overcame the anticoagulant activity of up to 5 units (about 0.05 mg) of heparin. Since excessive amounts of ascorbic acid were also clot-inhibitory, careful titration was required, as shown in Table 1.

Table 1.—Titration of Antiheparin Effect of Ascorbic Acid

Tube*	Heparin (units)	Ascorbic acid (mg)	Clot time (min)
1	0	0	5
2	5	0	None†
3	5	2	None
4	5	4	None
5	5	6	24
6	5	8	9
7	5	10	12
8	5	12	38

*Solutions of heparin (50 μ/ml) and of ascorbic acid (100 mg/ml) were mixed in the clotting tube before 1 ml of normal dog blood was added.
†None in 1 hour.

Table 2.—Effect of Ascorbic Acid Added to 1-ml Heparinized Dog Blood

Tube	Heparin (units)	Ascorbic acid (mg)	Clot time (min)
1	0	0	6
2	5	0	None in 1 hr
3	5	4	20
4	5	6	6
5	5	8	6
6	5	10	9
7	5	12	14

The same result was obtained if the heparin was put into the tube first, the freshly drawn blood added, and then the solution of ascorbic acid (Table 2).

Table 3.—Inhibitory Effect of Ascorbic Acid Added Alone to 1-ml Normal Dog Blood

Tube	Ascorbic acid (mg)	Clot time (min)
1	0	6
2	2	7
3	4	9
4	6	9
5	8	11
6	10	13

Since ascorbic acid is also moderately anticoagulant (Table 3), it was possible to retard clotting by the addition of ascorbic acid to the freshly drawn blood. The inhibitory effect of the ascorbic acid was overcome by appropriate amounts of heparin (Table 4).

Table 4.—Effect of Heparin on Anticoagulant Action of Ascorbic Acid

Tube	Ascorbic acid (mg)	Heparin* (units)	Clot time (min): After heparin added	Clot time (min): Total
1	0	0	...	6
2	10	0	...	14
3	10	1	6	8
4	10	2	6	8
5	10	3	7	9
6	10	4	4	7
7	10	5	6	9
8	10	6	7	10
9	0	5	None‡	None

*The solution of heparin was added to the ascorbic acid-treated blood between 2 and 3 minutes after the blood was drawn and mixed with ascorbic acid.
‡In 1 hour.

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common in Harris' day. This keen observer has left us the following description: "The gums of scorbutic persons have a reddish-brown colour, their margins are imperfectly festooned and thick, their structure rather disposed to become turgid and ever ready in the slightest cause of local irritation to take on a morbid action. When thus excited, the blood accumulates in their vessels when . . . it gives to the gums a dark purple or brown appearance, they swell and become spongy, flabby and bleed from the slightest touch. To these symptoms supervene the exhalation of a fetid odour, the destruction of the bond of union between them and the necks of the teeth, separation and recession of the margins of the same, gradual wasting of the alveolar cavities, loosening and, not infrequently, the loss of several or the whole of the teeth."

Ever since the discovery that vitamin C affects the gingiva, dentists have used large doses of the vitamin to treat bleeding gingival margins, inflamed periodontal tissues and loose teeth. Such treatment seemed logical for, apart from a superficial similarity in the manifestations of scurvy and periodontitis, it was found that lack of vitamin C weakens the walls of small blood vessels and reduces collagen formation with consequent mobility and loss of teeth. This evidence was coupled with the fact that humans, who are highly susceptible to periodontal disease, cannot synthetize vitamin C whereas most animals, who have a low incidence of periodontal disease, can do so.

In 1933 Hanke[10] reported that orange juice and lemon juice were so effective in children that these juices should be included in the treatment of all periodontal disease. Though several others have claimed highly encouraging results from vitamin C therapy, no one has ever demonstrated a relationship between levels of the vitamin and periodontal disease, nor has supplementation of vitamin C proved beneficial.[11-15]

Though the clinical symptoms of periodontal disease bear only a superficial resemblance to scurvy, its prevention by daily ingestion of a few milligrams of vitamin C remains extremely popular. Actually, a daily intake in excess of 10 mg has no preventive or remedial value. In fact, unwarranted prescription of this vitamin lulls many into a false sense of security so that preventive measures and appropriate treatment for periodontal disease are neglected.

Vitamin C's marginal role in periodontal disease has been confirmed in several studies. One of these involved chronically ill mental patients who had a low serum ascorbic acid and a very high incidence of severe gingivitis and bleeding from the gingival crevice. Some of the patients were given an additional 500 mg of ascorbic acid daily for six weeks. Though their plasma ascorbic acid level rose from 0.254 to 0.81 mg per cent, the condition of their gingiva remained unaltered.[16]

In another experiment, Crandon[17] consumed a vitamin C deficient diet for five months without incurring gingival changes. Only after six months did he observe slight changes at the gingival margin.

Linghorne and McIntosh,[18] who examined air force personnel, found that gingival inflammation is common in healthy young adults and that large doses of vitamins, including ascorbic acid, have no clinical effect. Daily doses of ascorbic acid did, however, delay the recurrence of inflammation when gingivitis was treated locally. Their study lasted five months and embraced the effects of vitamin A, vitamin D, thiamine, riboflavine and nicotinic acid as well as vitamin C.

Coven[19] tested the effect of vitamin therapy in 267 children divided into four groups. Each group received prophylaxis plus (1) a placebo, or (2) vitamin C, or (3) vitamin B complex, or (4) multivitamin supplements for 28 days. Only group 4 experienced a statistically significant reduction of the periodontal index. The multivitamin supplement contained vitamin A, vitamin D, thiamine, riboflavine, niacinamide, pyridoxine, cyanocobalamin, calcium pantothenate, biotin and ascorbic acid.

Apart from fluoride, there is little evidence that trace elements influence periodontal disease. According to Russell,[20] there is less periodontal disease in areas where the drinking water contains an optimum amount of fluoride, and Englander et al[21] suggest that fluoridated water retards the progress of destructive periodontal disease.

In 1962 Russell[13] surveyed over 12,000 individuals in five countries and ascertained body levels of vitamin A, ascorbic acid, thiamine, riboflavine and methyl nicotinamide. He also measured dietary intakes of calcium, riboflavine, thiamine, ascorbic acid, total calories and protein. There was no correlation between periodontal health and group levels of vitamin A or ascorbic acid or in the urine excretion of these substances. Russell therefore concluded that nutritional disturbances are not involved in the aetiology of periodontal disease in the well developed countries. Four years later he modified this view by suggesting "that adequate nutrition might be a beneficial factor. This may be true despite our inability to demonstrate any consistent relation between disease levels and inadequate intakes of body fluid concentrations of such items as vitamin A or ascorbic acid, thiamine, riboflavine, niacin, calcium, protein, iron or total calories . . . findings establish a high probability that periodontal disease is not a manifestation of a specific nutritional deficiency (similar to beriberi or pellagra)."[22]

However, says Russell, the possibility remains that non-specific depletion may hasten the process of deterioration. Periodontal deterioration is largely prevented by effective personal and professional oral hygiene and when teeth are kept free of calculus.

Variations in the incidence of periodontal disease among different populations suggest that the disease is susceptible to control. However, nutrition does not seem to play a significant role in this regard.

Waerhaug[23] also tested the relationship between periodontal conditions and clinical signs of vitamin A and B deficiencies in various parts of the world but found no correlation between nutritional levels and the status of periodontal health. He concluded that vitamin deficiency is not an important aetiological factor in developing nations; and in more advanced countries, vitamin deficiencies have no public health importance in periodontal disease.

In the United States and in Canada, therefore, periodontal disease is not associated with nutritional deficiencies or dietary inadequacy. In other words, nutritional deficiency is not a cause of periodontal disease on the North American continent. Consequently, nutritional supplementation cannot logically be used for the prevention or treatment of commonly encountered periodontal disease.

Summary

Though systemic factors may participate, local irritants are largely responsible for the production of chronic periodontal disease. Nutritional deficiency is not an aetiological factor in periodontal disease encountered on the North American continent. Its prevention or treatment by means of nutritional supplements like vitamin C is therefore unwarranted unless a specific deficiency of the nutrient can be demonstrated.

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The Significance of the Evidence about Ascorbic Acid and the Common Cold

(vitamin C/double-blind studies/statistical significance)

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Contributed by Linus Pauling, August 9, 1971

ABSTRACT **Only four independent double-blind studies have been reported of the effect of ascorbic acid regularly ingested in daily amounts more than 100 mg, in comparison with a placebo, in decreasing the incidence and integrated morbidity of the common cold for subjects exposed to cold viruses in the ordinary way and without colds when the test period began. A statistical analysis of these four studies leads to rejection of the null hypothesis that ascorbic acid has no more protective power than the placebo at the 99.86% level of confidence for the incidence of colds and the 99.9978% level of confidence for the integrated morbidity.**

For many years there has existed the popular belief that ascorbic acid has value in providing protection against the common cold and in ameliorating the manifestations of this viral disease. This popular belief has, however, not been generally shared by physicians, authorities on nutrition, and official bodies.

I was puzzled by the contraction between the popular belief and the official opinion, so I studied the published reports of controlled trials of ascorbic acid in relation to the common cold. On the basis of this study and of some general arguments about orthomolecular medicine (1) (the preservation of good health and the treatment of disease by varying the concentrations in the human body of substances that are normally present in the body and are required for health) and the process of molecular evolution (2), I reached the conclusion that ascorbic acid, taken in the proper amounts, decreases the incidence of colds and related infections, and also decreases the severity of individual colds. These arguments were presented in my book "Vitamin C and the Common Cold" (3). The evidence and arguments presented in this book were not convincing to some physicians and authorities on nutrition. Many statements contradicting my conclusions were made. Although my analysis of the published accounts of controlled studies in this field seems to me to be clear and straightforward, I have decided that, because of the importance of the question, it is desirable to publish a more detailed account of the evidence, including a more thorough statistical analysis of the controlled trials that have been performed. No statistical analysis of the body of evidence has previously been published.

THE NATURE OF THE STATISTICAL ANALYSIS

Each of the reports discussed in this paper describes a study of two groups of subjects selected at random from one population. The subjects in one group were administered the active substance (L-ascorbic acid, vitamin C) in certain amounts once or more every day, and those in the second group were administered an apparently identical inactive material, a placebo. The studies were double-blind, with neither the subjects nor the investigators knowing which subjects received the ascorbic acid and which received the placebo, that knowledge being kept by some other person until all of the information had been collected.

The question that I attempt to answer by analyzing the published reports is the following: Does the regular administration of ascorbic acid at a rate greater than 100 mg per day over a period of time beginning before the subjects have contracted a cold, and with the subjects exposed to cold viruses under ordinary living conditions, have an effect different from that of a placebo in decreasing the incidence and the severity of the common cold? A comparison with a placebo, with the subjects not knowing which group they are in, is essential because of the well-known "placebo effect" of even inactive medications.

The statistical methods used in the analysis are the conventional ones, for the most part Student's t-test or the calculation of χ^2 and then of the probability P(one-tailed) that the observed difference in effect of ascorbic acid and placebo (or a larger difference) would be obtained by chance alone in two groups taken at random from a uniform population if the null hypothesis of equal effectiveness of ascorbic acid and placebo were true. I have chosen to give P(one-tailed) rather than P(two-tailed) because no one contends that the placebo (usually citric acid) has a greater effect than ascorbic acid in preventing or ameliorating the common cold; the difference of opinion is between those people who state that ascorbic acid is no better than a placebo and those who say that it is better. Moreover, in none of the studies discussed did the investigators find a greater protective effect of the placebo than of ascorbic acid; in every study ascorbic acid is reported to provide greater protection than the placebo against the common cold, and the question to be answered is the level of confidence with which the reported results can be accepted and the null hypothesis of equal effectiveness of placebo and ascorbic acid can be rejected.

The following analysis includes every published double-blind study, whether statistically significant or not, of the comparative effects on the common cold of ascorbic acid and a placebo for two groups of subjects selected by some random process from a single population exposed to cold viruses in the ordinary way (contact with other people), with the ascorbic acid, in amounts over 100 mg per day, and placebo administered regularly over a period of time, beginning before colds were incurred. The common cold is considered to be the disease

or complex of diseases described by the investigators themselves as the common cold. Observations for other diseases are not included.

The results of the studies are usually reported in one or more of three aspects: first, the incidence of colds (number of colds per person in unit time, usually taken as the period of the study); second, the average severity of individual colds (as measured by days of illness per cold or number of days when symptoms were recorded); and third, the integrated morbidity (the product of the other two). In the following discussion I have assigned to each investigation a value of P for the incidence and a value for the integrated morbidity, based as reliably as possible on the reported observations, and then have combined the values for all of the published studies satisfying the criteria given above to obtain two values for the level of confidence at which the null hypothesis of equal effect of ascorbic acid and placebo is to be rejected. These two values, for incidence and for integrated morbidity, are of course not independent of one another.

THE WORK OF RITZEL

A study of the effect of 1 g of ascorbic acid per day was reported by G. Ritzel, a physician with the medical service of the School District of the City of Basel, Switzerland (4). The study was carried out in a ski resort with 279 skiers during two periods of 5–7 days. The conditions were such that the incidence of colds during these short periods was large enough (about 20%) to permit results with statistical significance to be obtained. The subjects were roughly of the same age and had similar nutrition during the period of study. The investigation was double-blind, with neither the participants nor the physicians having any knowledge about the distribution of the ascorbic-acid tablets and the placebo tablets. The tablets were distributed every morning and taken by the subjects under observation, so that the possibility of interchange of tablets was eliminated. The subjects were examined daily for symptoms of colds and other infections. The records were largely on the basis of subjective symptoms, partially supported by objective observations (measurement of body temperature, inspection of the respiratory organs, auscultation of the lungs, and so on). Persons who showed cold symptoms on the first day were excluded from the investigation.

After the completion of the investigation, a completely independent group of professional people was provided with the identification numbers for the ascorbic-acid tablets and placebo tablets, and this group performed the statistical evaluation of the observations.

The number of colds was 31 for the placebo group of 140 subjects and 17 for the ascorbic-acid group of 139 subjects. [The number of colds was not given explicitly in the paper. However, the number of days of illness for each of the two groups was given (80, 31), and the average number of days of illness per cold (2.6, 1.8). The only integral values for the number of colds allowed by these numbers are 31 for the placebo group and 17 for the ascorbic-acid group.] The incidence of colds is accordingly 0.221 per person for the placebo group and 0.122 for the ascorbic-acid group, a decrease of 45% for the ascorbic-acid group. The value of χ^2 is found to be 4.81, with P(one-tailed) < 0.015. This investigation accordingly shows with statistical significance that the null hypothesis that ascorbic acid has only the same effect as the placebo in decreasing the incidence of colds is to be rejected.

The group receiving ascorbic acid showed only 39% as many days of illness, per person, as the group receiving the placebo, and the number of individual symptoms per person was only 35% as great for the ascorbic-acid group as for the placebo group. The investigator states that the statistical evaluation of these differences by two-by-two tables gives a significant difference, $0.001 < P$(two-tailed) < 0.01. Each of these observations is a measure of the integrated morbidity. Information (values of standard deviation) permitting independent calculation of P is not given in the paper. I accept the value P(one-tailed) < 0.005 for the level of rejection of the null hypothesis with respect to the integrated morbidity.

THE WORK OF COWAN, DIEHL, AND BAKER

Another careful study of ascorbic acid and the common cold was reported by Cowan, Diehl, and Baker in 1942(5). The principal work was done during the winter "cold season" of 1939–1940. The subjects were all students in the University of Minnesota, who volunteered to participate in this study because they were particularly susceptible to colds. Persons whose difficulties seemed to be due primarily to chronic sinusitis or allergic rhinitis, as shown by examination of the nose and throat and consideration of symptoms of allergy, were excluded from the study. The subjects were assigned alternately and without selection to an experimental group and a control group. The subjects in the control group were treated exactly like those in the experimental group, except that they received a placebo instead of the ascorbic acid. The subjects were instructed to report to the Health Service whenever a cold developed, so that special report cards could be filled in by a physician. Dr. Cowan has informed me that the study was a double-blind one, with neither the subjects nor the physicians knowing which group a subject was in. Each subject was interviewed every 3 months in order to check the completeness of the reports.

The study was continued for 28 weeks. Of the 233 students initially in the ascorbic acid group, 183 received 200 mg per day throughout the period of 28 weeks, and 50 received 200 mg per day for 2 weeks, followed by 100 mg per day, except on inception of a cold, when an additional 400 mg per day for 2 days was administered. This group numbered 208 subjects at the completion of the study, 25 having dropped out. If the composition of the group remained unchanged, the average intake of ascorbic acid was 180 mg per day. The students in the control group initially numbered 194, of whom 155 completed the study. The reported average number of colds per person during the period of study was 2.2 ± 0.08 (SD 0.12) for the control group, and 1.9 ± 0.07 (SD 0.10) for the ascorbic acid group. The difference between the average number of colds in the control group and in the experimental group is given by the authors as one-third of a cold and also as 0.3 ± 0.11 (SD 0.16).

The authors state in their paper that "The actual difference between the two groups during the year of the study amounts to one-third of a cold per person. Statistical analysis of the data reveals that a difference as large as this would arise only three or four times in a hundred through chance alone. One may therefore consider this as probably a significant difference, and vitamin C supplement to the diet may therefore be judged to give a slight advantage in reducing the number of colds experienced."

Because the authors rounded off the numbers giving the actual numbers of colds per person, the difference is not

Table 1. *The weight of evidence for rejecting the null hypothesis of equal effectiveness of ascorbic acid and placebo*

Investigators	Daily intake of ascorbic acid, (mg)	Value of P(one-tailed) at which the null hypothesis is rejected: Incidence	Integrated morbidity
Ritzel	1000	0.015	0.005
Cowan, Diehl, and Baker	180	0.02	0.02
Wilson and Loh	200	0.025	0.025
Franz, Sands, and Heyl	205	0.44	0.008
χ^2 (8 df)		25.24	35.46
Combined P		0.0014	0.000022

known exactly. It is probably 0.33 or 0.34, which correspond (Student's t-test) to the values 0.04 or 0.03 for P(two-tailed), in agreement with the statement by the authors. This difference represents a decrease by 15% in the incidence of colds in the ascorbic-acid group as compared with the control group.

I accept the value P(one-tailed) < 0.02 for rejection of the null hypothesis with respect to the incidence of colds.

The average number of days lost from school per person in the placebo group was reported as 1.6, and in the ascorbic-acid group as 1.1, giving a decrease of 31% in integrated morbidity. Values of the standard deviation are not given in the paper. I accept as a conservative estimate of the level of rejection of the null hypothesis with respect to the integrated morbidity the same value, P(one-tailed) < 0.02, as with respect to incidence.

THE WORK OF WILSON AND LOH

Wilson and Loh (Department of Pharmacology, University of Dublin) have published a report of a double-blind study of subjects in a girls' boarding school during a winter period of 6 months (6). Of the 108 subjects, 57 received ascorbic acid (200 mg per day) and 46 received placebo tablets. The investigators state that ascorbic acid significantly reduced the incidence, duration, and severity of symptoms of colds, in comparison with the placebo. They do not give information permitting an independent statistical analysis to be carried out. I assume that their statement about significance has the usual meaning, P(two-tailed) < 0.05, and I accordingly assign the corresponding value $P < 0.025$ for rejection of the null hypothesis with respect to both incidence and integrated morbidity.

THE WORK OF FRANZ, SANDS, AND HEYL

A double-blind study of ascorbic acid and the common cold was carried out by Franz, Sands, and Heyl of Dartmouth Medical School during the 3-month period from February to May 1956, with 89 volunteer medical students and students and student nurses as subjects (7). The subjects were divided in a random way into four groups, three of 22 subjects and one of 23 subjects. One group received tablets containing ascorbic acid, the second ascorbic acid and a bioflavonoid (naringin), the third a placebo, and the fourth naringin only. The daily amount of ascorbic acid was 205 mg and that of the bioflavonoid was 1000 mg. Symptoms of colds were systematically recorded. The results for the bioflavonoid groups, with or without ascorbic acid, were the same as for the corresponding groups without bioflavonoid. The authors concluded that the administration of a bioflavonoid had effect neither on the incidence or the cure of colds nor on the ascorbic-acid concentration in the blood.

The incidence of colds in the ascorbic-acid group (with or without bioflavonoid) was 4.6% less than for the control group (placebo or bioflavonoid only), 14/44 versus 15/45, which corresponds to rejection of the null hypothesis at the level P(one-tailed) < 0.44. The only other results reported by the investigators is the incidence of serious colds (not cured or improved in 5 days), which I take as a measure of the integrated morbidity. This incidence was 1/44 for the ascorbic-acid group and 8/45 for the control group, significantly different ($\chi^2 = 5.88$, P(one-tailed) < 0.008). The authors state that this difference is statistically significant (0.05 level).

THE TOTAL EVIDENCE FOR THE EFFECTIVENESS OF ASCORBIC ACID

Each of these four double-blind studies of ascorbic acid has given the statistically significant result that the hypothesis that ascorbic acid administered in amounts over 100 mg per day to subjects who have not yet caught cold and are subjected to ordinary conditions of exposure to cold viruses show the same incidence and integrated morbidity of colds and related infections as placebo subjects is to be rejected, with one exception—the observation of Franz, Sands, and Heyl on incidence (Table 1). I now ask what the weight of the total body of evidence is.

There is no doubt that the four studies, carried out by different investigators in different places and at different times, are independent of one another. Moreover, these four studies constitute the entire set of published double-blind comparisons of ascorbic acid (more than 100 mg per day) and placebo administered to subjects who have not yet caught cold and are subjected to exposure to cold viruses in the ordinary way. We may, accordingly, combine the four values of P to obtain a measure of the significance of all of the evidence in rejecting the null hypothesis. Fisher's method (Table 1) leads to χ^2 (8 degrees of freedom) $= -2 \Sigma \ln P_i = 25.24$, $P = 0.0014$ for incidence and 35.46, $P = 0.000022$ for integrated morbidity. I conclude that the evidence about incidence leads to rejection of the null hypothesis of equal effect of ascorbic acid and placebo at the 99.86% level of confidence and the evidence about integrated morbidity leads to rejection of the null hypothesis at the 99.9978% level of confidence.

THE AMOUNT OF PROTECTION

To within the experimental error, the effect of ascorbic acid, relative to that of the placebo, can be represented by exponential expressions: incidence of colds $= 1 - \exp(-0.60a)$; severity of individual colds $= 1 - \exp(-0.29a)$; integrated morbidity $= 1 - \exp(-0.99a)$, with a the intake of ascorbic acid in grams per day. The coefficients are fitted to the values reported by Ritzel. The equation gives integrated morbidity (relative to placebo) of 61% for an intake of 500 mg per day, 37% for 1 g per day, and 14% for 2 g per day. The values are, of course, expected to depend somewhat on the nature of the population and environment.

OTHER STUDIES

Dahlberg, Engel, and Rydin (8) did a double-blind study with an average daily intake of about 90 mg of ascorbic acid. They observed a decreased incidence of the common cold by 4%, not statistically significant. (Decrease in incidence of 6% is given

by the exponential expression.) Cowan, Diehl, and Baker (5) also performed a second double-blind study, with a daily intake of 50 mg for one group and 25 mg for another, and observed no difference in incidence (to ±2%) from the placebo group. (Decreases in incidence of 3% and 1.5%, respectively, are given by the exponential expression.) These are the only double-blind studies that have been done with small daily amounts of ascorbic acid. They were not designed in such a way as to provide results with statistical significance, which would have required about three times as many subjects as were used.

THE WORK OF WALKER, BYNOE, AND TYRRELL

The study by Walker, Bynoe, and Tyrrell (9) is often mentioned as having shown that ascorbic acid has no protective value against the common cold. Of the 91 subjects, 47 received 3 g of ascorbic acid per day for 3 days before inoculation with viruses (a mixture of rhinoviruses, influenza B virus, or BS14 virus) and for 6 days after inoculation, and 44 subjects received a placebo. The incidence of colds was only 6% less for the ascorbic-acid group (18/47) than for the placebo group (18/44); this difference is not statistically significant. The investigators concluded that there is no evidence that the administration of ascorbic acid has any value in the prevention or treatment of colds produced by five known viruses. In fact, their study rejects with statistical significance ($P < 0.05$) a protective effect greater than 40%, but not a smaller effect, for subjects inoculated with viruses in the way used. The results of this study suggest that inoculation with viruses is not equivalent to ordinary exposure to viruses.

CONCLUSION

An analysis has been made of all published results of double-blind controlled studies of the effect of ascorbic acid in daily amounts over 100 mg on the incidence, severity, and integrated morbidity of the common cold in populations that receive the ascorbic acid (or a placebo) regularly, beginning before colds have been incurred, and with the subjects exposed to cold viruses in the ordinary way (contact with other people). The observations reject with high statistical significance the null hypothesis that under these conditions ascorbic acid has the same effect as a placebo. Ascorbic acid in a daily amount of 1000 mg is reported to decrease the incidence of colds by about 45% and the integrated morbidity by about 63%. These values are used in formulating exponential expressions for the amount of protection as a function of the intake of ascorbic acid. No controlled study under these conditions has given results rejecting with statistical significance the hypothesis that this amount of protective effect occurs. A smaller protective effect (not statistically significant) is reported when the colds are induced by inoculation with a virus suspension.

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Vitamin C and the Common Cold

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CHAPTER 4

The Properties of Ascorbic Acid

Ascorbic acid is an essential food for human beings. As noted earlier, an intake of about 10 mg per day is enough to provide protection against scurvy for most people, but to achieve the best health a much larger intake is probably needed.

The optimum intake of ascorbic acid—that is, the daily amount of this food that leads to the best of health—is not known, and no doubt it varies from person to person. It is my opinion that for most people the optimum daily intake is somewhere between 250 mg and 10 g.

These amounts are much larger than the daily dietary allowance recommended in 1968 by the Food and Nutrition Board of the National Research Council. The recommendation of this

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Board, said to be designed for the maintenance of good nutrition of practically all healthy people in the United States, is 35 mg per day for infants, 40 mg per day for children, increasing to 55 mg per day for women and 60 mg per day for men. In making its recommendation the Board stated that the minimal daily intake of ascorbic acid needed to prevent scurvy is about 10 mg, and that the somewhat larger amounts recommended should provide a generous increment for individual variability and a surplus to compensate for potential losses in food. The idea that beneficial effects would result from a larger intake of ascorbic acid was rejected, on the basis of reports that improvement in physical and psychomotor performances of men had not been improved by supplements of between 70 mg and 300 mg of ascorbic acid per day, and that the occurrence of bleeding gums in military personnel was not affected by supplements of 100 mg or 200 mg per day for periods of three weeks. There are, however, many published reports about beneficial effects of ascorbic acid ingested in larger amounts. Some of these reports are discussed later in this chapter and, with respect to the common cold and other infections, in the following chapter and Appendix III.

Ascorbic acid is not a dangerous substance. It is described in the medical literature as "virtually nontoxic." Guinea pigs that were given one half of one percent of their body weight of ascorbic acid per day for a period of days showed no symptoms of toxicity. Human beings are reported to have eaten 40 g per day for a month (Stone, 1967) and as much as 100 g per day for a few days (Herjanic and Moss-Herjanic, 1967) with no symptoms of toxicity. A large amount (several grams) of ascorbic acid eaten without other food may cause an upset stomach and diarrhea in some people (hence the recommendation in Chapter 10 that it usually be taken at the end of a meal), but more serious side effects have not been reported.

Ascorbic acid may be described as no more toxic than ordinary sugar (sucrose), and far less toxic than ordinary salt (sodium chloride). There is no reported case of the death of any person from eating too much ascorbic acid, nor, indeed, of serious illness from this cause.

Ascorbic acid is a white, crystalline powder, with large solubility in water. Its solution has an acidic taste, resembling that of orange juice.

Ordinary ascorbic acid is also called L-ascorbic acid. There is another substance, D-ascorbic acid, that is closely related to L-ascorbic acid; the two substances contain the same atoms, bonded together in essentially the same way, but with a spatial relationship corresponding to reflection in a mirror. The letters D and L indicate righthanded (dextro) and lefthanded (levo). Only L-ascorbic acid has vitamin-C activity. The name ascorbic acid without a prefix is used only in referring to L-ascorbic acid, vitamin C itself.

Ascorbic acid is found in many foodstuffs. Large amounts, 100 mg to 350 mg per 100 g (that is, between 0.10 and 0.35 percent of the weight of the food) are contained in green peppers, red peppers, parsley, and turnip greens. Orange juice, lemon juice, lime juice, grapefruit juice, tomato juice, mustard greens, spinach, and brussels sprouts contain a good quantity of ascorbic acid, from 25 mg to 100 mg per 100 g. Green peas and green beans, sweet corn, asparagus, pineapple, tomatoes, gooseberries, cranberries, cucumbers, and lettuce contain from 10 mg to 25 mg per 100 g. Somewhat smaller amounts, less than 10 mg per 100 g, are found in eggs, milk, carrots, beets, and cooked meat.

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The ascorbic acid in foodstuffs is easily destroyed by cooking at high temperatures, especially in the presence of copper, iron, and other metals. Cooked foods usually retain only about half of the ascorbic acid present in the raw foods. The loss of the vitamin can be kept to a minimum by cooking for a short period of time, with a minimum amount of water, and with the water not discarded, because it has extracted some of the vitamin from the food.

A good ordinary diet, including green vegetables and orange or tomato juice, may provide 100 mg to 300 mg of ascorbic acid per day. To obtain larger amounts of this important food the pure substance, crystalline ascorbic acid, may be ingested.

Pure ascorbic acid (Ascorbic Acid U.S.P., L-Ascorbic Acid, Vitamin C) is available in some drug stores and food stores as a powder, fine crystals, or coarse crystals, and also, with a binder or filler added, as tablets. This ascorbic acid is sometimes described as synthetic ascorbic acid. It is identical with the ascorbic acid present in natural foodstuffs, and it is, in fact, usually made from a natural sugar by a process involving two chemical reactions. The usual starting material is dextrose, which is also called glucose, grape sugar, honey sugar, corn sugar, or starch sugar. It is present in honey and other natural foods. Its chemical formula is $C_6H_{12}O_6$. It is converted into L-ascorbic acid, $C_6H_8O_6$, by oxidation reactions which remove four hydrogen atoms to form two molecules of water.

Many animals are able to manufacture their own ascorbic acid; they do not require ascorbic acid (vitamin C) as an essential food, and they never suffer from scurvy. These animals manufacture the ascorbic acid in their bodies (in the liver or the kidney) from dextrose by essentially the same reactions that are used to make ascorbic acid in the laboratory and on a commercial scale.

Ascorbic acid in the human body and in other animals seems to have many functions. These functions have been studied in the guinea pig and the monkey, both of which, like human beings, require ascorbic acid in their food, as a vitamin. It has been found that an insufficient supply of this essential food causes the animal to show symptoms of scurvy, including intramuscular and subcutaneous hemorrhages, tenderness of joints, a general weakening of connective tissue (skin, tendons, walls of blood vessels), lethargy, loss of appetite, and anemia. Ascorbic acid is needed for the healing of wounds, including burns. With a low intake of ascorbic acid wounds heal only slowly and the scar tissue is weak, so that the wounds break open again easily. Increase in the intake of ascorbic acid leads to rapid healing and to the formation of strong scar tissue (see Figure 1, on the next page).

It has been found that a large intake of ascorbic acid increases the capacity of resistance of the guinea pig, the rat, and the monkey to a cold environment (Dugal, 1961). In human beings, a fall in the concentration of ascorbic acid in the blood has been observed to follow exposure to the stress of surgery, accidental wounds, and burns, indicating the need for a larger supply of the vitamin. Ascorbic acid in increased amounts has been used in the treatment of burns, injuries, infections, rheumatic disease, and allergies (Holmes, 1946; Yandell, 1951).

In 1964 Dr. James Greenwood, Jr., clinical professor of neurosurgery in Baylor University College of Medicine, reported his observations on the effect of an increased intake of ascorbic acid in preserving the integrity of intervertebral discs and preventing back trouble. He recommended the use of 500 mg per day with an increase to 1000 mg per day if there were any discomfort or if work or strenuous exercise were anticipated. He said that evidence from most patients

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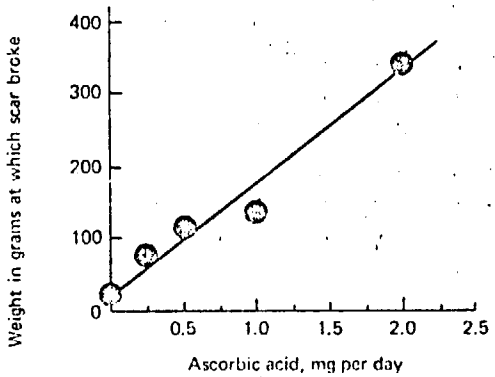

FIGURE 1
Strength of scar tissue in guinea-pig skin in dependence on the amount of ascorbic acid in the diet (0, 0.25, 0.5, 1, and 2 mg per day). The scars had been formed during a 7-day period after the cuts, 1/4-inch long, had been made. It is seen that the scar tissue is four times as strong for an intake of 2 mg per day as for 0.25 mg per day (Bourne, 1946). Similar results for human beings have been reported by Wolfer, Farmer, Carroll, and Manshardt (1947)

indicated that muscular soreness experienced with exercise had been greatly reduced by these doses of ascorbic acid, but increased again when the vitamin was not taken. He concluded, from observation of over 500 cases, that "it can be stated with reasonable assurance that a significant percentage of patients with early disc lesions were able to avoid surgery by the use of large doses of vitamin C. Many of these patients after a few months or years stopped their vitamin C and symptoms recurred. When they were placed back on the vitamin the symptoms disappeared. Some, of course, eventually came to surgery."

It has also been reported that the cancers that often appear in the bladders of cigar smokers and other users of tobacco

regress if the patient ingests a sufficient amount of ascorbic acid, 1 g per day or more. Schlegel, Pipkin, Nishimura, and Schultz (1970) found the ascorbic-acid level of the urine to be about half as great for smokers as for nonsmokers, and to be low for patients with bladder tumors. They also found with mice that implantation in the bladder of a pellet containing 3-hydroxyanthranilic acid (a derivative of the amino acid tryptophan) caused bladder tumors to develop if the mice were receiving a normal diet, but not if they had extra ascorbic acid in their drinking water. The authors suggest that the ascorbic acid prevents the oxidation of 3-hydroxyanthranilic acid to a cancerogenic oxidation product. They state that "there seems to be reason to consider the beneficial effects of an adequate ascorbic acid level in the urine (corresponding to a rate of intake of 1.5 g per day) as a possible preventive measure in regard to bladder tumor formation and recurrence." They also call attention to investigations indicating that ascorbic acid may have a beneficial effect on the aging process of atherosclerosis, the hardening and thickening of the walls of the arteries (Willis and Fishman, 1955; Sokoloff and others, 1966).

Patients with various infectious diseases have been reported to benefit from treatment with ascorbic acid. Baetgen in 1961 reported the successful treatment of epidemic hepatitis in 245 children with use of 10 g of ascorbic acid per day. Several investigators, most recently Hindson (1968), have reported that prickly heat is cured by the ingestion of 0.5 to 1 g per day.

An interesting investigation of the relation between intelligence, as indicated by the results of standard mental ability tests, and the concentration of ascorbic acid in the blood plasma has been reported by Kubala and Katz (1960). The subjects were 351 students in four schools (kindergarten to college) in three cities. They were initially divided into the higher-

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ascorbic-acid group (with more than 1.10 mg of ascorbic acid per 100 ml of blood plasma) and the lower-ascorbic-acid group (less than 1.10 mg per 100 ml) on the basis of analysis of blood samples. By matching pairs on a socio-economic basis (family income, education of father and mother), 72 subjects in the higher-ascorbic-acid group and 72 in the lower-ascorbic-acid group were selected. It was found that the average intelligence quotient of the higher-ascorbic-acid group was greater than that of the lower-ascorbic-acid group in each of the four schools; for all 72 pairs of subjects the average IQ values were 113.22 and 108.71, respectively, with an average difference 4.51. The probability that a difference this great would be found in a similar test on a uniform population is less than 5 percent; hence the observed difference in average IQ of the two groups is statistically significant.

The subjects in both groups were then given supplementary orange juice during a period of six months, and the tests were repeated. The average intelligence quotient for those in the initially higher-ascorbic-acid group had increased very little (by only 0.02), whereas that for the lower group had increased by 3.54 IQ units. This difference in increase is also statistically significant (probability in a uniform population less than 5 percent.

The study was continued through a second school year with 32 pairs (64 subjects), with similar results. The relation between the average intelligence quotient and the average blood-plasma ascorbic-acid concentration for these 64 subjects tested four times during a period of 18 months is shown in Figure 2. These results indicate that the intelligence quotient is raised by 3.6 IQ units when the blood-plasma ascorbic-acid concentration is increased by 50 percent (from 1.03 to 1.55 mg per 100 ml). This increase would for many people result from

increasing the intake of ascorbic acid for an adult by 50 mg per day (from 100 mg to 150 mg per day).

Kubala and Katz conclude that some of the variance in intelligence-test performance is determined by the "temporary nutritional state of the individual, at least with regards to citrus or other products providing ascorbic acid." They suggest that "alertness" or "sharpness" is diminished by a decreased intake of ascorbic acid.

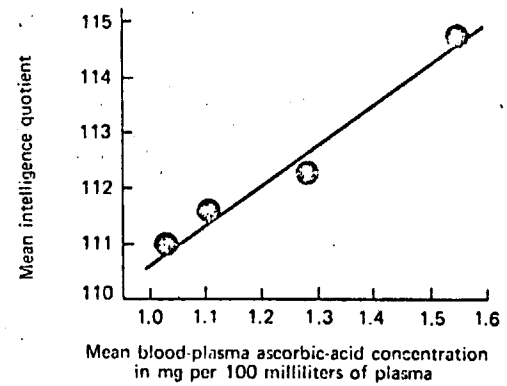

FIGURE 2
Relation between mean intelligence quotient (average IQ) and mean concentration of ascorbic acid in the blood plasma for 64 school children. Four tests were made of each child, over a period of 18 months. The plasma ascorbic-acid concentration was changed by giving all the subjects extra orange juice during certain months. (Redrawn from Figure 1 of Kubala and Katz, 1960).

There is no indication in Figure 2 that maximum mental ability has been reached at the value 1.55 mg of ascorbic acid per 100 ml of blood plasma. This concentration corresponds for a 70-kg (154-pound) adult to the daily ingestion of 180 mg of ascorbic acid. I conclude that for maximum mental per-

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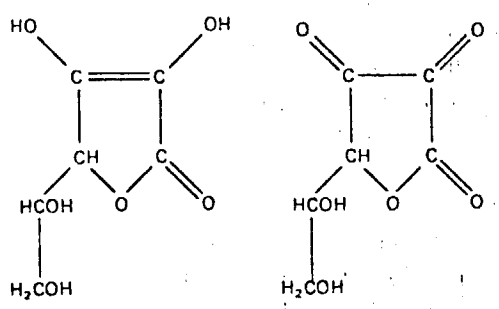
formance the daily allowance of ascorbic acid should be at least three times the 60 mg recommended by the Food and Nutrition Board of the U.S. National Research Council, and at least nine times the 20 mg recommended by the corresponding British authority.

The ways in which ascorbic acid functions in the human body are not well understood. It is a strong reducing agent, and is readily converted into dehydroascorbic acid, $C_6H_6O_6$, by oxidizing agents:



Ascorbic acid Dehydroascorbic acid

This reaction is reversible (dehydroascorbic acid is easily reduced to ascorbic acid), and it is likely that the reducing power of ascorbic acid and the oxidizing power of dehydroascorbic acid are responsible for some of the physiological properties of the substance.

Many of the results of deprivation of ascorbic acid, mentioned above, involve a deficiency in connective tissue. Connective tissue is largely responsible for the strength of bones, teeth, skin, tendons, blood-vessel walls, and other parts of the body. It consists mainly of the fibrous protein collagen. There is no doubt that ascorbic acid is required for the synthesis of collagen

in the bodies of human beings and other animals. Collagen differs from other fibrous proteins in having a rather large content of the amino acid hydroxyproline. There is evidence that ascorbic acid is required for the conversion of proline into hydroxyproline. This is an oxidation reaction, and there is evidence also that ascorbic acid is involved in some other oxidation reactions in the tissues.

In his book *The Advancing Front of Medicine* (1941) an able science writer, George W. Gray, who was associated with the Rockefeller Foundation, wrote the following statement about ascorbic acid:

> Recent studies show that vitamin C is essential to the formation of the colloidal substance which serves as a pliable cement to bind tissue cells together. In healthy tissue, this binding material shows under the microscope as a clear jelly streaked with darker bands of firmer texture, like the reinforcing strips of steel in concrete. But in the absence of sufficient vitamin C, the bands do not form, the intercellular substance becomes more liquid, less binding, and the cells show a tendency to separate. The hemorrhages which accompany scurvy are consequences of this weakness in the intercellular substance. The cells forming the walls of small blood vessels separate, and through the gaps the blood leaks out. Microscopic studies show that as soon as vitamin C is administered to a scurvy patient, the bands reappear in the intercellular substance, and the separated cells once more join into continuous tissue.

Part of the mechanism of protection against disease is the destruction of bacteria by certain white cells in the blood, the phagocytes. In order to be effective in this way, the phagocytes must have a certain concentration of ascorbic acid in them. This fact provides a partial explanation of the effectiveness of ascorbic acid in providing protection against bacterial infec-

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tions. The mechanism of its effectiveness against viral infections, such as the common cold, is not yet known. I have, however, formulated the hypothesis (which has not yet been tested by experiment) that the effectiveness of ascorbic acid in providing protection against virus diseases results from its function in the synthesis and activity of interferon in preventing the entry of virus particles into the cells. The discovery of interferon was reported in 1957 by Isaacs and Lindenmann. It is a protein that is produced by cells infected by a virus and that has the property of spreading to neighboring cells and changing them in such a way as to enable them to resist infection. In this way the interferon ameliorates the disease. I am sure that it will not be long before this hypothesis about the way in which ascorbic acid provides protection against virus diseases will be verified by experiment, or disproved by discovery of the actual mechanism. In the meantime we may make use of ascorbic acid for improving health in the ways indicated by experience, even though a detailed understanding of the mechanisms of its action has not yet been obtained.

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Vitamin C Status of Cigarette Smokers and Nonsmokers[1, 2, 3]

OMER PELLETIER[4]

CIGARETTE SMOKERS have been reported to be more prone to a variety of diseases (1–3), several of which are associated with lower vitamin C levels (4–8).

Vitamin C is absolutely necessary for the synthesis of our most abundant protein, collagen (9), and faulty collagen biosynthesis arising from vitamin C deficiency will eventually be manifested as scurvy. Although a minimal intake of 10 mg vitamin C/day (10) has been reported to prevent scurvy, this is not necessarily adequate for maintenance of good health and for optimal regulation of all the biochemical functions of vitamin C.

Vitamin C is required in the brain for the synthesis of neurotransmitters such as norepinephrine (11) and serotonin (12), and a role for the vitamin in the adrenal glands, which produce catecholamines and steroids, is demonstrated by the enlargement of adrenals deficient in vitamin C (13). People on low vitamin C intakes cannot utilize tyrosine normally (13); vitamin C deprivation in guinea pigs has marked effects on DNAse I and II (14, 15); and the utilization of nutrients such as iron and other vitamins may be improved by vitamin C (13).

Interest in the vitamin C status of cigarette smokers has given rise to several studies (16). Some reports of lower vitamin C levels in cigarette smokers were not convincing because the specificity of the analytical methods was doubtful; samples were taken without control of recent intakes and vitamin C levels were not related to previous intakes.

In a preliminary study, we used a very specific analytical method (16, 17) and avoided nutritional interference by control of diet. The results supported indications of lower vitamin C levels in smokers (16). Results published simultaneously by Brook and Grimshaw (18) concurred.

The present study involved 14 smokers and 14 nonsmokers having similar characteristics and dietary habits. The previous vitamin C intakes were related to blood levels and to excretions of a test dose. Strict dietary restrictions were used during the experiments and the excretions of 58- to 95-mg doses were measured.

MATERIALS AND METHODS

Twenty-eight persons from the Food and Drug Directorate in Ottawa participated in the study done in November 1968. The restrictions consisted of avoiding fruits, juices, fruit drinks, and instant breakfasts. Controlled lunches and dinners consisted of TV dinners plus bread and cakes. Other restrictions included alcoholic beverages, aspirin, and laxatives. Each person kept a complete record of all foods and drinks consumed during the experiment.

The subjects ate a TV dinner on the evening before the blood samples were taken at 8:30 AM

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[2] This work is part of a thesis in partial fulfillment of the requirements for the Ph.D. Degree of O. Pelletier, under the direction of Dr. C. Godin, Department of Biology.

[3] Presented in part at the 53rd annual meeting of the Federation of American Societies for Experimental Biology, April 13–18, 1969, Atlantic City, N.J.

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before breakfast (*day 0*). Vitamin C was determined on the day of sampling (16, 17).

On the next day (*day 1*) at 8:30 AM the subjects were given 1.1 g vitamin C and a fairly high dosage of water-soluble vitamins. They were told to drink at least one glass of water every 2 hr. The percent of the dose excreted in the urine until 4:30 PM was measured (16, 17). The same dosage was given at 4:30 PM and the urine collected until 8:30 AM the next day. These vitamin doses were continued for 5 days without dietary restrictions in order to saturate the individuals. Restrictions were resumed during the last 3 of the 5 days of desaturation and until the end of the experiment. After desaturation (*day 12*), blood vitamin C was determined and the excretion of vitamin C intakes ranging from 58 to 95 mg, given twice a day, was measured for 3 days. The two 95-mg intakes included other water-soluble vitamins. Finally (*day 15*), two loading doses were given in order to estimate the vitamin C retained from the previous loads.

The average daily vitamin C intakes were based on all the information supplied by the subjects and were evaluated by using food tables (19), amounts declared on labels of foods, and typical determinations of some vegetables such as boiled, mashed, or fried potatoes, and potato chips.

RESULTS

There was no significant difference between the smoker and nonsmoker groups with regard to age, weight, consumption of tea, coffee, and alcohol and intakes of vitamin C.

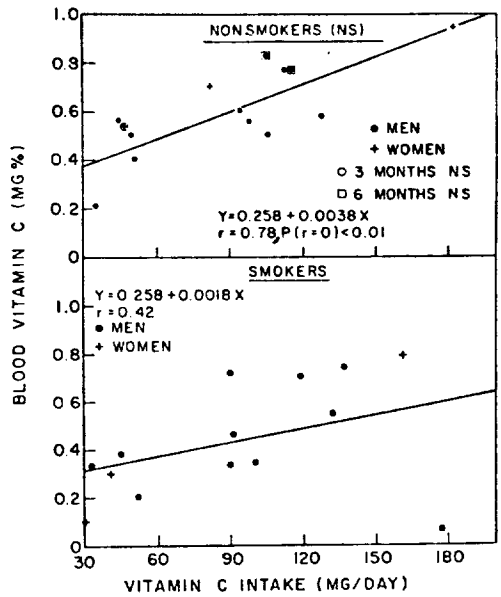

FIG. 1. Relationships between blood vitamin C (mg/100 ml) and intake of vitamin C (mg/day) of smokers and nonsmokers.

As shown in Table I, before the load tests, vitamin C blood levels of the smokers were 30% lower than those of the nonsmokers. The smokers excreted significantly less of the first vitamin C load than the nonsmokers, indicating a greater retention. The second load produced no difference. After saturation and desaturation, the blood vitamin C of each group increased to about the same level. The amounts of the vitamin excreted from the

TABLE I

Vitamin C in blood and excretion of 1.1-g doses (±SE)

	Day	Smokers	Nonsmokers	*P*
Blood, mg/100 ml	0[a]	0.42 ± 0.06	0.60 ± 0.05	<0.05
Excreted, %	1[b]	10.0 ± 2.5	20.1 ± 3.5	<0.05
Excreted, %	1[c]	24.4 ± 3.6	26.5 ± 4.4	NS
Blood, mg/100 ml	12[a]	0.88 ± 0.05	0.81 ± 0.04	NS
Excreted, %	15[b]	25.6 ± 2.5	27.6 ± 2.5	NS
Excreted, %	15[c]	41.7 ± 4.4	35.4 ± 3.4	NS
Retained, %	15[b]-1[b]	14.9 ± 3.6	5.9 ± 2.1	<0.05
Retained, %	15[c]-1[c]	14.5 ± 4.1	6.9 ± 4.3	NS

[a] Fasting. [b] 8:30 AM dose. [c] 4:30 PM dose.

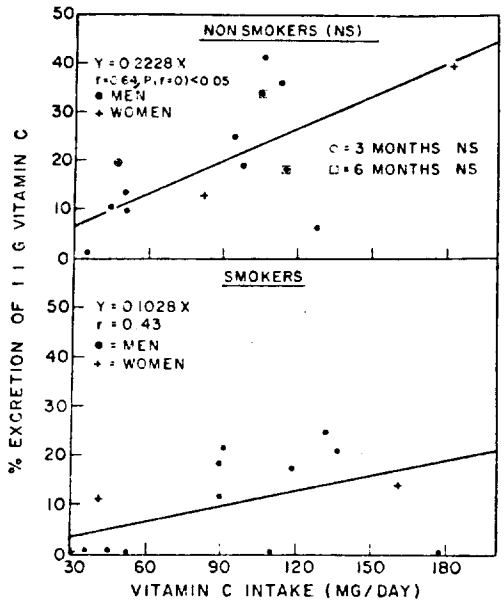

FIG. 2. Relationships between percentage excretion of 1.1 g vitamin C and intake of vitamin C (mg/day) of smokers and nonsmokers.

load doses were also higher than on the 1st day with no significant differences between groups. According to the difference in excretion between the 8:30 AM loads at the beginning and end of the experiment, the smokers retained significantly more vitamin C. They also retained more from the 4:30 PM load, but the difference was not statistically significant because there were several individual variations. One smoker resigned on the last day of the experiment and his nonsmoker partner was excluded so that the values for the excretion on the last day for the retention were calculated for 13 subjects/group.

In order to ascertain that the initial lower vitamin C levels of the smokers were not due to the difference in the intakes, regression straight-line fitting of the relationship between intakes, initial blood levels, and excretion of the first load was done. Figure 1 shows the best straight-line fits, from the least-square point of view, of the intakes as related to blood level. The slopes are obviously different. The correlation coefficient was significant for the nonsmokers but not for the smokers.

As shown in Fig. 2, the relationships between vitamin C intake and percentage excretion of the first load test showed about the same pattern for the difference between the two groups and the same correlation coefficient as was obtained for blood levels.

Figure 3 shows a good relationship between blood levels of all the subjects and excretion of the first load test. The correlation coefficient, 0.73, was highly significant. A regression straight-line analysis showed no difference between groups.

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

Table II shows the percentage excretion of 58- to 95-mg doses of vitamin C given

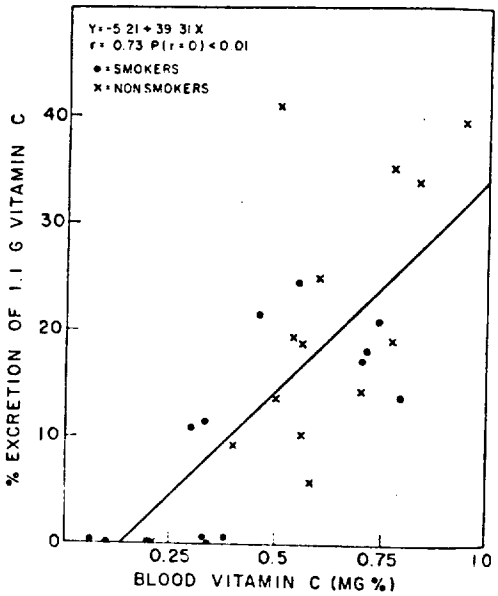

FIG. 3. Relationship between percentage of 1.1 g vitamin C and blood vitamin C (mg/100 ml).

TABLE II
Percent excretion (± SE) of vitamin C intake after saturation

Intake	Smokers	Nonsmokers	P
58 mg[a]	11.4 ± 1.6	14.4 ± 2.8	NS
61 mg[b]	17.9 ± 2.8	27.8 ± 3.4	<0.05
95 mg[a]	12.3 ± 2.1	25.6 ± 3.4	<0.005
95 mg[b]	25.6 ± 4.2	39.4 ± 2.7	<0.01
62 mg[a]	28.3 ± 4.3	43.1 ± 5.1	<0.05
61 mg[b]	38.2 ± 5.2	66.0 ± 7.6	<0.005

[a] 8:30 AM. [b] 4:30 PM.

twice a day after the desaturation period. The excretion of the first dose was low for both groups, but from all the other doses the smokers excreted about 40% less vitamin C than the nonsmokers.

DISCUSSION

What are the best criteria for assessing the vitamin C status of individuals? The vitamin content of white blood cells may be a good index of body stores, but such a determination is difficult (20). A person visiting six laboratories in Scotland asked each to estimate his leukocyte ascorbic acid and the figures obtained varied widely, from 15 to 54 μg 10^8 WBC (20). There is no reason why blood vitamin C levels could not reflect the body stores of persons having a regular diet if the samples are taken with the precautions used in this study. The relationship obtained between vitamin C intakes and blood levels indicated that a fair prediction of the blood levels of nonsmokers from their intakes would be possible, but predictions for the smokers would be misleading as indicated by the wide variation.

Several types of load tests have been previously proposed (21) but they have not gained in popularity because of their complexity. In the present study, the 1.1-g vitamin C load test was simple and gave a good relationship with the intakes of nonsmokers and with the blood levels of both groups. A larger load given in proportion to body weight would probably differentiate the lower levels more accurately. The accuracy would be improved by expressing the percentage excreted per gram creatinine for a given period.

The present study demonstrated that, in general, the lower vitamin C blood levels of cigarette smokers and their lower excretion or greater retention of a test dose were not caused by lower vitamin C intakes. No characteristic other than cigarette smoking was found to be related to the lower vitamin C levels of the smokers.

After the saturation and desaturation period, the urinary excretion of about 40% less vitamin C by the smokers after intakes varying from about 60–95 mg twice a day could mean that the smokers utilized more vitamin C or that less vitamin C was effectively available from the same intakes. A significant difference in the utilization of vitamin C by the organs and tissues appears unlikely, because in a preliminary study (16), when five smokers and five nonsmokers saturated with vitamin C were placed on a deficient diet for 13 days, the blood and plasma levels of both groups decreased at the same rate, and there was no significant difference in the subsequent retention of vitamin C from load tests.

Since the absorption mechanism may be causing less vitamin C to be available for utilization, this possibility will be further investigated.

SUMMARY

The vitamin C status of 14 cigarette smokers and 14 nonsmokers was evaluated before and after saturation for 6 days with 2.2 g L-ascorbic acid. The smokers' group initially had lower vitamin C blood levels and a lower urinary excretion after a 1.1-g test dose than the nonsmokers, but no difference was found after the saturation and desaturation period; thus, the smokers initially retained more vitamin C.

The relationship between the vitamin C

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intake and blood levels, or urinary excretion of the first test dose, indicated that the differences in the vitamin C status were not due to different intakes.

The vitamin C excreted during 8 hr after a 1.1-g load correlated well with the vitamin C intake of the nonsmokers and with the blood vitamin C levels of both smokers and nonsmokers. This type of load test should be useful for determining the vitamin C status of individuals.

Following elimination of excess vitamin C after saturation, intakes of vitamin C ranging from about 60 to 95 mg were given twice a day for 3 days, and it was found that in general the smokers excreted 40% less vitamin C than the nonsmokers. This indicates that less vitamin C is effectively available for utilization by the smokers, or that the smokers are utilizing vitamin C differently.

The author wishes to thank Dr. C. Godin, Biology Department, Ottawa University, and Dr. T. K. Murray, Chief, Nutrition Research Division, Food and Drug Directorate, Ottawa, for their encouragement and useful discussions in planning the experiments and preparing the paper. The excellent technical assistance of R. Brassard and R. Madère, the professional advice and assistance of D. A. Lyon for the regression analyses, and the collaboration of the volunteers from the Food and Drug Directorate are gratefully acknowledged.

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Vitamins, Sugars and Ethanol Metabolism in Man

It is generally accepted that the rate-limiting step in the metabolism of alcohol (ethanol) in man is the conversion of alcohol into acetaldehyde[1-3]. This reaction is believed to occur chiefly in the soluble cytoplasm of liver cells[4,5], and is catalysed by alcohol dehydrogenase (ADH)[6], NAD acting as hydrogen acceptor[7,8]. The part played by the interesting ethanol-oxidizing system recently discovered[9] in the microsomes of liver cells may be significant, but requires further evaluation. The availability of unreduced NAD in the cell sap seems to be important[10,11], because NADH competes with NAD for binding sites on the enzyme ADH and, in sufficient concentration, may inhibit the rate of ethanol dehydrogenation[12]. The NADH produced in the initial oxidative step in ethanol metabolism must therefore be continually reoxidized by various oxidation-reduction systems for the reaction to proceed[13]. In the cell sap of liver cells the reoxidation of NADH may be coupled to the reduction of pyruvate to lactate[3,8], and to the reductive synthesis[14], elongation and saturation of fatty acids; but perhaps the most important route for reoxidation of the NADH involves the mitochondrial flavoprotein-cytochrome electron transfer system coupled with phosphorylating oxidation[15-17]. The "carrier" of the hydrogen equivalents from NADH across the relatively impermeable mitochondrial membranes may be substances like alpha-glycero-phosphate[17], beta-hydroxybutyrate[18,19], malate and glutamate[20], which serve to "shuttle" hydrogen from the extramitochondrial NADH, across the mitochondrial membranes, for oxidation in the intra-mitochondrial compartment.

In theory, the B vitamins nicotinamide and riboflavin participate in the metabolism of alcohol, for they are implicated in the formation of NAD and flavin-adenine dinucleotide (FAD), respectively. It is also possible that ascorbic acid and the tocopherols, which can be involved in oxidation-reduction reactions, affect alcohol oxidation. There has been a report that pyridoxine increases the rate of alcohol metabolism in man[21]. Sugars, which yield pyruvate and participate in fatty acid synthesis, may also facilitate ethanol oxidation by means of NADH reoxidation pathways. Some workers have, in fact, claimed that the administration of vitamins and sugars can increase the rate of alcohol metabolism and of "sobering up" in man and laboratory animals, but the published results to date are equivocal[22].

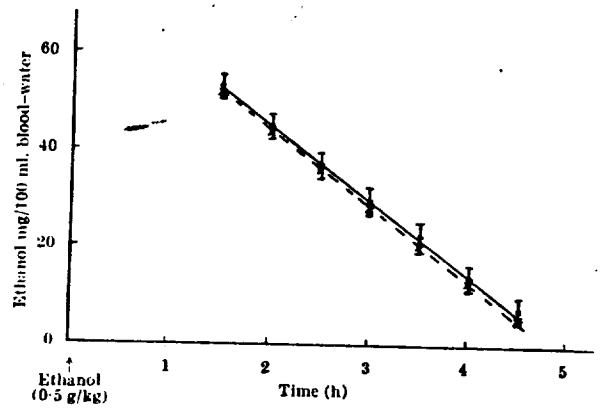

Fig. 1. Control experiments (●—●). Plasma alcohol concentrations (mean ± S.E.M.) of fifteen men given 0·5 g ethanol/kg body weight on three occasions. The effect of vitamin supplements for 10 days on this dose of alcohol in the same subjects is shown (× - - - ×).

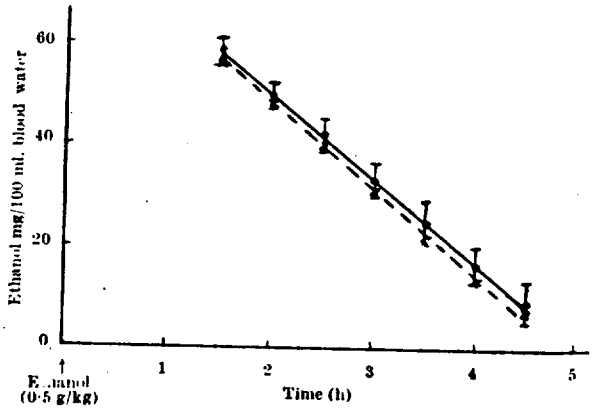

Fig. 2. Control experiments (●—●). Plasma alcohol concentrations (mean ± S.E.M.) of four men given 0·5 g of ethanol/kg body weight on three occasions. The effect of a large dose of ascorbic acid (700 mg) nicotinamide (150 mg) and pyridoxine (20 mg), taken with this quantity of alcohol in the same subjects is shown (× - - - ×).

I have therefore studied the effects of vitamin and sugar supplements on the rate of metabolism of standard doses of alcohol in normal healthy male volunteers. After an overnight fast of 10–14 h, eighteen normal well nourished men, aged 19–52 years (mean age 30 years), who were all occasional moderate drinkers, were given 0·5 g ethanol/kg body weight, administered orally in pure lemon juice as described earlier[23], at intervals of 3–5 days. Venous blood and urine samples were obtained at half-hourly intervals for triplicate analyses of ethanol and water. Ethanol was determined in Cavett flasks by a modification of the method of Williams, Linn and Zak[24], and water content of the specimens by a gravimetric method using samples dried at 105° C. The concentrations of alcohol, expressed in terms of the amounts in plasma water and urine water, were plotted against time, and the slopes of the rectilinear curves after equilibration throughout the total body water (after 1–1·5 h) were taken as indices of the rate of ethanol metabolism. These were found to be relatively constant on each of three occasions in fifteen subjects. These fifteen men were each given vitamin supplements at the following concentrations daily for 10 days: vitamin B1 (thiamine hydrochloride, 10 mg); vitamin B2 (riboflavine, 4 mg); vitamin PP (nicotinamide, 40 mg); vitamin B6 (pyridoxine hydrochloride, 4 mg); vitamin C (ascorbic acid, 200 mg); vitamin A (retinol, 5,000 IU); vitamin D (calciferol, 2 mg); vitamin E (tocopherols, 20 mg). On the fifth and tenth day of vitamin supplementation, the ethanol experiments were repeated, the daily dose of the vitamins being taken together with the alcohol. Fig. 1 shows the results obtained. No significant effects on the slopes of the alcohol curves were produced in any of the subjects by the vitamin supplements. In four men the ethanol experiments were repeated 5 days later, but the subjects received much larger doses of the vitamins together with the alcohol (ascorbic acid, 600 mg; nicotinamide, 150 mg; pyridoxine, 20 mg). These larger doses of vitamins had no effect on the slopes of the alcohol lines (Fig. 2). It was concluded from these experiments that the vitamin supplements were without significant effect on the rate of metabolism of alcohol in the subjects tested.

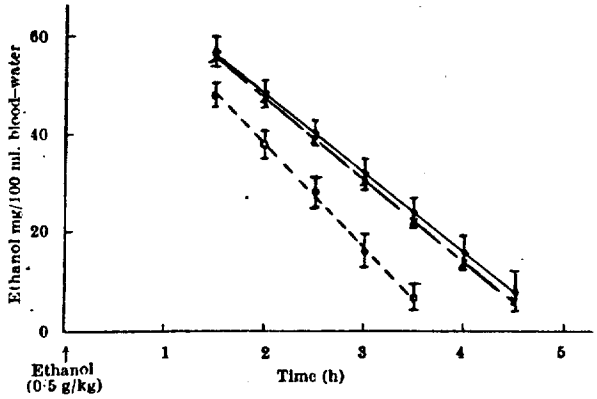

Fig. 3. Control experiments (●—●). Plasma alcohol concentrations (mean ± *S.E.M.*) of twelve men given 0·5 g ethanol/kg body weight on three occasions. The effect of 30 g glucose (— - —) and of 30 g fructose (○ - - - ○) (± *S.E.M.*) taken with this dose of alcohol in the same subjects.

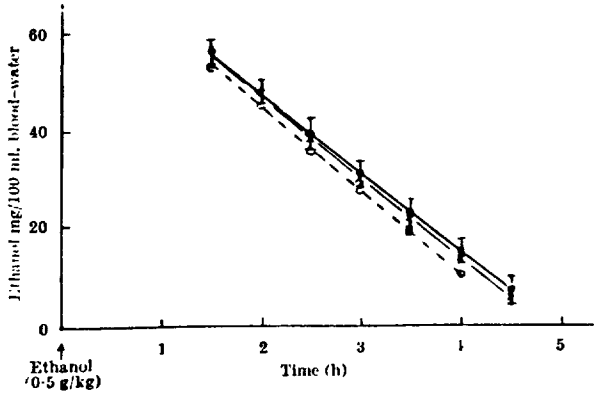

Fig. 4. Control experiments (● — ●). Plasma alcohol concentrations (mean ± *S.E.M.*) of twelve men given 0·5 g of ethanol/kg body weight on three occasions. The effect of 30 g galactose (× — — ×), and of 30 g sucrose (○ - - - ○), taken with this dose of alcohol in the same subjects.

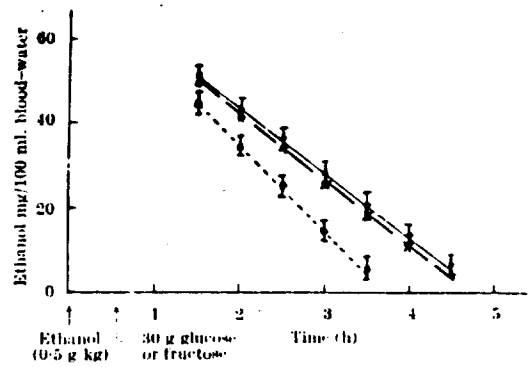

Fig. 5. Control experiments (● — ●). Plasma alcohol concentrations (mean ± *S.E.M.*) of eight men given 0·5 g ethanol/kg body weight on three occasions. The effect of 30 g glucose (× — — ×), and of 30 g fructose (● - - - ●), taken 30 min after this dose of alcohol in the same subjects.

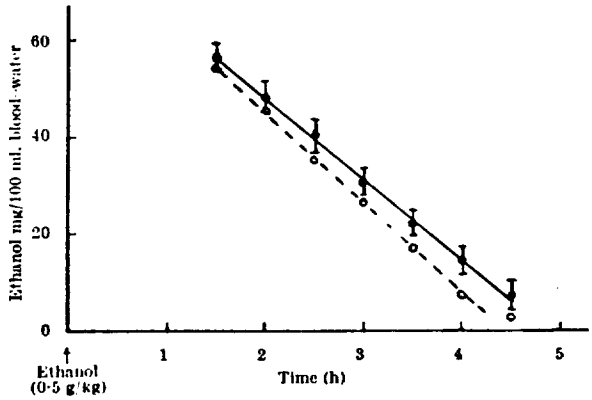

Fig. 6. Control experiments (●—●). Plasma alcohol concentrations (mean ± *S.E.M.*) of six men given 0·5 g ethanol/kg body weight on three occasions. The effect of 60 g sucrose (○ - - - ○) taken with this dose of alcohol in the same subjects.

The alcohol experiments were repeated in twelve men, but 30 g doses of sugars (glucose, fructose, galactose and sucrose, each sugar given on different days) were taken with the alcohol at intervals of 3–5 days. The results are shown in Figs. 3 and 4. Glucose and galactose were without effect, fructose produced a significant increase of 18–40 per cent, and sucrose caused a very slight increase in the rate of alcohol metabolism. These doses were used because earlier experiments showed that some individuals experience abdominal discomfort with larger doses of fructose. In eight subjects the alcohol experiments were again repeated, but the sugars (glucose and fructose, administered on different days) were received 30 min after the alcohol was taken. Glucose was again ineffective (Fig. 5), but fructose increased the rate of metabolism of the alcohol by 15–30 per cent.

Because the slight effect on the alcohol curve induced by sucrose was probably caused by its fructose moiety, the effect of a 60 g dose of sucrose was similarly studied in six subjects (Fig. 6). This dose of sucrose produced a greater increase on the rate of alcohol metabolism than

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VITAMINS

Determination of Ascorbic Acid in the Presence of Ferrous and Stannous Salts

By O. PELLETIER and A. B. MORRISON (Food and Drug Research Laboratories, Department of National Health and Welfare, Ottawa, Canada)

Ferrous and stannous ions in the presence of traces of the oxalic acid found in canned fruit juices and drinks interfere with 2,6-dichlorophenol - indophenol titration of ascorbic acid in various media. A method is presented in which these ions, in buffered solution, are oxidized by air and chelated with EDTA before ascorbic acid is determined by titration. The method has given accurate results in samples containing ferrous and stannous ions.

A recent survey (1) shows that fruit drinks and juices kept refrigerated in nonlacquered cans gave a pseudo increase in ascorbic acid due to the presence of interfering reducing ions which leached from the cans and were titrated with 2,6-dichlorophenol-indophenol. The interference was encountered when either metaphosphoric acid or an acetate buffer at pH 0.65 (2, 3) was used as a diluting solution. Chapman, *et al.* (3) investigated several methods for ascorbic acid, with particular emphasis on interference of ferrous sulfate and other reducing ions in pharmaceutical products. The method of Lugg (4), which uses formaldehyde to condense with ascorbic acid, was not included in their study since the many titrations required for a single determination were not practical for routine use. Lugg (4) suggested that, in the presence of great quantities of ferrous sulfate, the difference in rates of aerial oxidation of ascorbic acid and ferrous salts in metaphosphoric acid at pH 3.5 might be used to advantage. This did not seem satisfactory, since ferric and traces of other metal ions could cause oxidation of ascorbic acid. Chapman, *et al.* (3) reported that methods using hydrogen peroxide (5, 6) to react with the reducing ions gave low recoveries when ferrous sulfate was present. Diluting solutions composed of 8% acetic acid or 2.5% H_2SO_4 were satisfactory in presence of ferrous salts, provided that sufficient care was taken to allow for the instability of ascorbic acid in these solutions. The 2,4-dinitrophenylhydrazine method of Roe, *et al.* (7) is unaffected by reducing cations, but lacks the simplicity and speed of titration methods.

In the studies reported here, various media were used to determine ascorbic acid with 2,6-dichlorophenol-indophenol in the presence of ferrous and stannous ions. Since fruit

juices or drinks may contain oxalic acid, its effects on ascorbic acid values were investigated. A method is presented in which ferrous and stannous ions are oxidized with air in the presence of ethylenediaminetetraacetic acid and the resulting ferric and stannic ions are chelated to prevent them from oxidizing ascorbic acid.

METHOD

Apparatus and Reagents

(a) *Suction apparatus.*—Use 50 ml Erlenmeyer flasks equipped with side arm. Insert a 5 mm i.d. glass tube through a rubber stopper so it extends approximately 5 mm into 17 ml of solution in the flask. Connect four flasks with rubber tubing of equal length to 2 "Y" tubes connected directly to a water aspirator or a vacuum pump. Adjust the air flow through the system so air will bubble moderately in each flask.

(b) *Buffer.*—To 1200 ml H_2O add 77 ml conc'd. HCl and 500 ml 1.0*N* sodium acetate. Mix well and adjust to pH 0.65, if necessary, with either 1.0*N* HCl or 1.0*N* sodium acetate, as required. Dilute to 2 L with H_2O.

(c) *Ethylenediaminetetraacetic acid solution.*—Add 20 g of ethylenediaminetetraacetic acid (EDTA) to 200 ml H_2O. Add 14 ml 10*N* NaOH, and shake or stir until dissolved. If necessary, adjust the pH to 6.0 ± 0.5 with 10*N* NaOH or HCl. Dilute to 250 ml with H_2O.

(d) *2,6 - Dichlorophenol - indophenol solution.*—Dissolve 250 mg sodium 2,6-dichlorobenzenone-indophenol in ca 200 ml warm H_2O. Stir until completely dissolved, filter, and make to 1 L with H_2O. Place in a brown bottle and store at about 3°C.

(e) *Ascorbic acid standard solution.*—Accurately weigh 50 mg ascorbic acid (preferably USP Reference Standard) and dilute to 100 ml with buffer. Prepare fresh daily.

(f) *Ferrous sulfate solution.*—Dilute 250 mg $FeSO_4.7H_2O$ to 100 ml with buffer.

Determination

(a) *Preparation of samples and titration.*—Into 100 ml volumetric flasks, add 50 ml buffer and a sample of juice or fruit drink containing 4–7 mg ascorbic acid. Dilute to 100 ml with distilled H_2O. When assaying tomato juice, remove the colored particulate matter by filtration. Add 1.0 ml standard solution to each of 2 Erlenmeyer flasks with side arm ("A"). Add 1.0 ml of buffer to two similar flasks ("B"). Add 10 ml sample solution, ca 1 ml 0.25% $FeSO_4.7H_2O$[1], and 5 ml EDTA solution to each flask, and mix by swirling. Bubble air simultaneously in all flasks by suction for 10 min. Wash glass tube with 1–2 ml H_2O.

Titrate each flask with 2,6-dichlorophenol-indophenol to a permanent end point, i.e., one which persists for at least 30 seconds. Prepare reagent blanks ("C") similarly using 7 ml $FeSO_4$ reagent (f), 5 ml H_2O, and 5 ml EDTA. For colored solutions, a photoelectric colorimeter at 545 mμ may be used to detect a positive absorbance deflection for determining the titration end point (1).

(b) *Calculations.*—The amount of ascorbic acid in 100 ml of solution of juice or drink is calculated from the following formula:

$$0.5 \times (100/x \text{ ml aliquot}) \times (1/10) \times (\text{titrations } B - C/\text{titrations } A - B)$$

Results and Discussion

Effects of Various Media.—The effect on ascorbic acid values of 2.5 mg $FeSO_4.7H_2O$ and 3.0 mg $SnCl_2$ in presence and absence of 20 mg anhydrous oxalic acid was tested with various media; the results are reported in Table 1. With metaphosphoric acid, interference was caused by ferrous sulfate only; with acetic acid and H_2SO_4, interference was caused by ferrous sulfate and stannous chloride in the presence of oxalic acid. In the acetate buffer, a quantitative recovery was obtained only with ferrous sulfate alone. These results show that none of the above solutions was entirely satisfactory for direct titrations. In the proposed method acetic acid and H_2SO_4 were not tried, but the acetate buffer was preferred to metaphosphoric acid because it produced clearer solutions after air oxidation in presence of EDTA.

Aerial Oxidation in Presence of EDTA.—Aeration in the absence of EDTA caused oxidation of ferrous sulfate, stannous sulfate, and ascorbic acid. EDTA was added to chelate the ferric and stannic ions resulting from

[1] To samples containing an appreciable amount of ferrous ions, add a smaller amount of $FeSO_4.7H_2O$, determined as follows: Add ca 100 mg metaphosphoric acid to 10 ml sample solution and titrate with 0.05% aqueous solution of methylene blue to a blue end point. Similarly titrate 1.0 ml 0.25% $FeSO_4.7H_2O$ plus 4 ml buffer and 5 ml H_2O. $FeSO_4.7H_2O$ to be added (ml) = $\frac{\text{titration } FeSO_4.7H_2O - \text{titration of sample}}{\text{titration } FeSO_4.7H_2O}$

Table 1. Effects of various media on per cent recoveries of 1 mg ascorbic acid in presence of interfering ions

Media	2.5 mg $FeSO_4 \cdot 7H_2O$		3.0 mg $SnCl_2$	
	No Oxalic Acid	20 mg Oxalic Acid	No Oxalic Acid	20 mg Oxalic Acid
Metaphosphoric acid, 3%	192.5	—	102.5	102.5
Acetic acid, 8%	100	194.9	102.5	189.9[a]
Sulfuric acid, 2.5%	100	133.1[a]	100	116.2[a]
Acetate buffer, pH 0.65	100	186.1	284.8[a]	—

[a] These values represent an end point lasting only 10 seconds.

oxidation and thus prevent ascorbic acid oxidation. With only 1 ml of EDTA solution, aerial oxidation of ferrous sulfate was not rapid, but 3 ml or more of EDTA resulted in the rapid disappearance of reducing ferrous ions. The disappearance of the stannous ions during aeration in presence of EDTA was complete only when ferrous sulfate was present. This effect may be due either to direct oxidation of stannous ions by ferric ions prior to their chelation or to an enhancement in the chelation of stannic ions. The removal of the reducing metal ions was directly dependent on aerial exposure and was complete in 8 minutes. Extension of this time to 12 minutes resulted in no loss of ascorbic acid. The aeration was carried out in 7 ml of buffer at pH 0.65 but variation in volume from 6 to 10 ml and in pH from 0.5 to 0.8 did not affect the results.

Validity of the Proposed Method Applied to Pure Solutions.—From 0.25 to 1.5 mg ascorbic acid gave a linear response in the titration curve which passed through the origin after correction for the reagent blank. To these amounts of ascorbic acid, $FeSO_4 \cdot 7H_2O$ was added in amounts of 2.5 to 10.0 mg, and assays were conducted by the above procedure. Titration curves were linear and passed through the origin with up to 10 mg $FeSO_4 \cdot 7H_2O$. Above this amount, there was a slight interference from ferrous sulfate. Stannous chloride was similarly tested from 0.5 to 2.0 mg. In the absence of ferrous sulfate, $SnCl_2$ resulted in high titrations, but in the presence of 2.5 mg $FeSO_4 \cdot 7H_2O$ it caused no interference. Figure 1 shows typical titration curves of ascorbic acid in the presence of 2.5 mg $FeSO_4 \cdot 7H_2O$, with and without 1.5 mg $SnCl_2$. Oxalic acid added at the rate of 10 mg per flask had no effect on the titration curves with $FeSO_4 \cdot 7H_2O$ in the presence or absence of $SnCl_2$.

Application of Proposed Method to Fruit Juices and Drinks.—The proposed method was applied as described to fruit juices and drinks in the presence of various amounts of $FeSO_4 \cdot 7H_2O$ and $SnCl_2$, and the results are reported in Table 2. The presence of 5.0 mg $FeSO_4 \cdot 7H_2O$, including the 2.5 mg required in the method, caused no significant increase in apparent ascorbic acid values. Additional amounts up to 10.0 mg $FeSO_4 \cdot 7H_2O$ resulted in a 14% increase in apparent ascorbic acid values for orange-pineapple drink but did not affect values found with the other samples. Amounts of $SnCl_2$ up to 2.0 mg in the presence of 2.5 or 5.0 mg of $FeSO_4 \cdot 7H_2O$ had no effect on ascorbic acid values, except those found for the orange-pineapple drink where the presence of 2.0 mg $SnCl_2$ plus 2.5 and 5.0 mg $FeSO_4 \cdot 7H_2O$ resulted in recoveries of 91.5 and 94.8%, respectively. Amounts of $SnCl_2$ up to 1.5 mg had no effect on the values found for this product. These results show that a limit of 2.5 mg

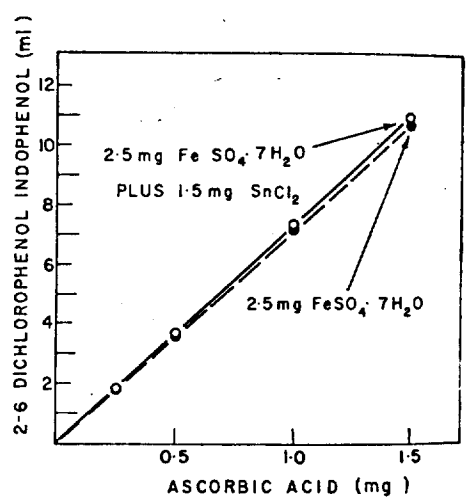

Fig. 1—Titration of ascorbic acid by the proposed method in presence of $FeSO_4$, with and without $SnCl_2$.

Table 2. Per cent recoveries of ascorbic acid in fruit juices determined by the proposed method in presence of ferrous sulfate[a] and stannous chloride

	mg $FeSO_4 \cdot 7H_2O$			mg $SnCl_2$ (plus 2.5 mg $FeSO_4 \cdot 7H_2O$)		mg $SnCl_2$ (plus 5.0 mg $FeSO_4 \cdot 7H_2O$)	
	5.0	7.5	10.0	1.5	2.0	1.5	2.0
Orange juice	100.6	—	102.4	—	98.8	99.4	102.1
Grapefruit juice	100.9	—	101.6	—	99.9	—	99.6
Orange-pineapple drink	100	103.8	114	99.6	91.5	98.8	94.8
Orange drink	100	—	99.5	—	100.2	—	101.3

[a] The amounts of ferrous sulfate include the 2.5 mg recommended in the procedure.

$FeSO_4.7H_2O$ and 1.5 mg $SnCl_2$ is applicable to fruit juices and drinks.

Comparison of Proposed Method and Direct Titration.—Fresh juices or fruit drinks kept in glass containers were assayed by the proposed method and direct titration at pH 0.65 (3); both gave essentially the same results (Table 3). With canned products, however, the proposed method gave lower values for orange juice, grapefruit juice, and orange-pineapple drink when the products were assayed immediately after the cans were opened (Table 4). Tomato juice, on the other hand, gave comparable results by both methods. Part of the samples were transferred to glass jars and kept refrigerated, along with the portion left in the cans. Assays were conducted 2 days and 7 days afterward, and the results are summarized in Table 4. For samples kept in the cans, the direct titration at pH 0.65 (3) gave higher values for orange juice, grapefruit juice, and orange-pineapple drink. In two of these samples, apparent ascorbic acid values increased during the storage period as a result of interference from reducing ions dissolving from the can into the fluid. It may be concluded, therefore, that the proposed method is valuable for determining ascorbic acid in samples stored in nonlacquered cans. Although not entirely specific, the method eliminates interference from iron and tin which may be present in canned fruit juices and drinks.

Table 3. Ascorbic acid content of fruit juices and drinks in glass containers as determined by the proposed method and direct titration at pH 0.65

Product	Proposed Method (mg/100 ml)	Direct Titration (mg/100 ml)
Orange juice	48.2	48.2
Grapefruit juice	33.3	33.5
Orange drink I	18.3	18.4
Orange drink II	20.1	20.0

Table 4. Ascorbic acid in fruit juices or drink refrigerated in their original containers and in glass jars, as determined by two procedures

		Nonlacquered Cans		Glass Jar	
Product	Days After Opening Container	Direct Titration (mg/100 ml)	Proposed (mg/100 ml)	Direct Titration (mg/100 ml)	Proposed (mg/100 ml)
Orange juice	0	37.1	34.3	37.1	34.3
	2	37.4	33.3	36.7	34.3
	7	36.6	37.2	32.5	31.6
Grapefruit juice	0	45.0	43.5	45.0	43.5
	2	58.5	43.7	44	43.7
	7	59.5	43.2	40.8	40.2
Orange-pineapple drink	0	32.2	28.1	32.2	28.1
	2	41.2	29.9	30.6	29.6
	7	40.6	29.05	26.6	27.4
Tomato juice	0	25.8	25.0	25.8	25.0
	2	25	25.1	25.0	25.1
	7	21	21.7	18.3	19.0

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VITAMIN C (ASCORBIC ACID, CEVITAMIC ACID)*

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The year has been one of consolidation and critical appraisement of previous claims.

Chemistry.—Papers upon the organic chemistry of this vitamin are naturally few. Haworth, Hirst & Jones (1) have taken exceptional care to prove that *d*-gluco-ascorbic acid really has the structure assigned. The *l*-form is slightly active. Several physico-chemical points are now clearer. A slight increase in the earlier pK values of Birch & Harris and Karrer *et al.* by Ball (2) gives pK 4.21 instead of 4.17. A careful study of oxidation-reduction potentials has been made by Borsook, Davenport, Jeffreys & Warner (3). They recognise at least three stages *in vitro* corresponding to the previously recognised stages of degradation (4) but they emphasise that the correctness of their facts does not depend upon this interpretation. The first stage, A, can be studied at a pH lower than 5.0 (at pH 5.0 $E_0' = +0.127$) and corresponds, presumably, to the reversible transformation ascorbic acid $\rightleftharpoons$ dehydroascorbic acid. The oxidised (and only the oxidised) dehydro form passes very readily through two other stages, the transformation occurring with increasing rapidity as the pH becomes greater than 6.0. The products of both changes are more strongly reducing than A. The changes correspond to the transformation from dehydroascorbic acid to diketogulonic acid (B) ($E_0' = +0.015$ at pH 5.5), and to the further degradation (C) to decomposition products previously characterised as *l*-threonic acid and oxalic acid. Stage A was measured electrometrically at pH < 5, equilibrium being slowly attained, and stages B and C with oxidation-reduction indicators. Values for A have been confirmed by Ball (2) using as carrier traces of dye which induces a more rapid attainment of equilibrium. He criticises the figures for B and C upon the ground that no true reversibility is shown and that velocities do not necessarily indicate the true values. Only stage A is normally important biologically[1] and the importance of keeping ascorbic acid reduced

* Received January 26, 1938.

[1] Cf. *Ann. Rev. Biochem.*, 6, 354 (1937)

in the tissues is clearly shown by the extreme lability of the dehydro form at body pH. The only case in which dehydroascorbic acid has been proved to exist as such is in the apple (5), though many living systems rapidly reduce this form. Stage B can, at present, be reversed only by chemical means (4), and there is no evidence that B and C have biological significance. In the paper by Borsook *et al.* (as elsewhere) is to be found clear evidence that glutathione (originally shown by Szent-Györgyi to interact) is one of the main constituents of blood responsible for the stability of ascorbic acid. No dehydroascorbic acid exists in blood; its alleged presence is an artifact due to oxidation *in vitro* by the precipitants at the moment of precipitation of blood proteins in the presence of blood corpuscles (3, 6, 7). Reference may be made to the work of Fischer (8) on the behaviour of ascorbic acid *in vitro* with haemoglobin compounds and cyanide in which an adsorption compound with oxyhaemoglobin is obtained (cf. 9).

Kon (10) finds that visible light destroys the vitamin C in milk, an observation of practical importance. A clue to the mechanism of this has been given by Hopkins (11). In the presence of light *in vitro* the fluorescent lactoflavin rapidly causes destruction of the vitamin, there being a simultaneous degradation of lactoflavin to lumichrome, i.e., an interaction of two vitamins. Another action *in vitro* of ascorbic acid has received attention, namely the deaminating action upon amino acids (12, 13). There is no evidence that it is of biological significance.

Methods of estimation.—Papers upon this subject are very numerous and various methods have been tried (3, 14, 15, 16, 17) though there is not much change in the position since last year's review. The most practical method for urine still seems to be acidification to pH 3 and titration as soon as practicable, though the presence of other reducing substances may interfere (18, 19). In plant juices the total reducing power to 2,6-dichlorophenolindophenol probably corresponds to the ascorbic acid present, but in animal-tissue extracts, particularly those of liver tissue, it may be as much as 20 per cent too high.

Ascorbic acid oxidase.—There is still much interest in the capacity of oxidising ascorbic acid possessed by plant extracts, one of the first facts to be described by Szent-Györgyi. The properties of a series of such oxidases have been reported by Johnson & Zilva (20). They conclude that there are slight differences in matters of detail in

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respect to their activities, pH range, and behaviour to cyanide. In the cabbage, cauliflower, cucumber, and marrow the enzymes oxidise *l*-ascorbic acid directly, but in the apple and potato the phenolases present oxidise it in the presence of catechol or the juice. Tauber (21) has described the oxidation of ascorbic acid by plant peroxide-peroxidase systems when there is present some substance capable of acting as a carrier by forming a quinone, but it must be remembered that any oxidising system which forms an oxidant of the correct potential should be capable of oxidising unprotected ascorbic acid *in vitro*.

In another paper (22), following up the previous finding of Zilva that the enzyme is not specific for *l*-ascorbic acid, Johnson and Zilva find that the members of a series in which the ring is to the right of the carbon chain (namely, *l*-ascorbic acid, *d*-arabo-ascorbic acid, *l*-gluco-ascorbic acid, and *l*-galacto-ascorbic acid) are directly oxidised by cucumber oxidase at much higher rates than are their enantiomorphs (for formulae see 22). In the slowly oxidisable group oxidation is faster for those with six than with seven carbon atoms. The facts suggest that the asymmetric carbon atoms outside the ring also condition antiscorbutic activity but not the direct enzyme oxidation of *l*-ascorbic acid and its six-carbon analogues.

In contrast to the specificity implicit in the above, Stotz, Harrer & King (23) regard the enzyme as an association resembling Bertrand's artificial laccase. Minute traces of copper previously have been reported[2] to catalyse actively ascorbic acid oxidation, and Stotz *et al.* state that they can imitate the enzymatic oxidation with copper + gelatin or copper + albumin. The argument is supported with evidence that copper inhibitors reduce the activity of artificial and natural enzymes in a similar way (a discrepancy for cabbage juice is explained), that there is a general resemblance of activity-pH curves, that heating to 100° C. for five minutes destroys the activity, and that similar inactivation is effected by treatment with acid at pH 2.0. They report only 60 to 70 per cent inactivation as a result of two hours treatment with acid, while Tauber *et al.* (24) report total inactivation of the natural enzyme in fifteen minutes. In the case of inactivation of the artificial enzyme, the copper is presumably removed from the solution by precipitation of the protein or converted to some inactive association compound. The case made out by these

[2] Cf. *Ann. Rev. Biochem.*, 5, 370 (1936).

authors requires consideration but it would have been improved by publication of copper estimations on the natural enzyme; it does not yet seem to account for the apparent specificity.

Mode of action.—There is a strong feeling that vitamin C acts by virtue of its reducing properties. Furthermore there is evidence that its absorption by tissues of the rat and guinea pig is specific as compared with inactive compounds of the series (25). However, there has been no progress in demonstrating an effect of the vitamin upon ascorbic-avitaminous tissues. The claims originally made by Harrison (26) and by Quastel & Wheatley (27) for an action, *in vitro*, of ascorbic acid upon scorbutic and normal liver tissues have turned out, upon closer examination, to be fallacious. Stotz, Harrer, Schultze & King (28) conclude that the rates of oxygen consumption and carbon dioxide evolution of guinea-pig liver (but not kidney) tissues increase with onset of scurvy and that there is no increased oxygen consumption, as the result of adding ascorbic acid, over and above that due to oxidation of the ascorbic acid itself. There is, therefore, nothing described yet with these tissues analogous to the catatorulin effect of vitamin B_1, but there is the older observation (27) that addition of ascorbic acid to rat-liver slices partially removes the inhibition of acetoacetate production caused by iodoacetate. Scheunert & Schieblich (29) have found that the livers of rats upon diets free of vitamins A, B, C, and D still contain sufficient ascorbic acid to cure guinea pigs; hence the synthesis of ascorbic acid is independent of these factors.

Ascorbic acid and toxins.—Related to the above is the question of the role of ascorbic acid in toxic conditions. There is indication that the ascorbic acid content of the adrenals of guinea pigs is diminished in diphtheria intoxication (30). Papers still appear indicating that the general level of nutrition of guinea pigs as influenced by the quantity of the food intake and by ascorbic acid is significant. For instance, Sigal & King (31), using the glucose-tolerance test, showed that sub-lethal injections of diphtheria toxin caused a greater carbohydrate disturbance in guinea pigs receiving just enough ascorbic acid to prevent scurvy than in those receiving a more generous supply. But the ascorbic acid dosing of animals not subject to scurvy, e.g., mice (32) and rabbits (33), has been shown to be without effect on the course of intoxications or infections.

In a recent paper Zilva (34) discusses critically the literature upon the alleged antitoxic action of ascorbic acid in diphtheria. He

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THE IMPORTANCE OF VITAMIN C IN BODILY DEFENSES

I. The Anti-anaphylactic Effect of Vitamin C in the Prevention of Pollen Reactions

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The effect of Vitamin C on adverse reactions to drugs is extremely interesting. The effect of ascorbic acid on the sensitivity to neoarsphenamine is well known, and has been repeatedly described.[1,5,9] It is unknown whether the patients exhibiting these actions are deficient in Vitamin C, or whether Vitamin C detoxifies the neoarsphenamine in a well-saturated patient. Recently, I described a case of rheumatic fever that rapidly became intolerant to salicylates and had a low ascorbic acid content of the serum, this patient was quickly relieved of his disability by adequate intake of Vitamin C.[6] This was also shown to be true for several cases exhibiting intolerance to sulfonamides,[7] and more recently to stilbestrol.[8] These experiments have largely been verified.[4]

Holmes[3] recently published evidence that hay fever patients may be relieved of their disability in great measure by giving Vitamin C in large doses, on the average of about 500 mg. per day. More recently, also, he has shown that Vitamin C will also protect against food allergies.[2]

This short communication is concerned with the protection of an extremely sensitive ragweed patient against adverse reactions to pollen injections.

The patient, a woman thirty-five years of age, was markedly sensitive to ragweed pollen, and was given pre-seasonal treatment with pollen antigen (Lederle). When 200 pollen units were given, the patient developed hives all over her body, swelling of the eyes and tongue and marked wheezing. This reaction was relieved in about fifteen minutes by epinephrine. Attempts to increase this amount slightly brought forth increasingly severe reactions.

The following week the 200 pollen units of ragweed were given along with ½ c.c. of sodium ascorbate, (100 mg. in 2 c.c.) in the same syringe. No reaction occurred. Thereafter, at two-day intervals, increasing doses of ragweed pollen were given with ½ c.c. of sodium ascorbate. In addition, 100 mg. of ascorbic acid were given three times a day by mouth.

The patient volunteered that she had not had citrous fruits for many years because of sensitivity to orange and grapefruit. Finally, a dose of 1500 units of ragweed pollen antigen combined with ½ c.c. of sodium ascorbate was given without incident.

Ascorbic acid was thus able to reduce the exquisite sensitivity to ragweed pollen antigen in this patient. It was thought desirable to find out whether this phenomenon was a local effect of ascorbic acid on the pollen antigen. On a ragweed-sensitive patient, the following skin tests were carried out by intradermal injection:

Ragweed .02 c.c.; ragweed .02 c.c. plus .05 c.c. of sodium ascorbate (described above); histamine hydrochloride 1/1000 solution, .02 c.c.; histamine hydrochloride (1/1000 solution) .02 c.c. plus .05 c.c. of sodium ascorbate. There was no observable difference between ragweed and ragweed with sodium ascorbate or between histamine and histamine with sodium ascorbate. Thus, whatever detoxification occurs is not local in nature.

The liver is the site of detoxification for most noxious substances in the body. Usually they are rendered harmless by conjugation in the liver cells. It is certainly by design that the liver, next to the adrenal glands, contains the most ascorbic acid of any organ in the body. The ascorbic acid is undoubtedly one of the substances concerned with detoxification.

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of the degree of saturation (47). Others (39, 44) believe with justification that such values may be unreliable and that dosing until constant excretion is reached provides the only true measure of the condition. Recently Wright *et al.* (48) state that saturation is best measured by vascular injection of ascorbic acid. Abbasy *et al.* (46) find that their patients excrete relatively less than do normal persons in response to three test doses. In some cases the results are striking, and some of the facts are supported by other evidence. Finkle (47), using an injection technique, found low saturation as well as low excretion in lupus erythematosus and also in rheumatoid arthritis. These results led them to the general view that there is a greater apparent utilisation of ascorbic acid in these cases, but it must be noted, as Parsons (49) has pointed out, that the findings of Abbasy *et al.* do not apply specifically to rheumatism. He and his colleagues found that rheumatic patients tended to excrete more than "infected" controls.

(*c*) The implicit interpretation that there is a direct relation between these states of apparent unsaturation and the disease does not seem clear, and it cannot yet be accepted at its face value. The results are not really concordant with careful observations by Sendroy & Schultz (50, 51) who do not think that dosing with ascorbic acid improves the rheumatic condition. This view is supported by Parsons (49) and by Baumann & Rappolt (14) for other infections. There is no evidence from animal experimentation that saturation, as such, matters.[4] In fact it has yet to be demonstrated that any person requires an excess of vitamin C beyond a wise nutritional margin of at least twice the indispensable minimum to protect against scurvy and latent scurvy. The latter has been redefined in careful work by Göthlin (52) using capillary-fragility tests as 23 to 29 mg. per 60 kg. for the normal person. For pregnant and nursing women the amount would be increased to three times. For the latter 50 mg. per day is given by Baumann & Rappolt (14) and 80 to 100 mg. per day by Widenbaner & Kühner (53). "Gastric" cases need more. It is to be noted that metabolic conditions certainly influence excretion. Less is stated to be excreted in urine under alkaline than under acid conditions; conversely, that the liver retains more. Drugs as well as anaesthetics also influence excretion (54, 55, 56).

[4] As a qualification to this Perla (45) reports one unpublished experiment indicating that extra vitamin C protected guinea pigs against trypanosome infection. If the basic diet was adequate in vitamin C this experiment would certainly support the idea that partial saturation was helpful.

The number of papers upon vitamin C and related subjects is very large this year and it has been impossible in this short review to do adequate justice to much of interest. Reference may be made for instance to Glick (57) for microtechnical achievements and determinations in the developing shoot, to Zilva & Morris (58) for proof that the vitamin-C content of canned vegetables and fruit can be improved by adding vitamin C, to Perla & Marmorston (45) for a review of vitamin C in pathology, and to Giroud *et al.* (62) for the distribution of ascorbic acid in tissues of the ox and horse.

Vitamin P.—Considered to influence permeability especially, and identified by Szent-Györgyi (64) and collaborators as citrin and hesperidin, this vitamin has not yet established itself beyond criticism. Starting from the view that the factor was active clinically in man (purpura conditions) Szent-Györgyi and associates thought at first that it prolonged the life of guinea pigs and lowered the intensity of the pathological lesions. This could not be confirmed by Zilva (59), who stated that he could reproduce the animal symptoms by traces of ascorbic acid below the curative dose. Later, Szent-Györgyi and colleagues took the view that the factor required small traces of ascorbic acid for its activity (60). Moll's experiments (61) have confirmed Zilva in finding no curative effects with hesperidin, etc., nor interactions with small doses of vitamin C in guinea pigs. The efficacy of vitamin P in guinea pigs is, therefore, unconfirmed. Whether there are any clinical effects is undecided; it is certainly curious that Warburg & Elmby (63) state they have cured scorbutic patients (and raised serum ascorbic acid) by the use of lemon juice in some cases not cured by ascorbic acid. Such suggestions make it unwise to pronounce sweepingly against the existence of a capillary factor for man.

[We wish to thank Miss Hume and Miss Copping for facilities provided, Dr. Zilva for criticism, and A. Harrison for secretarial help.]

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points out that a diminution of ascorbic acid in the adrenals or other organs of guinea pigs suffering from diphtheria is probably secondary and that even if destruction of the toxin by the acid occurs *in vitro* it is not of significance *in vivo* since many substances carry out such inactivation *in vitro*. Using guinea pigs he attacks again the problem of whether or not ascorbic acid can modify the action of diphtheria toxin *in vivo*. Known amounts of diphtheria toxin were injected into animals (*a*) depleted, (*b*) saturated, or (*c*) injected with ascorbic acid. In these three conditions the plasma "apparent" ascorbic acid was (*a*) practically nil, (*b*) 0.5 mg. per 100 cc., or (*c*) from 32 to 3.5 mg. per 100 cc. for periods of from one to four hours after injection. The tables show no differences among the groups. Animals with or without ascorbic acid reacted identically to one minimum lethal dose of toxin.

In addition, attempts to prove an undoubted action of ascorbic acid on toxins *in vitro* have been seriously criticised by Sigal & King (35) who state that when ascorbic acid is carefully neutralised to pH 6.4 to 7.4 before being mixed with diphtheria toxin there is no inactivation. The published data of other workers do not appear to exclude the possibility that the alleged inactivation was caused by the non-specific acid or reducing properties of ascorbic acid. No experiments using the closely related but non-antiscorbutic substances have been reported.

Such evidence appears to the reviewers to be convincing, though of course it does not exclude the possibilities that ascorbic acid may be catabolised to a greater extent by diseased animals or that it may have an inhibitory effect upon the growth of microorganisms *in vivo*. But this is speculative. It is quite clear to an impartial observer that no case has yet been made out for ascorbic acid as an antitoxic agent *per se*, that is apart from its influence as an antiscorbutic agent.

Metabolism and the significance of saturation.—Much attention still continues to be devoted to the relation between the amount of ascorbic acid in the plasma and in the urine. Most of the work is of a purely clinical nature, but the question is also of scientific interest. The facts established in previous years are briefly as follows: The ingestion of constant doses of ascorbic acid by man or animal gives rise after a variable time to an increased excretion in the urine[3] which

[3] Proved originally by the urine's curative action on guinea pigs. Recently, ascorbic acid has been isolated from urine as the 2,4-dinitrohydrazone (36, 37).

tends to reach an approximately constant value (38, 39, 40); with large doses this constant amount may be some 300 mg. in twenty-four hours, but it is always much less than the amount ingested. The fate of the disappearing ascorbic acid is unknown, but Wright *et al.* (41) and Bernstein (42) have reported the excretion of ascorbic acid in sweat. This is not only important for miners, who often suffer from sub-scurvy, but it might also help to explain the greater disappearance of ascorbic acid in patients with diseases causing increased sweating. There is a low urinary excretion of ascorbic acid in scurvy, and the excretion is raised after the ingestion of ascorbic acid. There is a tendency for the excretion to rise more rapidly after large oral doses in conditions in which much ascorbic acid has previously been given, though the excretion falls upon the cessation of administration. Thus there is evidence of "saturation." In guinea pigs, even when two to three times the necessary daily protective dose is given the selective tissues contain only traces of ascorbic acid (43). To get maximum tissue saturation ten times the protective dose is necessary. Yet both groups of guinea pigs become scorbutic at approximately the same time after having been placed on a scorbutic diet; hence the tissue vitamin C hardly behaves as a store. In patients all signs of scurvy disappear during ascorbic acid dosing some time before there is any evidence of saturation (44). Normal individuals do not respond to test doses of ascorbic acid with increased excretion at equal rates.

The present problems are: (*a*) whether the normal urinary excretion is a guide to the general state of the organism; (*b*) whether the measure of saturation is significant; (*c*) whether non-scorbutic degrees of "unsaturation" are dangerous to health. This last point is to be distinguished from such questions as to whether a scorbutic animals get infected more easily than a vitamin A or D deficient animal. [See Perla & Marmorston (45) for a review of these questions.] In regard to (*a*) Abbasy, Hill & Harris (46) have stated from massed experiments upon hospital patients that rheumatic, rheumatoid, arthritic, and tubercular patients show upon the whole low excretion values even when being dosed with liberal supplies of orange juice, the amounts of which were controlled in one paper. Finkle (47) found generally low excretory values in hospital patients.

As for (*b*) there are two definitions of saturation. Some consider that the rise in excretion following one or, at the most, three test doses of large amounts of ascorbic acid is sufficient to give evidence

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A STUDY OF TISSUE RESPIRATION AND CERTAIN REDUCING SUBSTANCES IN CHRONIC FLUOROSIS AND SCURVY IN THE GUINEA PIG

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Attention has been called to the marked similarity of the symptoms of fluorosis and scurvy in the guinea pig (17). A suggestion was made that chronic fluorine poisoning interfered in some manner with ascorbic acid or its function in the animal body. Studies on the distribution of a reducing substance (vitamin C) in the tissues of fluorine-fed cows as well as respiration of the suprarenal cortex indicated that ascorbic acid or vitamin C and respiration were disturbed in chronic fluorine poisoning (19). It is known that cattle synthesize vitamin C. The vitamin C content in the suprarenal gland and anterior lobe of the hypophysis increases in the fluorine-poisoned rat (18), another species known to synthesize vitamin C. These observations suggested that the mechanism of fluorine toxicosis and the mode of action of vitamin C could be profitably studied further in a species susceptible to scurvy.

Szent-Györgyi (24) in 1928 showed that hexuronic acid, now called ascorbic acid (vitamin C), played a rôle in certain biological oxidation-reduction mechanisms and suggested that vitamin C might have a similar function in the animal body (25). Because ascorbic acid reduced hemin to a green chromagen in air, von Euler and Malm (4) concluded that it functioned as a catalyst in oxidation-reduction enzyme systems. The evidence of Purr (20) and of Karrer and Zehender (11) suggests that ascorbic acid is capable of activating intracellular proteinases and in this respect it resembles glutathione and cysteine. Purr suggested that protein decomposition was dependent upon the presence of vitamin C,

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and that the interaction of glutathione and ascorbic acid regulates the intracellular protein metabolism.

The distribution of ascorbic acid has been studied by various laboratories. Birch, Harris, and Ray (3), Bessey and King (1), Svirbely (23), and others have shown by the use of chemical methods that ascorbic acid is widely distributed throughout the body, and that it is highest in the more actively metabolizing tissues. The suprarenal cortex has long been known to be relatively rich in ascorbic acid. More recently Harris and Ray (6), and Huszak (10) have shown that the medulla of the suprarenal gland is likewise rich in ascorbic acid.

The distribution of glutathione in scorbutic animals has not attracted much attention. Sato and Ohata (21) concluded from a few scorbutic guinea pigs that glutathione was increased in the adrenal gland (about 43 per cent) over a like number of controls. Since the number of animals was few and the controls lost considerable weight during the experiment, an accurate interpretation of the data is difficult. Wolff and Manjean (27) reported glutathione values for a few organs from normal guinea pigs.

The importance of glutathione in cellular respiration has been shown by Hopkins and Elliott (9). Birch and Dann (2) studied the distribution of ascorbic acid and glutathione in animal tissues and suggested that ascorbic acid and glutathione might be two linked factors in one system of oxidation in the animal cell.

There is a paucity of data on the effect of scurvy upon tissue respiration. Söderström and Törnblom (22) measured the oxygen consumption of normal and scorbutic guinea pigs under basal conditions and found a reduction of O_2 consumption per minute per unit of body surface but concluded that their animals were too few in number to warrant the conclusion that the reduced O_2 consumption was due to vitamin C deficiency. Contrary to these results Mosonyi and Rigó (16) found the gaseous metabolism of scorbutic guinea pigs increased above normal. The administration of ascorbic acid reduced the gaseous metabolism in scorbutic guinea pigs and increased it in normal animals. Harrison (7) found that the rate of oxygen uptake, measured in an atmosphere of oxygen, of slices of liver from scorbutic guinea pigs was less than that of liver slices from normal animals, and that the *in vitro* addition of ascorbic acid increased the rate of oxygen uptake of

the scorbutic liver but did not increase the uptake of the normal liver. On the other hand it is known that NaF inhibits cellular and tissue glycolysis.

The hypertrophy of the suprarenal gland during fluorine toxicosis and scurvy (17, 18) indicates that the vital processes of the gland itself may be affected. Hartman (8) and Lockwood and Hartman (15) have called attention to the supplementary effect of cortin to diets low in vitamins C and B_1. Thus a summary of the literature indicates that ascorbic acid (vitamin C) as well as glutathione may be concerned with tissue respiration and because of their oxidation-reduction properties function as proteolytic enzyme activators, that both substances have a systemic distribution which is most pronounced in the vitally active organs, and that little or no data are available on the influence of fluorosis upon these substances and their respective physiological functions.

The aim of this study was to determine the effect of fluorosis and of scurvy upon the distribution of the 2,6-dichlorophenolindophenol and the iodine-titratable substances (ascorbic acid) (glutathione), and upon the tissue respiration of several tissues from guinea pigs.

EXPERIMENTAL

Guinea pigs varying in weight from 140 to 350 gm. were divided into seven lots. The first three lots had ten animals each, while Lots IV to VI inclusive contained five animals each and Lot VII contained six animals. The basal ration used was the diet formerly used in this laboratory and consisted of rolled oats 69 parts, autoclaved alfalfa hay 25 parts, casein 5 parts, and 1 part NaCl.

The animals were fed the basal diet and 1 cc. of orange juice for 4 days preliminary to starting the experiment proper, in order to accustom them to handling and to their quarters. They were then placed upon the following regimen.

Lot I. Basal ration only (negative controls)
" II. " " + 3 cc. orange juice daily
" III. Diet of Lot II + F (25 mg. per kilo of body weight)
" IV. Basal ration + 6 cc. orange juice daily
" V. Diet of Lot IV + F (25 mg. per kilo of body weight)
" VI. Basal ration + 12 cc. orange juice + 25 mg. F per kilo of body weight
" VII. Basal ration + pure ascorbic acid (1 to 2 mg. daily for 25 to 28 days)

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The influence of scurvy and fluorosis upon the distribution of ascorbic acid and glutathione is shown in Table I. An inspection of the data in Table I shows that scurvy produces a decline in the amount of ascorbic acid present per unit of weight in all the tissues studied. A decrease of approximately 50 per cent occurred in the liver, kidney, and pancreas. Practically complete disappearance occurred in the anterior lobe of the hypophysis. Because of the smallness of the gland, the material available for

TABLE I

Influence of Fluorosis and Scurvy upon Reducing Substances in Tissues of Guinea Pigs

	Lot I. Basal ration (scorbutic)	Lot II. Basal + 3 cc. orange juice daily	Lot III. Basal + 3 cc. orange juice + NaF daily	Lot IV. Basal + 6 cc. orange juice daily	Lot V. Lot IV diet + NaF	Lot VI. Basal + 12 cc. orange juice + NaF
Average vitamin C values expressed in mg. per gm. fresh tissue						
Liver	0.039	0.074	0.074	0.155	0.161	0.232
Kidney	0.024	0.051	0.041	0.079	0.064	0.115
Pancreas	0.033	0.066	0.089	0.139	0.134	0.259
Hypophysis (anterior lobe)	Trace	0.544	0.491	0.747	0.300	0.518
Mg. glutathione calculated from iodine titration and expressed in mg. per gm. fresh tissue						
Liver	6.73	5.73	6.76	5.01	5.50	6.72
Kidney	3.68	3.79	3.67	3.72	3.69	3.52
Pancreas	3.03	2.83	3.08	2.84	2.76	7.89

analysis in this tissue makes the error admittedly large. However, a consistent reduction in the ascorbic acid content of the gland was obtained in the animals fed fluorine. In view of the vital hormonal relationships associated with this gland the importance of this result should not be overlooked. Fluorine did not apparently influence the ascorbic acid content of the other tissues. The ingestion of increased quantities of ascorbic acid, or vitamin C, causes a corresponding rise in the ascorbic acid content in the tissues studied, regardless of fluorine ingestion.

Scurvy and fluorine toxicosis were found to be alike in that they

caused an increase in the amount of reducing substance, or substances reacting with iodine (presumably glutathione) in the liver. The extent of the increase was approximately 18 per cent at ordinary levels of vitamin C feeding, and about 10 per cent when the vitamin C feeding was doubled. No appreciable change was apparent in the iodine-titratable substances in the kidney or pancreas, although the latter seemed to be slightly increased in the fluorine-fed animals.

TABLE II

Ascorbic Acid (Vitamin C) Found in Suprarenal Gland. Data Averaged by Lots

	Weight of suprarenal gland		Ascorbic acid per gm. fresh tissue	Total ascorbic acid present in suprarenal	
	Single gland	Both glands		Single gland	Both glands
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
Lot I. Basal ration (scorbutic)	102.7	169.8	0.150	0.015	0.025
Lot II. Basal + 3 cc. orange juice	92.1	157.7	0.474	0.044	0.075
Lot III. Lot II diet + NaF	96.0	202.9	0.277	0.027	0.056
Lot IV. Basal + 6 cc. orange juice	87.5	180.0	0.476	0.042	0.086
Lot V. Basal + 6 cc. orange juice + NaF	113.0	240.0	0.528	0.060	0.127
Lot VI. Basal + 12 cc. orange juice + NaF	110.6		1.012	0.112	
Lot VII. Basal + crystalline ascorbic acid	67.4	138.6	0.25	0.017	0.035

Since ascorbic acid has been intimately associated with the suprarenal gland, the data obtained on the distribution of ascorbic acid in this gland in chronic fluorine toxicosis and scurvy have been compiled in Table II. It is seen that the ascorbic acid content of the suprarenal gland was relatively and absolutely decreased in scurvy. The first animals removed from the experiment were in the early stages of scurvy, or fluorosis, as the case happened to be. Withholding the first two animals removed from the experiment in Lot I from the average used to show the mg. of

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ascorbic acid per gm. of fresh tissue reduces the value from 0.15 mg. to 0.10 mg. of ascorbic acid per gm. of tissue. Similarly animals fed the fluorine exhibited various degrees of severity of fluorotic symptoms when sacrificed. While not entirely uniform and of the same grade of fluorine toxicosis, no animal was assayed unless fluorotic symptoms had appeared.

Symptoms of fluorosis usually began to appear between the 22nd and 26th days, and generally developed into a well defined syndrome of fluorine toxicosis within 10 to 15 days. Lot V which also received fluorine appears to disrupt the trend of the data in the first three lots. However, two animals from this lot were removed in the early stages of the development of fluorosis and it was found that the ascorbic acid values of the suprarenal gland were well above the average of our control lot and contained respectively, 0.815 and 0.70 mg. of ascorbic acid per gm. of fresh tissue. If these two animals are disregarded the ascorbic acid content decreases from 0.528 mg. per gm. to an average of 0.353 mg. per gm. of tissue for the lot which may be considered at least near the border line of scurvy. 12 cc. of orange juice maintained the ascorbic acid content of the suprarenal gland but did not prevent the appearance of the clinical symptoms of fluorosis. These symptoms of fluorosis simulating those of scurvy were in most cases present at the time the animals were sacrificed in this lot.

We regret that insufficient material for analysis makes a clear cut conclusion concerning the influence of fluorosis and scurvy upon the glutathione content of the suprarenal gland exceedingly hazardous. The glutathione content seemed to be increased in the fluorine-poisoned animals. Since the glutathione was calculated from the excess iodine above that required to titrate the vitamin C, or ascorbic acid, the latter when considerably reduced in amount, as in the case of fluorosis, materially influences the amount of the glutathione present in the gland.

Table III gives the range of values and the averages for the rate of respiration and the cyanide inhibition for the liver tissue as measured in glucose, lactate, and succinate substrates and in plain buffer. When the average results are determined in glucose substrate little difference is observed among the various lots. The oxygen uptake averages close to 870 c.mm. per gm. per 2 hours and the cyanide inhibition is about 60 per cent. The oxygen

uptake for Lot VII is somewhat lower than for the other groups. Similarly the average results for oxygen uptake and cyanide inhibition measured in lactate substrate, succinate substrate, and in plain buffer show little variation among the different lots, though a difference is observed in the values obtained with the various substrates.

TABLE III

Oxygen Uptake of Liver Tissue from Guinea Pigs. Measured at 37° in Phosphate-Ringer's Solution Buffer, pH 7.3

Values are reported in c.mm. of O_2 per gm. of tissue, wet weight, per 2 hours.

	O_2 uptake, no substrate	Per cent CN inhibition	O_2 uptake, glucose	Per cent CN inhibition	O_2 uptake, lactate	Per cent CN inhibition	O_2 uptake, succinate	Per cent CN inhibition
Lot I. Basal ration (scorbutic)	915 (2)*	55	950 (8)	60	1120 (7)	45	1980 (3)	31
Lot II. Basal + 3 cc. orange juice	990 (2)	50	800 (8)	49	1200 (7)	51	1940 (1)	
Lot III. Lot II diet + NaF	890 (2)	52	850 (7)	52	1088 (5)	45	1680 (2)	21
Lot IV. Basal + 6 cc. orange juice	840 (1)	52	830 (3)	62	990 (4)	43	1710 (1)	15
Lot V. Basal + 6 cc. orange juice + NaF	820 (1)	56	930 (4)	70	1120 (3)	52	1850 (2)	22
Lot VI. Basal + 12 cc. orange juice + NaF			850 (4)	56	1150 (4)	50		
Lot VII. Basal + crystalline ascorbic acid			760 (3)	52				

* The numbers in parentheses indicate the number of animals used for calculating the values obtained.

The results of the rate of oxygen consumption and cyanide inhibition determined in plain buffer are very similar to the values obtained in glucose buffer. It appears as though the addition of glucose *in vitro* does not increase the rate of oxygen consumption of liver tissue when measured over a period of 2 hours.

The data presented in Table III indicate that scurvy and fluorine poisoning have relatively little effect on the rate of oxygen uptake and the cyanide inhibition of liver tissues of guinea pigs

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as measured under the conditions described in this paper. These results do not confirm the conclusions made by Harrison (7), that the rate of oxygen uptake of liver tissue from scorbutic guinea pigs is lower than that of liver from normal animals. However, the results obtained are not strictly comparable with Harrison's (7)

TABLE IV

Effect of Ascorbic Acid (Karrer), in Vitro, on Oxygen Uptake of Liver Tissue from Guinea Pigs. Measured at 37° in Glucose-Phosphate-Ringer's Solution Buffer, pH 7.3

Values are reported as c.mm. of O_2 per gm. of tissue, wet weight, per 2 hours.

	Guinea pig No.	No ascorbic acid added		0.12 mg. ascorbic acid added		Change on addition of ascorbic acid	
		O_2 uptake	Cyanide inhibition in per cent of total	O_2 uptake	CN inhibition in per cent of total	Absolute	Per cent
Lot I. Basal ration (scorbutic)	9*	980	54	1040	46	+60	+6
	10*	850	55	1050	74	+200	+24
Lot II. Basal + 3 cc. orange juice	8*	1300	55	820	34	−480	−37
	9	900	54	720	11	−180	−20
	10	740	51	770	52	000	00
Lot III. Lot II diet + NaF	8	1020	73	1050	66	+30	+3
	9	860	40	940	40	+80	+9
Lot VII. Basal + crystalline ascorbic acid							
(Link)	1	710	41	780	53	+70	+10
(Karrer)	1	640	70	640	47	00	00
(Bauman)	1	930	46	990	58	+60	+6

* These determinations were made in plain phosphate-Ringer's solution buffer; no added substrate.

data, since he made his measurements in plain phosphate buffer on slices of liver tissue rather than on minced tissue and in an atmosphere of oxygen rather than of air.

Harrison also reported that the *in vitro* addition of ascorbic acid brought about an increase in the oxygen uptake of scorbutic liver slices. Even though the results reported in Table III show little difference in the rate of oxygen consumption of the liver tissue

from the scorbutic animals as compared with the normal animals, it would be of interest to observe the effect of *in vitro* addition of ascorbic acid on the oxygen uptake measurements, especially since a diminution of vitamin C in the liver tissue was detected in the scorbutic group (Lot I). A few experiments on the effect of *in vitro* addition of 0.12 mg. of ascorbic acid on the rate of oxygen consumption of liver tissue have been carried out and the results are given in Table IV. These determinations as well as all of the other determinations of oxygen uptake on liver tissue were made on samples of liver weighing in the region of 200 mg. The addition of 0.12 mg. of ascorbic acid represents a concentration of 5 times the average amount found in 200 mg. of normal liver tissue. Harrison in his experiments added 0.25 mg. of ascorbic acid and used tissue slices averaging 200 mg. From Table IV it is readily seen that the effect of addition of ascorbic acid is not very pronounced and is quite variable. There appears to be a slight tendency to increase the respiration of the liver from the scorbutic guinea pigs and not to affect or to decrease slightly the respiration of the liver from the normal guinea pigs. The data presented are insufficient from which to draw definite conclusions.

Table V gives the average time required for extracts of the liver tissue to decolorize methylene blue in the presence of various substrates and the effect of cyanide on the decolorization time.

Examination of Table V readily shows that the oxidation of succinate occurs much more rapidly than does the oxidation of lactate, and the oxidation of lactate occurs more rapidly than the oxidation of glucose or formate. The decolorization time of methylene blue is not decreased significantly by the addition of glucose or formate as compared to the decolorization time in plain buffer. A few experiments not listed in Table V were performed with tartaric acid, maleic acid, arabinose, and xylose as substrates. With none of these substrates was the decolorization time decreased as compared to the time required when no substrate (plain buffer) was used, and with tartaric acid and maleic acid a definite inhibiting effect on the decolorization of methylene blue was observed. In all cases cyanide increased the time required for decolorization.

Comparing the decolorization time of the liver extracts from the various groups of guinea pigs, one observes no great difference.

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The scorbutic group (Lot I) seems to be slightly more active in decolorizing the dye. This effect is observed with all of the substrates and also with the plain buffer. Fluorine poisoning tends to slow down the decolorization of methylene blue in succinate substrate as may be seen when the fluorine-fed lots (Lots III and V) are compared with their normal control lots (Nos. II and IV). With Lot VI, the guinea pigs fed on the high orange juice level, there is a definite trend toward more rapid decolorization of methylene blue in all of the substrates used. Even though the guinea pigs in Lot VI received fluorine, the high level of orange

TABLE V

Decolorization of Methylene Blue by Extracts of Livers from Guinea Pigs. Experiments Run at 37°, pH 7.3, in Thunberg Tubes

Values are reported in minutes for 80 per cent decolorization.

	No. of guinea pigs	Average decolorization time of methylene blue in min. Various substrates				
		Succinate	Lactate	Glucose	Formate	No substrate
Lot I. Basal ration (scorbutic)	8	6	20	43	55	64
" II. " + 3 cc. orange juice	9	7	29	64	61	72
Lot III. Lot II diet + NaF	9	8	27	51	38	58
" IV. Basal + 6 cc. orange juice	5	6	40	56	86	90
" V. " + 6 " " " + NaF	5	7	30	67	83	88
Lot VI. Basal + 12 cc. orange juice + NaF	4	5	16	38		

juice fed (12 cc. per guinea pig per day) seemed to counteract the inhibiting effect observed with succinate buffer in the fluorine-fed lots on the lower levels of orange juice (Lots III and V).

The indophenol oxidase content was estimated by the ability of the liver extracts to cause the oxidation of the Nadi reagent to indophenol. The researches of Keilin (12–14) have shown that indophenol oxidase is the active agent in the oxidation of cytochrome and thus it assumes an important part in the oxidative mechanisms of the tissues. It was observed that the relative concentration of indophenol oxidase is distinctly decreased in the liver extracts prepared from the scorbutic guinea pigs and the

guinea pigs in the fluorine-fed groups as compared with the two control lots (Lots II and IV). When the oxidase tests were conducted on extracts heated at 45° for ½ hour, it is observed that the general effect was to decrease the sensitivity of the test, in other words the indophenol oxidase of the liver tissue is slightly heat-labile when held at 45° for ½ hour.

Since a decrease in the indophenol oxidase content as measured by the oxidation of the Nadi reagent was observed in the liver extracts from the scorbutic and the fluorine-fed animals, it was of interest to observe the cytochrome spectrum. Slices of liver, kidney, and heart tissue were reduced with sodium hydrosulfite, placed between two glass slides, and examined with a microspectroscope for the cytochrome absorption spectrum. In tissues from animals in all lots a distinct absorption spectrum of reduced cytochrome was observed. By such simple qualitative examination of the absorption spectrum little indication is given as to the relative amount of cytochrome; however, it could readily be observed that the concentration of cytochrome was greatest in heart tissue, though there was no decided decrease in any of the components of the absorption spectrum in the tissues from guinea pigs in the various lots.

Because ascorbic acid is concentrated in the suprarenal gland and because in scurvy the amount of ascorbic acid in this gland is markedly decreased, it would be of interest to compare the rates of oxygen consumption of suprarenal tissue from scorbutic and normal animals and with fluorine-poisoned animals.

The literature contains few reports of the rate of oxygen consumption of the suprarenal tissue. Von Euler, Myrbäck, and Larsson (5) reported that the oxygen uptake of the medulla of the suprarenal gland of beef cattle was greater than that of the cortex. Similar observations on dairy cows were recorded by Phillips and Stare (19). No observations on the rate of oxygen consumption of the suprarenal tissue from guinea pigs were found in the literature.

In Table VI are given the range of values and the averages for the rate of oxygen uptake and the cyanide inhibition of the suprarenal tissue as measured in glucose-phosphate-Ringer's solution buffer. When the average rate of oxygen consumption of the tissue from the scorbutic group (Lot I) is compared with that of

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the two normal groups (Lots II and IV), it is quite obvious that scurvy produces a marked decrease in the oxygen consumption of the tissue of the suprarenal gland. The average rate of oxygen uptake of the tissue from the scorbutic group was 455 c.mm. per gm. per 2 hours, a decrease of 55 per cent as compared with the controls receiving 3 cc. of orange juice per day (Lot II), and compared with the controls receiving 6 cc. of orange juice per day (Lot IV) a decrease of 66 per cent. A decrease in the rate of respiration of the same order is observed in the average values for

TABLE VI

Oxygen Uptake of Suprarenal Tissue from Guinea Pigs. Measured at 37° in Glucose-Phosphate-Ringer's Solution Buffer, pH 7.3

Values are reported as c.mm. of O_2 per gm. of tissue, wet weight, per 2 hours.

	No. of guinea pigs	Average values	Average cyanide inhibition in per cent of total
Lot I. Basal ration (scorbutic)	8	455	10
" II. " + 3 cc. orange juice	8	1000	33
" III. Lot II diet + NaF	8	540	45
" IV. Basal + 6 cc. orange juice	4	1335	69
" V. " + 6 " " " + NaF	4	525	100
" VI. " + 12 " " " + NaF	4	605	91
" VII. " + crystalline ascorbic acid			
(Link)	2	930	
(Karrer)	2	1135	
(Bauman)	2	680	

the suprarenal tissue from the three fluorine-fed groups (Lots III, V, and VI). The suprarenal tissue from the guinea pigs in Lot VII gives a rate of oxygen consumption equally comparable with the values of the two normal groups. The low average value for the two guinea pigs in Lot VII is considerably lower than for the two other ascorbic acid-fed groups. This low figure is due largely to the low result of one of the guinea pigs which for some reason objected strenuously to swallowing the ascorbic acid, and it is probably not due to a less active ascorbic acid preparation.

When the effect of cyanide on the rates of oxygen uptake of

the suprarenal tissue is compared, considerable difference is observed among the various lots. Cyanide produced only a 10 per cent inhibition in the scorbutic group. If ascorbic acid functions prominently in the oxidative metabolism of the suprarenal gland, one would expect little cyanide inhibition on the rate of oxygen uptake of tissue from scorbutic animals, since the ascorbic acid, even though its reversible oxidation-reduction characteristic is inhibited by cyanide, is practically nil in the suprarenal tissue from scorbutic guinea pigs. When Lots II and III are compared, both of which were on the lower level of orange juice and one of which received fluorine, the cyanide inhibition is found to increase in the fluorine-fed group. This same phenomenon is observed in comparing Lots IV and V which were fed with the higher level of orange juice. Likewise, in the other fluorine-fed group (Lot VI) a high cyanide inhibition was observed. It should be mentioned that a considerably greater variation was obtained between the cyanide inhibition values of the suprarenal tissue of the individual guinea pigs of the various lots as compared with the variation in the rates of oxygen uptake. Too much emphasis should not be placed on these findings until they are repeated, though it appears that in fluorine poisoning there is a tendency to increase the cyanide-inhibited respiration factor, and curiously, with the exception of Lot III, the extent of the cyanide inhibition is roughly proportional to the amount of ascorbic acid found in the suprarenal tissue.

Since such a marked decrease was observed in the rates of oxygen uptake of the suprarenal tissue of scorbutic and fluorine-poisoned guinea pigs as compared with normal controls and because the ascorbic acid content of this tissue is very low in scorbutic animals, it would be of interest to study the effect of *in vitro* addition of ascorbic acid on the rate of oxygen consumption. A few experiments of this order were performed. It was observed that the rate of respiration of the suprarenal tissue from two scorbutic guinea pigs (only two animals available) was increased very materially by the *in vitro* addition of ascorbic acid. With animals in Lots II and III the effect observed was variable. With Lot VII the same phenomenon was observed as was with the *in vitro* addition of ascorbic acid to the liver tissue from this lot; namely, the *in vitro* addition of the same preparation of ascorbic acid as

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fed to the animals (Karrer's preparation) did not cause an increase in the rate of oxygen consumption; however, when this ascorbic acid was added to the suprarenal tissue obtained from the guinea pigs in Lot VII, which were fed other ascorbic acid preparations, a considerable increase in the rate of oxygen uptake was observed. The results on the effect of *in vitro* addition of ascorbic acid on the rate of oxygen uptake of suprarenal tissue were too few in number to warrant drawing any generalizations, but they point to an interesting study.

DISCUSSION

The nature of the oxidative mechanisms involved in the chemical reactions constituting tissue respiration is extremely complex, and our present knowledge of the subject is quite meager. The facts emerging from the experimental data reported in this paper indicate that the activity of certain cellular oxidative mechanisms is intimately associated with the syndromes of scurvy and fluorine poisoning. A correct interpretation of the data relative to scurvy and fluorine toxicosis is limited by the extent of the experimentation and by the limited knowledge of the intracacies of the respiration of animal tissues.

The results obtained with the liver tissue were consistent and a logical interpretation of them may be presented. The total respiration as represented by the rate of oxygen consumption remained fairly constant in the animals from the various groups. The inhibiting effect of cyanide on the rate of oxygen uptake was similarly quite constant. Qualitative tests for indophenol oxidase with the Nadi reagent were markedly less in the livers of the scorbutic and the fluorine-poisoned animals as compared with the concentration found in the livers from the normal control animals. According to Keilin (14) indophenol oxidase functions in oxidizing the cytochrome reduced in the dehydrogenation of the tissue metabolites. A decrease in the indophenol oxidase content of a tissue would lower the effectiveness of the oxidase-cytochrome system and unless the activity of other oxidative systems was not increased one would expect the rate of oxygen consumption to be diminished. Since indophenol oxidase is inactivated by cyanide any decrease in respiration from a lowering of the oxidase could be detected by a lowering of the cyanide-inhibited respiration

fraction. It was found that the rate of oxygen consumption and the cyanide inhibition of the rate of oxygen consumption of the liver tissue from scorbutic and fluorine-poisoned animals were not significantly changed from the values obtained with tissue from normal animals, therefore the experimentally observed decrease in indophenol oxidase must have been compensated for by an increase in the activity of some other component of the cyanide-inhibited respiration fraction.

The glutathione content of liver tissue increased in scurvy and in fluorine toxicosis. In normal tissue respiration glutathione may function in a manner similar to cytochrome. The oxidation of reduced glutathione is dependent on metals which are poisoned by cyanide, thus glutathione is a part of the cyanide-inhibited respiration fraction. The observed increase in glutathione content suggests that it has assumed the function formerly performed by the oxidase-cytochrome system. If this assumption were true it would be predicted that the total respiration would remain the same and that the fraction inhibited by cyanide would remain the same. The data show this to be the case. In so far as these results are concerned no other component seemed to be involved in the respiration of the liver tissue. Lowering the vitamin C content of the liver by scurvy produced no marked changes in oxygen uptake. The addition of pure ascorbic acid to minced liver did not significantly change the oxygen uptake in tissue obtained from either the scorbutic, the fluorine-poisoned, or the normal animals.

The results with the decolorization of methylene blue by extracts prepared from the livers from the animals on the different diets do not differ enough to draw any correlations with the syndromes of scurvy or fluorine poisoning. These results indicate that apparently scurvy or fluorine toxicosis has no effect on the hydrogen-activating systems.

In the case of the suprarenal gland the quantity of material available for study from each animal was so small as to handicap seriously a complete study such as that made upon the liver. No material was available for testing this tissue with Nadi's reagent. The quantity available for iodine titration was too meager for reliable results. Thus the lack of complete and accurate data relative to these two substances is somewhat disappointing. Had

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complete data been possible perhaps it would permit of a more rational interpretation.

The results obtained with the suprarenal gland in other respects are extremely interesting. The most likely explanation for the hypertrophy of the gland, it seems to us, is a compensatory effort on the part of the organism to maintain an indispensable function. We are not unmindful of the somewhat prevalent opinion that the suprarenal function is concerned with water metabolism and that the increase in weight might be due to additional fluids in the gland. Regardless of what the ultimate cause may be shown to be, we are chiefly concerned with the fact that its weight does increase.

Other pertinent facts concerning the suprarenal gland have been obtained. The oxygen uptake fraction inhibited by cyanide is very low in the case of scurvy and high in fluorine poisoning. Apparently the efficiency of the gland is decreased in either syndrome. As to the mechanism involved in the respiration of the suprarenal gland, we cannot state. It seems not unlikely that the following offered merely as a suggestion may in part rightfully interpret our results. The ascorbic acid content of the suprarenals decreases relatively and absolutely in scurvy and fluorine toxicosis. Since the addition of ascorbic acid to suprarenal tissue *in vitro* caused the return of normal oxygen uptake, it suggests immediately that ascorbic acid seems to function as an oxidation-reduction intermediary agent. The cyanide action upon oxygen uptake would support this view. The cyanide-inhibited portion of respiration is decreased in scurvy, perhaps due to the decrease in ascorbic acid. This would be in conformity with Szent-Györgyi's (24) finding that the oxidation-reduction activity of ascorbic acid was inhibited by cyanide. In the fluorine-fed animals the CN-inhibited portion of respiration was definitely increased. The vitamin C content of the suprarenal gland in these cases, while appreciably lowered, was not depleted to the extent that it was in the case of scurvy. If this quantity of vitamin C were functional it might account for the CN inhibition of respiration. However, the slight response of glutathione in the fluorine-fed animals would also tend to increase this portion of the respiration of the gland. On the other hand large quantities of orange juice fed in Lot VI (12 cc. daily) indicate that the ascorbic acid content of the gland can be maintained in fluorosis and yet the

total O_2 uptake is significantly decreased from the normal. Thus the evidence in the case of fluorine points to one of two alternative conclusions. Either the fluorine inactivates the ascorbic acid as far as the animal economy is concerned, or the fluorine specifically inhibits an enzyme system in which ascorbic acid is involved. The latter point of view seems more in keeping with the known facts concerning fluorine action in the body. Thus it would seem plausible to suggest that ascorbic acid has a function in the respiration of the suprarenal gland. In scorbutus the ascorbic acid eliminates the major portion of this system while in fluorosis some enzyme link of the oxidative system is inhibited.

Other observations made in these studies require some comment. It seems not unimportant that the ascorbic acid content of the anterior lobe of the hypophysis should follow the general relationship to scurvy and fluorosis that were found to exist in the suprarenal gland. The known important hormonal function of this gland requires no comment. Should the relationship to cellular metabolism be found to follow those shown to exist for the suprarenal gland, the action of fluorine and ascorbic acid would be further elucidated. The similarity of the action of fluorine and scurvy are again demonstrated. The symptomatic likeness was mentioned previously. In these studies the effect of fluorine and scurvy upon the liver was alike in that both syndromes eliminated the indophenol oxidase in so far as this could be detected by Nadi's reagent. Both diseases cause the suprarenal gland to hypertrophy. The oxygen uptake of the suprarenal tissue was uniformly decreased in both diseases approximately 50 per cent. The CN-inhibited portion of the total respiration was dissimilarly affected but the likelihood of this result being caused by a disturbance in the same system may explain this difference. The ascorbic acid content was decreased in scurvy and fluorosis. In the latter the decrease was much less marked but nevertheless uniformly lowered. There was a tendency for glutathione to increase in the fluorine-fed groups as compared to scorbutic and normal control animals. In these respects chronic fluorosis and scurvy have been found to exert much the same physiological response in the liver and suprarenal gland. The symptoms of scurvy cannot be directly associated with the decrease of ascorbic acid in the liver but may be directly associated with the decrease of ascorbic acid in the suprarenal gland.

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SUMMARY

1. Ascorbic acid is widely distributed in the tissues of the normal guinea pig and is concentrated in the anterior lobe of the pituitary and in the suprarenal gland. It may be decreased to subfunctional levels and increased to relatively high values by varying the ascorbic acid content of the diet.

2. The rate of oxygen uptake of liver tissue and the inhibiting effect of cyanide is not significantly different for liver tissue obtained from scorbutic, fluorine-poisoned, or normal guinea pigs.

3. Decolorization time of methylene blue by extracts prepared from liver tissue from scorbutic, fluorine-poisoned, or normal guinea pigs is not significantly different.

4. The rate of oxygen uptake, the inhibiting effect of cyanide, and the decolorization time of methylene blue are all dependent on the substrate in which they are measured. Succinate is most rapidly oxidized, lactate next, and formate and glucose differ little from plain buffer solution without added substrate.

5. The indophenol oxidase content as qualitatively estimated by the coloration of the Nadi reagent is markedly diminished in the liver tissue of scorbutic and fluorine-fed guinea pigs as compared with normal controls, and the glutathione content increases, suggesting a compensating action on the part of glutathione.

6. Cytochrome is present in the heart, kidney, and liver tissue from scorbutic, fluorine-poisoned, and normal guinea pigs.

7. The rate of oxygen uptake of the suprarenal tissue from scorbutic and fluorine-poisoned guinea pigs is about half that found in the tissue from normal control animals. In the case of two scorbutic animals (only two animals available) the oxygen uptake was increased to the normal value by the *in vitro* addition of ascorbic acid. The cyanide-inhibited fraction of the oxygen uptake of the suprarenal tissue is very low in scorbutic animals, high in fluorine-poisoned animals, and in general is proportional to the amount of vitamin C in the tissue.

8. Ascorbic acid is definitely associated with a considerable portion of the cyanide-inhibited intracellular respiration of the suprarenal gland of the guinea pig.

9. The similarity between scurvy and fluorosis is thus confirmed and extended by these researches. The evidence obtained indicates strongly that ascorbic acid deficiency and the deleterious effect of chronic fluorine toxicosis result primarily from disturbances in specific phases of cellular respiration.

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Effect of Peroral Ascorbic Acid on Blood Methemoglobin of Newborn Infants

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The methemoglobin content of healthy adults expressed as per cent of total hemoglobin has been observed to range from 0.4–0.8%. The methemoglobin of infants has been found to be higher than that of adults, and many investigators have reported that prematures have the highest methemoglobin values [5, 6, 9]. Ascorbic acid lowers the blood methemoglobin content in patients with methemoglobinemia [3, 4, 10, 12].

The purpose of the present work was to study the concentration of methemoglobin in the blood of full-term newborn infants during the first day of life and the effect of ascorbic acid on the methemoglobin level.

Material and Methods

Eighty-one full-term newborn infants, 43 boys and 38 girls were studied in March–May 1967. The birth weights were 2350–4900 g and the Apgar score at birth was 2–10. No infant had generalized cyanosis, and later all infants were found healthy on physical examination.

At the age of ½–24 h, 0.5–1.5 cc of venous blood was taken from the femoral vein of each child. The blood methemoglobin content expressed as per cent of total hemoglobin was determined within one hour of taking the sample. Every other infant, 39 in all, was given 50 mg Ascorbic acid daily *per os* (Ascorbin drops, Orion). The control series (40 infants) received no ascorbic acid. After 4–7 days, a new blood sample was taken and the methemoglobin concentration was determined.

The determination of methemoglobin was performed by one of the authors with Beckman B spectrophotometer [8]:

Methemoglobin has a typical absorption maximum at 630 mμ. When methemoglobin is transformed to cyanmethemoglobin with potassium cyanide, the absorption maximum mentioned above partially disappears. A control solution is obtained when the hemoglobin is completely oxidized to methemoglobin by potassium ferricyanide. The decrease in extinction recorded after potassium cyanide treatment is compared with the latter extinction drop, from which the methemoglobin content in per cent of total hemoglobin can be calculated.

Results

The methemoglobin contents in various groups is expressed in table I. The mean methemoglobin concentration during the first day of life in 81 newborn infants was 0.75% of total hemoglobin (range 0.21–1.58%). The methemoglobin level seems to be independent of the infant's age during the first 24 h of life.

Mildly asphyctic children (with Apgar score 2–7 60 sec *post partum*) had mean methemoglobin concentration 0.95% during the first day of life. The methemoglobin of these infants remained higher than that of the normal series also in the later samples (table II).

The values in table I reveal no significant difference between the methemoglobin value during the first day of life and the value at the age of one week, with or without the ascorbic acid treatment.

Fig. 1 demonstrates that the methemoglobin level of 4–7 day depends slightly on the methemoglobin value of the first day of the infant's life.

Fig. 2 shows the methemoglobin percentages of both sample groups in the same plot. The straight line drawn through the mean

Table I. Methemoglobin values during the first day of life and at the age of 4–7 days

	No. cases	Mean metHb-%	Standard error of the mean	Range
Newborn infants (0–24 h)	81	0.754	±0.037	0.21–1.58
Newborn infants (4–7 days treated with ascorbic acid)	39	0.767	±0.045	0.00–1.35
Newborn infants (4–7 days, control series)	40	0.744	±0.045	0.25–1.29

Table II. Methemoglobin content versus Apgar score

	No. cases	Apgar score	Mean metHb-%	Range
Newborn infants (0–24 h)	12	2–7	0.95±0.11	0.31–1.58
Newborn infants (4–7 days)	12	2–7	0.88±0.08	0.25–1.27
Newborn infants (0–24 h)	69	8–10	0.72±0.04	0.21–1.52
Newborn infants (4–7 days)	69	8–10	0.73±0.04	0.00–1.35

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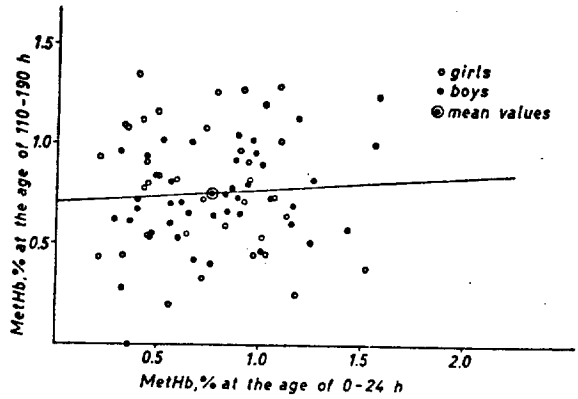

Fig. 1. The interdependence of the methemoglobin percentages of the two sample groups.

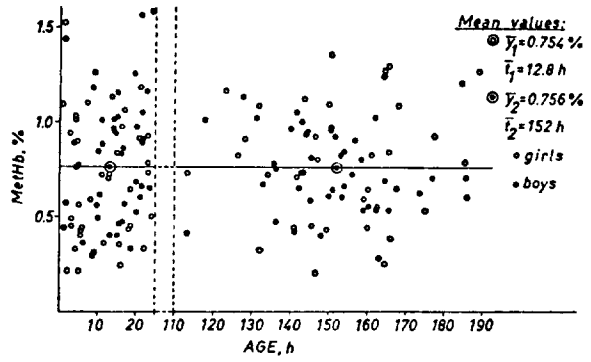

Fig. 2. Plot of methemoglobin concentration against the age during the first week of life.

values (y_1, t_1) = (0.754%, 12.8 h) and (y_2, t_2) = (0.756%, 152 h) is roughly parallel to the X axis.

In other words, the methemoglobin content in the blood of newborn infants during the first week of life remains roughly constant ($0.755 \pm 0.034\%$).

No correlation was established between the infant's birth weight and methemoglobin content. Neither were divergent methemoglobin values demonstrated in any infant whose mother was known to have taken drugs (e.g. sulfonamides) at the end of her pregnancy.

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Table III. Methemoglobin values reported by different authors

No. cases	Age	Mean metHb-%	Range	Reference
39	Newborn	0.47	0.2–1.3	KLEIHAUER [5]
27	1–4 months	0.82	0.3–1.5	KLEIHAUER [5]
39	1–10 days	1.5	0.00–2.8	KRAVITZ *et al.* [6]
8	1–12 months	1.2	0.07–2.4	KRAVITZ *et al.* [6]
35	1–14 years	0.79	0.00–2.4	KRAVITZ *et al.* [6]
81	0–24 hours	0.75	0.21–1.58	Present series
79	0–7 days	0.76	0.00–1.35	Present series

Discussion

Compared with earlier results (table III) the values obtained were of the same magnitude as those of KLEIHAUER [5]. The mean in the present study was a little higher than that of KLEIHAUER. On the other hand, our values were distinctly lower than those reported by KRAVITZ *et al.* for newborn and infants [6].

The differences in the values reported by different authors are suspected to arise not from inaccuracy of method but from environmental conditions such as the nitrate content of drinking water [5]. Higher methemoglobin values have been established in prematures than in full-term newborn [5, 6]. This has been ascribed to the tendency of fetal hemoglobin, more profuse in prematures, to oxidise more readily than adult hemoglobin. The present material included only one premature, weight 2350 g, whose methemoglobin was 0.50%. Higher than normal methemoglobin content of newborn has been attributed also to the lowered content of DPNH-dependent methemoglobin reducing diaphorase analyzed in cord blood sample [1, 2, 11]. As methemoglobin partly originates in intravascular hemolysis, destruction of the red cells of newborn also plays a role in the elevated methemoglobin concentration.

KRAVITZ *et al.* reported that the methemoglobin content rises during the first days of life [6]. We were unable to demonstrate this in our series. Our newborn with perinatal distress (Apgar score 2–7) displayed higher concentrations than our normal newborn (Apgar score 8–10). Drugs ingested by mother and duration of labour had no effect on methemoglobin content. No cyanosis was established in the children. Cyanosis caused by methemoglobinemia does not occur until levels of over 10% [5, 6].

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It has been assumed that ascorbic acid reduces methemoglobin directly, without any enzyme mechanism [1, 4]. MINDLIN has demonstrated the intestinal absorption of ascorbic acid in newborn infants [7]. Daily ascorbic acid doses of physiologic magnitude failed to lower the methemoglobin content in our series. This can be declared with the observation that fairly massive (70 g/l) ascorbic acid concentrations are necessary before a significant reduction occurs in the methemoglobin content *in vitro* [5].

Summary

The methemoglobin content of 81 healthy newborn was studied. It remained constant during the first week of life (0.755±0.034% of total hemoglobin). Perinatally distressed infants had significantly higher methemoglobin level (0.95%) than the normal series. The values obtained were compared with earlier reports. An ascorbic acid dose of physiologic magnitude (50 mg/day *per os*) had no influence on methemoglobin content.

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VITAMIN C IN CHRONIC LEAD POISONING.

AN EXPERIMENTAL STUDY.*

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IT has been suggested that vitamins may play a rôle in heavy metal poisoning, especially with reference to the nervous system. Williams and Spies[5] are of the opinion that, while thiamin does not improve the condition of patients with chemical neuritis produced by tri-ortho-cresyl phosphate, there are theoretical grounds for believing that large doses of vitamin B_1 would act as a prophylactic.

Holmes, Campbell and Amberg[4a,b] treated several cases of chronic lead poisoning in factory workers and painters with large doses of vitamin C (100 to 200 mg. of ascorbic acid daily above the normal intake) and presented data to show that the vitamin treatment was more effective in removing the symptoms of lead poisoning and restoring the blood picture, than is the usual therapy with calcium or the institution of more adequate methods for reducing exposure hazards. They claim that those painters who normally have a high vitamin C intake do not develop lead poisoning; the vitamin acts as a detoxifying agent by forming poorly ionized, but soluble, lead compounds, and conversely that lead leaves less vitamin available for physiologic purposes. This is reflected in the lower urinary excretion levels of ascorbic acid as well as resemblance of the tooth and gingival changes of lead intoxication to subclinical scurvy.

These conclusions can be tested experimentally since guinea pigs respond somewhat like humans to both lead intoxication and vitamin C deficiency. Also, the technique for detecting vitamin C changes in the blood by measuring the complement titer as developed by Ecker, Pillemer, Wertheimer and Gradis[3] is available. Furthermore, it has been shown in this laboratory[2] that certain heavy metal salts may *in vitro* reversibly inactivate serum complement. It was also demonstrated that a direct correlation existed between the ascorbic acid content of serum and its complementary activity. Therefore it was of interest to discover whether chronic lead poisoning would lead to a weakening of complement. It is evident that the influence of lead poisoning on this relationship is worthy of further study.

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Experimental. Two experiments were conducted, one involving 46 guinea pigs and the other involving 24. The animals were placed on a diet of Purina rabbit chow which is adequate in all respects excepting vitamin C. This is present only in traces, and guinea pigs develop scurvy in 21 days on this diet. The food was not autoclaved in order to avoid destruction of other vitamins. In addition to this diet, each animal received, on alternate days, a hypodermic injection of ascorbic acid as the buffered commercial product, Cevalin, as follows:

Experiment 1.		Experiment 2.	
Mg. vitamin C.	No. animals.	Mg. vitamin C.	No. animals.
1	28	1	8
20	18	2	8
		20	8

As established in this laboratory,[1] 0.5 mg. of ascorbic acid daily is the minimum amount necessary for the well-being of guinea pigs *provided that environmental conditions are good*, while 10 mg. daily is the saturation level and represents the intake of the guinea pig in its normal habitat where it is accustomed to drastic environmental changes. The animals were stabilized for 1 month on this routine before lead was added to the diet.

Lead was administered as the basic carbonate, $2PbCO_3.Pb(OH)_2$, placed in capsules and inserted in the esophagus. The following table indicates the dose of the lead compound (80% Pb) in mg. per kg. of body weight, 2 out of 3 days for 1 month.

Experiment 1.—Dosage of Lead.

	Median lead dosage in mg.		Range lead dosage in mg.	
Vitamin C dosage.	Start.	End.	Start.	End.
0.5 mg. per day	74	95	63–88	70–108
10 mg. per day	64	69	55–73	57–78

The doses are variable due to the fact that each animal received 40 mg. of lead compound per day, and they reflect the different weights, and at the end of the experiment the loss in weight. There is sufficient overlapping in the ranges to give a complete picture, and the variations are compensated for by the variability in absorption from the intestines. However, in the second experiment the administration was maintained at 80 mg. of basic carbonate per kg. of body weight, every 2 out of 3 days for 1 month.

Results. 1. *Weight Changes.* During the period of stabilization when no lead was administered, the animals on the low vitamin intake showed variation in weight; approximately one-third gained up to 10%, an equal number lost to a similar degree, and the remainder did not change. All of the animals in the high vitamin groups gained up to 15% of the original body weight. From Table 1 it will be seen that after 1 month's administration of lead, the animals receiv-

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ing the high vitamin level fared better than those on the lower intake. Of significance was the loss of 20% or more of body weight in 25% of the animals maintained on the minimal antiscorbutic level, whereas

TABLE 1.—WEIGHT CHANGES IN THE LEAD-TREATED GUINEA PIGS.

Treatment.	% loss.			% gain.		
	1–10.	11–20.	21–30.	1–10.	11–20.	21–30.
0.5 mg. Cevalin per day plus 40 mg. lead carbonate 2 out of every 3 days for 32 days . .	11	9	6	1		
Same lead dose plus 10 mg. Cevalin per day	12	2	1	2	1	
0.5 mg. Cevalin per day plus 80 mg. lead carbonate per kg., 2 out of every 3 days for 34 days .	5	..	3			
Same lead dose plus 1 mg. Cevalin per day	2	2	1	2		
Same lead dose plus 10 mg. Cevalin per day	2	2	..	4		

in the animals on higher vitamin intakes this figure was approached only in isolated instances. The variations in weight changes encountered were not sufficiently great to rule out the effects of adding an offending agent to the diet of animals already on subminimal conditions for health.

2. *Changes in the Blood Picture.* In both experiments, the degree of plumbism attained, as measured by erythrocyte counts, hemoglobin estimation and number of stippled cells, was not materially different in the animals on different vitamin C intakes. The data are summarized in Table 2.

TABLE 2.—CHANGES IN THE BLOOD PICTURE OF GUINEA PIGS IN CHRONIC PLUMBISM.

Exp.	Animal.	Stabilization period.			After 1 month of lead.		
		R.B.C.*	% Hb.†	Stipple cells.‡	R.B.C.	% Hb.	Stipple cells.
		0.5 Mg. Cevalin Daily.					
1 . .	A 5451	5.85	90	0	4.25	60	4
1 . .	A 5425	5.20	80	0	2.90	60	6
1 . .	A 5032	5.15	90	0	2.75	50	35
1 . .	A 5465	4.95	90	0	3.95	60	22
2 . .	A 5387	4.20	75	0	3.85	60	36
		1 Mg. Cevalin Daily.					
2 . .	B 5389	5.00	75	0	3.55	60	48
2 . .	B 5384	5.35	70	0	2.90	50	20
		10 Mg. Cevalin Daily.					
1 . .	B 5453	5.10	90	0	3.70	65	21
1 . .	B 4853	5.20	85	0	3.95	90	31
1 . .	B 5404	5.05	90	0	3.30	55	14
1 . .	B 5464	5.05	80	0	3.95	60	9
1 . .	B 5459	5.80	100	0	3.65	60	26
1 . .	B 5472	5.45	80	0	3.45	70	15
2 . .	C 5380	5.35	70	0	2.30	50	52
2 . .	C 5379	4.90	75	0	3.15	50	58

* Millions of red blood cells. † Tallqvist estimation.
‡ Number per 20 high-power fields.

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3. *Changes in Complement Function and Serum Ascorbic Acid Levels.* Ecker, Pillemer, Wertheimer and Gradis[3] showed that a parallelism exists between the ascorbic acid concentration of the guinea pig serum and its complementary activity, and that the parallelism holds true until a definite level of ascorbic acid is attained at about 1 mg. per 100 cc. of serum. At this point the complementary activity reaches its maximal efficiency. In the course of this study it was also noted that this parallelism occurs even in the presence of chronic lead poisoning. The results are given in Chart 1. It will be seen that the degree of complementary activity

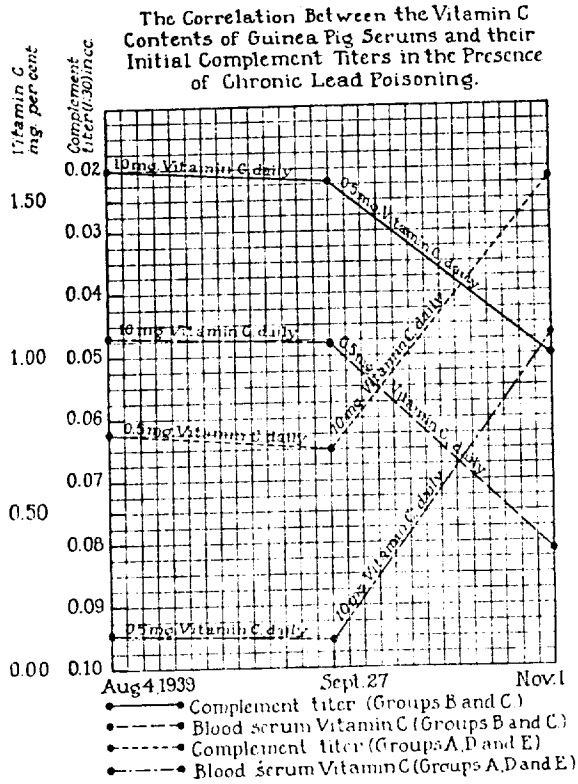

CHART 1

is dependent on the content of ascorbic acid in the serum. Holmes and his co-workers stated that the possible action of ascorbic acid in alleviating lead poisoning was the formation of a poorly ionized, but soluble, lead ascorbate complex. If this were true, it would be expected that a reduction of complementary activity as well as the blood serum ascorbic acid content would follow. However, this is not the case. The observations confirm the earlier work from this laboratory and indicate that the formation of a lead ascorbate complex does not occur.

4. *Deposition of Lead in the Organs.* The determinations of lead in the organs were made by the Kettering Laboratory of Applied Science, University of Cincinnati, Cincinnati, Ohio, and are here listed:

	No. 5379. 10 mg. vit. C/day.		No. 5392. 10 mg. vit. C/day.		No. 5381. 0.5 mg. vit. C/day.		No. 5383. 0.5 mg. vit. C/day.	
Tissue.	Mg. Pb per 100 gm.	Ratio kidney = 100.	Mg. Pb per 100 gm.	Ratio kidney = 100.	Mg. Pb per 100 gm.	Ratio kidney = 100.	Mg. Pb per 100 gm.	Ratio kidney = 100.
Kidney . .	15.0	100.0	20.0	100.0	5.0	100	23.0	100.0
Liver . . .	7.0	46.5	4.6	23.0	1.6	32	1.55	6.74
Bone . . .	7.8	52.0	16.0	80.0	10.0	200	14.4	62.6
Brain . .	0.58	3.87	0.17	0.85	0.2	4	0.25	1.1

The analytic data expressed as mg. of Pb per 100 gm. fresh tissue do not show any clear-cut effect of vitamin C on lead storage. They do suggest that the deposition of lead in the liver tends to be greater when the animal is on a saturation diet of vitamin C. The data were not sufficiently extensive to be conclusive. If the results are expressed as ratios (kidney = 100), the above mentioned effect is not so decisive. It is also worthy of note that, regardless of vitamin intake, the deposition of lead in the kidney was several times that of the liver. In acute and chronic lead poisoning in man the reverse is true.

5. *Paralysis, Convulsions and Death.* Here, the beneficial effects of a high ascorbic acid intake were striking and appear to be unequivocal. In both experiments, only 2 of the 26 guinea pigs on the high vitamin C régime developed clear-cut spasticities or paralyses, and none of the pigs died of lead poisoning during the period of observation. On the other hand, 18 of the 44 animals on the low ascorbic acid intake developed some form of neuro-plumbism and 12 died of typical lead poisoning. Of the 18 animals which developed neuro-plumbism, 4 showed signs of spasticity and clumsiness, 5 spasticity and paralysis, and 9 developed convulsions.

6. *Vitamin C as a Therapeutic Agent in Lead Poisoning.* When 15 of the animals originally on 0.5 mg. Cevalin daily had received lead for 1 month, and were in frank plumbism, they were taken off the lead diet, and 7 of these were retained on the low vitamin level and 8 were raised to the daily 10 mg. Cevalin level. Both groups showed a rapid weight gain, increase in erythrocyte count, and disappearance of stippled cells. The degree of recovery in 1 month was not strikingly greater in the high vitamin group as compared to the lower vitamin group. In contrast to its prophylactic action, ascorbic acid failed to show any beneficial effect on the spasticities and paralyses. The results are shown below:

WEIGHT CHANGES IN POISONED GUINEA PIGS TAKEN OFF LEAD.

Continued on 0.5 mg. Cevalin daily.		Raised to 10 mg. Cevalin daily.	
% stabilized weight restored.	No. animals.	% stabilized weight restored.	No. animals.
90–100	3	74	1
100–110	2	90–100	2
127	1	100–110	4
		117	1

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BLOOD PICTURE UNDER SIMILAR CONDITIONS.

% increased R.B.C.	% increased Hb.	Stippled cells.	% increased R.B.C.	% increased Hb.	Stippled cells.
10	25	0	40	16	0
24	20	0	60	50	0

Summary and Conclusions. 1. In two series of 44 guinea pigs on a subclinical scurvy intake of vitamin C, the degree of lead poisoning developed in 1 month by oral ingestion of basic lead carbonate was more severe when compared to the plumbism in two groups of 24 pigs on a saturation level of the vitamin.

2. The vitamin was not significantly protective against weight loss, anemia and stippling.

3. The vitamin was effective in preventing the onset of neuroplumbism.

4. No evidence has been found to show that lead has any effect on vitamin C utilization or its metabolism.

5. As a therapeutic procedure, vitamin C is inefficient as compared to removal of exposure to lead.

6. Complementary activity of the serum of the animals in lead poisoning was parallel to the ascorbic acid concentration of the serum, and was not influenced by the lead intake.

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Inhibitory Effect of L-Ascorbate on Tumor Formation in Urinary Bladders Implanted with 3-Hydroxyanthranilic Acid* (33916)

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Previous reports (1-3) have shown the following: (a) 3-hydroxyanthranilic acid (3-HOA) is unstable under certain simulated physiologic conditions and oxidatively decomposes. (b) 3-HOA is frequently unstable in urine, especially in urine of some tumor patients, and (c) 3-HOA in urine can be stabilized *in vitro* and perhaps *in vivo* by the presence of high levels of L-ascorbate, an antioxidant.

Since 3-HOA or perhaps an oxidative product(s) produces uroepithelial tumors when implanted into mice bladders (4, 5) and also is a suspected bladder carcinogen in man (6), this experiment was done to investigate the effect of elevated urinary levels of ascorbate in mouse urine on the carcinogenicity of 3-HOA implanted into mouse bladders.

Methods. A 2 × 2 factorial experiment was designed to test the interaction of ascorbate and 3-HOA on: (a) survival of mice, (b) number of malignant tumors induced, and (c) the total number of tumors induced.

Cholesterol pellets containing 3-HOA were prepared as described by Bryan *et al.* (7).

Swiss albino female mice (60-120 days old) were anesthesized with pentobarbital and ether. Pellets were then inserted into bladders of four groups of mice (A, B, C, D) by the technique of Jull (8) as modified by Allen *et al.* (4). Two groups (A and B) received pellets of cholesterol alone, and two groups (C and D) received cholesterol pellets containing 3-HOA. The total number of mice in each group treated originally and the number that survived 40 weeks after surgery

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TABLE I. Data Summary of the Effects of Ascorbic Acid and 3-Hydroxyanthranilic Acid and Their Interactions on the Development of Bladder Tumors in Mice.

Group no.	Chemical implanted in cholesterol pellets into mouse bladders	Ascorbate fed in drinking water (mg/100 ml)	No. of mice treated originally	No. of mice which survived 40 weeks	Tumors observed in 40 weeks		
					Premalignant	Malignant	Total
A	Cholesterol alone	None	58	49	3	2	5
B	Cholesterol alone	250	33	23	1	2	3
C	3-HOA[a]	None	70	46	1	8	9
D	3-HOA	250	51	37	1	2	3

[a] 3-Hydroxyanthranilic acid.

is shown in Table I.

After surgery the mice were returned to their cages and fed Purina mice pellets *ad libitum*. Mice in groups B and D only were fed L-ascorbate (250 mg/100 ml) in their drinking water *ad libitum*. Any further increase in concentration of ascorbate in the drinking water offered to groups B and D would have caused a decreased daily intake of water in groups B and D compared to that in groups A and C.

To evaluate the effects of the feeding of L-ascorbate in drinking water upon the urinary ascorbate level, two groups of mice were studied purely for the purpose of evaluating urinary ascorbate levels. One group was fed L-ascorbate (250 mg/100 ml) in their drinking water *ad libitum* and the other group was kept on the same food and was fed drinking water *ad libitum* but with no addition of L-ascorbate. Urinalysis (9) from these two groups of mice indicated that ascorbate concentration in the urine of the group receiving ascorbate in their drinking water was approximately 400 mg/100 ml as compared to 16 mg/100 ml in the group not receiving ascorbate. On the basis of this experiment groups of mice (B and D) that received ascorbic acid in the drinking water should have had a significantly higher ascorbate level in the urine than the groups (A and C) that did not receive ascorbate.

All surviving mice were killed at the end of 40 weeks after surgery. They were examined postmortem in a manner previously described by Jull [illegible] The bladder [illegible] tended by the injection of Bouin's solution and fixed for 24 hr before they were cut open and examined with a hand lens. Selected material from each mouse bladder was then removed for sectioning and microscopic examination. Tumors found were classified as malignant or premalignant, the malignant tumors showing frank invasion into submucosa and muscle.

Results. A summary of the data of the experiment is presented in Table I. These data were adjusted for initial distribution and treated statistically by a chi-square analysis. In some instances a Z test was made (10).

Prior to testing the interaction of 3-HOA and ascorbate and their effect on the number of malignant tumors induced and the total number of tumors induced, it was necessary to determine whether or not the probability of survival was the same for all four experimental groups. The results of an analysis of information (11) concerning survival is reported in Table II. The analysis showed that the number of mice surviving the surgical procedure was independent of the presence or absence of ascorbic acid or 3-HOA, and that

TABLE II. Analysis of Information on Survival of Mice Implanted with 3-Hydroxyanthranilic Acid and Fed Ascorbate.

Source	df	I	P
Vitamin C	1	0.20	ns[d]
3-HOA[a]	1	2.98	ns
Vitamin C + 3-HOA[c]	1	0.742	ns

[a] 3-Hydroxyanthranilic acid.
[b] Information.
[c] [illegible]
[d] Nonsignificant.

there was no interaction of these two factors in altering the probability of survival of the mice.

A chi-square test (after adjustment for initial distribution) of the interaction between 3-HOA and ascorbate in affecting the number of malignant tumors induced was significant ($0.05 < p < 0.10$). It was decided to test the equality of the proportion of mice with malignant tumors that received 3-HOA and the proportion of mice with malignant tumors that received 3-HOA and ascorbic acid using a Z test (10). The test was significant at the 0.05 level. One may conclude that urinary ascorbate inhibited the expression of the anticipated carcinogenic effect of 3-HOA. A similar analysis (chi-square) was done on the total number of tumors induced. There was no significant interaction between 3-HOA and ascorbate. However, in view of the results of the preceding analysis, it was decided to test the equality of the proportion of mice with total tumors that received 3-HOA and the proportion of mice with total tumors that received 3-HOA and ascorbic acid with a Z test. The results of this test indicates that elevated urinary levels of ascorbate significantly ($p < 0.07$) inhibited the expression of the anticipated carcinogenic effect of 3-HOA.

Discussion. From these data one may conclude that the probability of survival was the same for all four experimental groups and that ascorbic acid inhibited the carcinogenic effect of 3-HOA reported by Allen *et al.* (4) and Bryan *et al.* (5).

By what mechanism(s) ascorbate inhibits the carcinogenic effect of 3-HOA on mice bladders is of interest. Since 3-HOA is easily oxidized in urine but is stabilized by high levels of urinary ascorbate in such urine, one hypothesis to explain such mechanism is that urinary ascorbate simply acts as an antioxidant on urinary 3-HOA *in vivo* and prevents its oxidative decomposition as postulated by Schlegel *et al.* (12). If this hypothesis is true, urinary 3-HOA is not the primary bladder carcinogen, but rather some oxidative product(s) of 3-HOA.

Experiments are underway in this laboratory to test the inhibitory effect of ascorbate on the carcinogenicity to mice bladders of some of the other carcinogenic tryptophan metabolites.

Summary. A 2 × 2 factorial experiment was done to test the interaction of ascorbic acid and 3-hydroxyanthranilic acid on the development of malignant and total tumors in the urinary bladders of mice. An analysis of the data showed that the probability of survival was the same in all groups of mice and that ascorbic acid inhibited the anticipated carcinogenic effect of 3-hydroxyanthranilic acid.

Since the evaluation of the two groups of mice receiving 3-hydroxyanthranilic acid pellet implantation with and without oral administration of vitamin C was of utmost importance in our final evaluation of our results, we asked for an authoritative and unbiased opinion, and Dr. Charles E. Dunlap, M.D., Professor and Chairman, Department of Pathology, Tulane University School of Medicine, most graciously consented to review all of the slides. We gratefully acknowledge Dr. Dunlap's cooperation. The authors, also, gratefully acknowledge the assistance of M. Clinton Miller, Ph.D., Department of Biostatistics, Tulane University School of Medicine in the statistical analysis of data.

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Stabilization of Urinary 3-Hydroxyanthranilic Acid by Oral Administration of L-Ascorbic Acid.* (32547)

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Certain metabolites of dietary L-tryptophan produce uro-epithelial tumors when implanted into urinary bladders of mice(1). One such metabolite is 3-hydroxyanthranilic acid (3-HOA). This metabolite or a derivative thereof, if present in urine in sufficient concentration, could possibly produce tumors of the urinary bladder in man.

Therefore, data concerning true quantities of 3-HOA excreted in urine of tumor patients may be important in studying the etiology of tumors of the urinary bladder; yet, obtaining such data is complicated or prevented perhaps because of instability of 3-HOA in some urines. Data obtained in our laboratory(2,3) show that 3-HOA is unstable under certain simulated physiologic conditions, and is oxidatively decomposed in some samples of urine during incubation at 37°C for six hours. Since urine ordinarily remains in the bladder for at least six hours (overnight), and since voided samples of it are not always promptly analyzed for 3-HOA, these data suggest that amounts of 3-HOA found in some samples of voided urine are not necessarily a reliable index of true quantities formed, but rather may be the amounts remaining after decomposition of this metabo-

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lite in the interim between formation and analysis.

Consequently, some method for stabilization of urinary 3-HOA in such interim may be important to allow adequate estimation of its presence. Since we have observed, *in vitro*, that 3-HOA is stable in aqueous solutions containing L-ascorbate, we have investigated the possibility of use of this substance as an anti-oxidant for stabilization of urinary 3-HOA.

Methods. A consecutive series of 4 overnight (midnight to 7:00 A.M.) specimens of urine was collected from each of 5 tumor patients. After collection of the second specimen, each patient was given, orally, L-ascorbic acid, 500 mg, 3 times each day for 2 days to cause spillage into the urine of ascorbate during collection of the last 2 specimens. Concentration of ascorbate in each urine specimen was determined by the method of Hughes(4).

Since urine containing 3-HOA may remain in the bladder for periods of at least 6 hours (overnight), percentage recovery of 3-HOA added to urine samples was determined after this time period. A known amount (300/μg) of 3-HOA was added to 20 ml of urine in duplicate (Samples A & B) and allowed to incubate together with duplicate control samples (no 3-HOA added in samples C & D) for 6 hours at 37°C in air. After the incubation period, all samples of urine were analyzed for amounts of 3-HOA by the method of Michael *et al*(5). The mean amount of 3-HOA found in control samples C & D was subtracted from that found in samples A & B to correct for endogenous 3-HOA before calculation of percentage recovery of added 3-HOA. Results are expressed as percentages of 3-HOA remaining after 6 hours.

Results. A total of 20 urine samples from 5 tumor patients were analyzed for percentage recovery of added 3-HOA. Fig. 1 shows the mean percentage recovery of added 3-HOA from these urine samples together with mean urinary ascorbate concentrations in specimens 1-4. In specimens 1 and 2, collected before ascorbate administration, mean percentage recovery of added 3-HOA was about 50%, and the mean urinary ascorbate concentration relatively low, about 0.1 mg%. In specimens 3 and 4, collected during ascorbate administration, percentage recovery of added 3-HOA was about 100% and urinary ascorbate levels were elevated as expected.

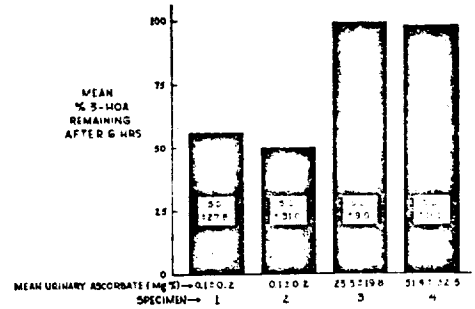

FIG. 1. Effect of elevated levels of urinary ascorbate on stability of 3-hydroxyanthranilic acid in urine from patients with tumors of the urinary bladder.

An analysis of variance was performed using a random complete block design where the blocks were the individual patients(6). The analysis indicated that percentage recovery of added 3-HOA in urine specimens 1 and 2 differed significantly from those in urine specimens 3 and 4 at the 0.01 level. There was no significant difference in recovery between specimens 1 and 2 or between specimens 3 and 4.

Discussion. The data from these experiments show that significant amounts of 3-HOA can decompose in urine samples of some tumor patients if such samples are allowed to incubate at 37°C for 6 hours, and that the presence of high levels of L-ascorbate in such samples inhibits this decomposition. The results suggest that significant amounts of urinary 3-HOA may decompose during its retention in the urinary bladder, and that such decomposition can be prevented by the oral administration of L-ascorbic acid.

Some investigators(7,8) have found high urinary levels of 3-HOA in patients with tumors of the bladder; yet others(9) observed lower urinary levels of 3-HOA in some of their patients than in normal subjects. Stabilization of urinary 3-HOA by oral administration of L-ascorbic acid before collection of urine specimens perhaps would give more reliable data concerning the urinary excre-

tion of 3-HOA by patients with tumors of the bladder.

Summary. A method is presented for the stabilization of urinary 3-hydroxyanthranilic acid (3-HOA) to enable the acquisition of more reliable data concerning the urinary excretion of 3-HOA by patients with tumors of the urinary bladder. The data show that significant amounts of 3-HOA can decompose in urine samples of some tumor patients if such samples are allowed to incubate at 37°C for 6 hours, and that the presence of high levels of L-ascorbate in such samples inhibits this decomposition.

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STUDIES IN STRESS INDUCED CHANGES ON RAT'S ADRENALS.

Part III.

EFFECT OF PRE-TREATMENT WITH ASCORBIC ACID.

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INTRODUCTION.

DURING stress, adrenal ascorbic acid and cholesterol are depleted. The role of cholesterol as a principle precursor of steroidal hormones is well established (Pincus, 1962). However, the exact role of ascorbic acid in the synthesis of cortical hormones is not clear. In this study, effect of pre-treatment with ascorbic acid on the rat's adrenal ascorbic acid and cholesterol content during stress has been studied and the possible role of ascorbic acid has been discussed.

MATERIALS AND METHODS.

Method of producing stress.—White male rats, weighing about 150 g. to 170 g., were used. They were divided into groups as mentioned below. After various treatments mentioned, they were subjected to stress by use of centrifugal motion applied by vertical spinning at 600 r.p.m. for one minute (Details are mentioned in Part I). Animals were sacrificed half an hour after the stress. They were divided into 8 groups as follows :

Group I : Unstressed control group (No treatment ; no stress).

Group II : Stressed control group (No pre-treatment).

Group III : Fed normal saline orally for six days, sacrificed on the 7th day without stress.

Group IV : Administered normal saline intraperitoneally for six days and sacrificed on the 7th day without stress.

Group V : Rats fed orally with ascorbic acid 10 mg./100 g. for 6 days prior to stress and sacrificed on the 7th day half an hour after stress.

Group VI : Rats administered ascorbic acid 10 mg./100 g. intraperitoneally for 6 days prior to stress and sacrificed on the 7th day half an hour after stress.

Group VII : Treated similarly to group V but not subjected to stress (unstressed for group V).

Group VIII : Treated similarly to group VI not subjected to stress (unstressed for group VI).

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TABLE I.

Effect of ascorbic acid administration.

Treatment.	Dose per 100 g. body weight.	Adrenal ascorbic acid, mg./100 g. of tissue :		Adrenal cholesterol, mg./g. of tissue :	
		Unstressed.	Stressed.	Unstressed.	Stressed.
Nil ..	*Nil*	445·0±4·5 (13)	252·0±9·1 (16)	33·65±1·5 (15)	22·13±0·61 (17)
Saline oral ..	1 ml. (for 6 days)	442·5±6·3 (6)	..	33·48±0·43 (6)	..
Saline intraperitoneal ..	1 ml. (for 6 days)	416·8±8·3† (6)	..	31·01±0·64* (6)	..
Ascorbic acid oral ..	10 mg. (for 6 days)	516·1±17·0 (7)	415·6±7·6 (8)	37·27±0·64 (7)	23·40±0·43 (8)
Ascorbic acid intraperitoneal ..	10 mg. (for 6 days)	442·2±18·1 (7)	367·1±13·9 (7)	32·38±0·71 (7)	22·77±0·73 (7)

Figures in parenthesis include number of adrenals for ascorbic acid and cholesterol.
±S. E. M. *$p<0·01$ †$p<0·05$.

TABLE II.

Ascorbic acid : Cholesterol ratio in the adrenals of normal and ascorbic acid administered animals.

	Ascorbic acid (mg./100 g. of tissue).	Cholesterol (mg./g. of tissue).	Ascorbic acid/ cholesterol.
Normals (without stress)	444·7	33·65	13·22
Ascorbic acid fed (orally)	516·1	37·27	13·85
Ascorbic acid given (intraperitoneally)	442·2	32·38	13·65

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Adrenal ascorbic acid and cholesterol were determined as already described (Pohujani *et al.*, 1969).

RESULTS.

Adrenal ascorbic acid and cholesterol were both depleted significantly in untreated rats, when subjected to stress. Treatment with oral ascorbic acid (10 mg./100 g.) chronically over a period of 6 days raised the level of adrenal ascorbic acid and cholesterol as compared to unstressed control group and orally saline fed groups. When ascorbic acid was administered intraperitoneally, these levels were not different from the unstressed control group. The group receiving saline intraperitoneally showed a significant low level of adrenal ascorbic acid ($p < 0{\cdot}05$) and adrenal cholesterol ($p < 0{\cdot}01$) as compared to unstressed control group. In the stressed groups, administration of ascorbic acid blocked the depletion of adrenal ascorbic acid, but did not block the depletion of adrenal cholesterol (Table I).

DISCUSSION.

It is likely that the procedure of intraperitoneal injection acted as stress, reducing adrenal ascorbic acid and cholesterol levels. This is supported by the results obtained with intraperitoneal saline group which also showed lower levels of adrenal ascorbic acid and cholesterol (Table I). Lowenstein and Zwemer (1946) have mentioned that the cortical steroids occur in combined form with ascorbibc acid in the adrenals. This is also postulated by Slusher and Roberts (1957). In the present series when ascorbic acid was administered orally over a period of six days, the levels of adrenal ascorbic acid and cholesterol were higher. It is likely that when larger amount of ascorbic acid is available, a greater reservoir of ascorbic acid-cholesterol combination is built up in the adrenals. The ratio of adrenal ascorbic acid to adrenal cholesterol (A : C) was found to be fairly constant in all groups (Table II) indicating that additional ascorbic acid combines with larger amount of cholesterol and raises the total adrenal ascorbic acid cholesterol complex in the gland. This supports the hypothesis that there occurs an ascorbic acid-cholesterol linkage in the adrenal gland. Under stress, this complex breaks up making cholesterol available for corticosteroid synthesis and the released free ascorbic acid passes out in general circulation. As the ascorbic acid is quickly washed out in the adrenal vein, its value is lowered in 30 minutes after stress, whereas cholesterol incorporation into steroid synthesis takes some time and hence the lowest values after stress are found at 2 hours (Pohujani *et al.*, 1969). After stress, in the ascorbic acid fed group, the depletion of adrenal ascorbic acid is much less compared to unstressed control group. This may be either because the amount of ascorbic acid in the adrenal gland before stress in the ascorbic acid fed group is higher compared to unstressed control group and hence the depletion observed is not as much as in unstressed controls. It is also likely that in the ascorbic acid fed groups large amount of ascorbic acid is released in general circulation and this comes back to adrenal cortex replenishing the depleted ascorbic acid. These possibilities are not worked out but if adrenal ascorbic acid content is measured at shorter time intervals —between 0 and 30 minutes, some explanation may be available. Changes in adrenal

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cholesterol after stress are same in both the groups (oral as well as intraperitoneal administered groups).

SUMMARY AND CONCLUSIONS.

Effect of pre-treatment with ascorbic acid, orally and intraperitoneally, on rat's adrenal ascorbic acid and cholesterol content under the conditions of stress have been discussed. From the present study, it may be concluded that there exists a possibility of a cholesterol ascorbic acid linkage in the adrenals. A constant ratio of adrenal ascorbic acid and adrenal cholesterol under varying conditions gives a better index of adrenocortical status.

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PROTECTIVE EFFECT OF ASCORBIC ACID, ISOASCORBIC ACID AND MANNITOL AGAINST TETRACYCLINE-INDUCED NEPHROTOXICITY[1]

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ABSTRACT

POLEC, ROMAN B., SAMUEL D. J. YEH AND MAURICE E. SHILS: Protective effect of ascorbic acid, isoascorbic acid and mannitol against tetracycline-induced nephrotoxicity. J. Pharmacol. Exp. Ther. **178**: 152–158, 1971. The marked azotemia and other evidence of renal damage induced in rats and dogs by rapid i.v. administration of pure tetracycline·HCl at 50 mg/kg was prevented by concomitant administration of ascorbic acid at 125 mg/kg or higher. D-Isoascorbic acid had a similar effect when tested in rats. Mannitol was protective in dogs but had no consistent benefit in rats. When injection of ascorbic acid into rats preceded that of tetracycline by 5 to 10 minutes, protection was marked; reversal of the order of injections with the same interval resulted in less protection. Ascorbic acid was ineffective against antibiotic toxicity when blood flow to rat kidneys was prevented by renal pedicle ligation. Ascorbic acid increased the rate of dialysis and the percentage of ultrafiltration of tritiated tetracycline from plasma. The protective effect of ascorbic acid, isoascorbic acid and mannitol (the latter in dogs) against the nephrotoxicity of tetracycline is postulated to be secondary to an osmotic diuretic effect. Decreased protein binding in the plasma by ascorbic acid may be a contributing factor.

In the course of another study, we noted that nephrotoxicity after i.v. injection of a solution of pure tetracycline·HCl was much greater than that occurring with a commercial therapeutic i.v. formulation. The major difference in composition of the two preparations is the large ascorbic acid content of the latter. This report summarizes our observations in rats and dogs on the effects of ascorbic acid and other agents on tetracycline toxicity and on *in vitro* binding with proteins.

METHODS. Tetracycline·HCl in a concentration of 25 mg/ml of normal saline or water was injected within 10 seconds into the tail vein of lightly etherized rats. Females, 200 g, of the Charles River CD strain were used. Six adult mongrel dogs weighing between 12 and 17 kg were used in repeated experiments with sufficient interval between to permit return of monitored blood chemistries to baseline levels. Tetracycline·HCl was given to unanesthetized dogs in 120 to 190 ml of sterile saline in a concentration of 4.3 mg/ml at a dose of 50 mg/kg administered *via* leg vein in 10 to 15 minutes. L-Ascorbic acid, D-isoascorbic (D-araboascorbic) acid or mannitol (prepared from 25% sterile water solution) was given in solution with the tetracycline unless otherwise indicated. Food was withheld from the rats and dogs for 24 hours after the i.v. infusion; water was supplied *ad libitum*. The rats were anesthetized with ether at 24 hours, blood was drawn by cardiac or aortic puncture and the animals were sacrificed. Dogs were given a stock diet after the one-day fast. Determinations were made of plasma urea nitrogen (Kaplan, 1965), creatinine (Kingsley and Schaffert, 1953), inorganic phosphorus (Fiske and SubbaRow, 1925), calcium and magnesium (by atomic absorption spectrophotometry) and sodium and potassium (by emission flame photometry). Tetracycline-7-^{3}H·HCl (New England Nuclear Corporation, Boston, Mass.; specific activity 0.25 mc/1.1 mg) was given in a dosage of 9 to 10 μc/rat with carrier tetracycline at 50 mg/kg in saline solution; urine was collected there-

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after for 24 hours and the urine and metabolic cage washings were brought to 40 ml with water. One-half milliliter of the latter was added to 17 ml of Diotol (Herberg, 1960) for counting and comparison with a standard solution of tritiated tetracycline. For determining radioactivity in plasma or in tissue homogenate, 0.2 ml of the sample was dissolved in 0.5 ml of hyamine hydroxide and then in 17 ml of Diotol. The samples were counted in a Packard scintillation counter. Tetracycline was determined in selected samples of plasma by the *Bacillus cereus* agar diffusion method and in urine by a turbidimetric method with *Staphylococcus aureus*. Dialysis was performed in bags, prepared from cellulose dialysis tubing (Arthur H. Thomas Company, Philadelphia, Pa., no. 4466-A2), in a constantly rotating drum against 5 liters of saline at 4°C with three or four bath changes in 22 hours.

RESULTS. STUDIES IN RATS. *Tetracycline effect on plasma urea nitrogen.* The marked azotemia which occurred after a single i.v. injection of 50 mg/kg of purified tetracycline·HCl was in marked contrast to the finding of normal or only slightly elevated urea nitrogen values when a similar dosage of a commercial therapeutic i.v. formulation was injected. In a typical experiment, when blood was obtained at 24 hours by cardiac puncture, the respective values for urea nitrogen were 88.8 ± 6.5 (six rats) and 12.2 ± 0.3 mg/100 ml (nine rats).

When tetracycline·HCl was injected in increasing amounts, a significant elevation in urea nitrogen levels occurred with dosages of 25 mg/kg or above (table 1). At the larger doses, the azotemia was associated with a rise in plasma creatinine, development of diuresis and histologic evidence of tubular necrosis.

TABLE 1

Plasma urea nitrogen in rats after varying doses of tetracycline (TC)

TC[a]	Urea Nitrogen[b]
mg/kg	*mg/100 ml*
0	10.1 ± 0.6 (7)[c]
12.5	11.3 ± 0.9 (5)
25.0	40.8 ± 10.0 (5)[d]
37.5	65.0 ± 6.6 (5)[d]
50.0	78.9 ± 11.3 (7)[d]

[a] Purified tetracycline·HCl in saline at 25 mg/ml injected within 10 seconds *via* tail vein.

[b] $\bar{X}$ ± S.E. at 24 hours.

[c] Number in parentheses indicates number of animals.

[d] $P < .01$ when compared with group given no TC.

TABLE 2

Effect of different levels of ascorbic acid on tetracycline-induced azotemia[a]

Ascorbic Acid	Urea Nitrogen[b]
mg/kg	*mg/100 ml*
0	78.9 ± 11.3 (7)[c]
15	54.4 ± 20.0 (4)
50	57.7 ± 20.4 (3)
75	62.4 ± 12.4 (8)
100	60.2 ± 20.0 (4)
125	24.8 ± 7.3 (5)[d]
150	14.9 ± 1.6 (36)[d]

[a] Tetracycline given i.v. at 50 mg/kg in solution with amounts of ascorbic acid indicated.

[b,c] See footnotes *b* and *c* in table 1.

[d] $P < .01$ when compared with group given no ascorbic acid.

Effect of ascorbic acid. Commercial i.v. antibiotic preparations contain ascorbic acid in amounts ranging from 250 to 300 mg/100 mg of tetracycline. The influence of ascorbic acid on the azotemic effect of tetracycline was tested by injecting ascorbic acid alone and in varying amounts in solution with pure tetracycline·HCl at 50 mg/kg. Ascorbic acid at 125 mg/kg was associated with a significant decrease in urea nitrogen; there was essentially complete protection at 150 mg/kg (table 2).

Time relations. When ascorbic acid was given 5 to 10 minutes prior to tetracycline, urea nitrogen values were within normal limits; the protection was appreciably less if the antibiotic preceded the vitamin by a similar interval (table 3). When tritiated tetracycline was administered, the tritium levels in plasma were directly related to the urea nitrogen levels of the various groups whereas the urine levels were inversely related.

Specificity of vitamin and reducing actions. Isoascorbic acid was as protective as ascorbic acid at 150 mg/kg when given with tetracycline (table 4). The plasma and urine levels of tetracycline as measured by tritium activity and microbiologic assay offer confirmatory evidence of the ability of the kidneys to excrete the antibiotic when accompanied by either of the hex-

TABLE 3

Time of ascorbic acid (C) administration, tetracycline (TC) nephrotoxicity and excretion

Treatment and Order[a]	Interval	Urea Nitrogen	Radioactivity	
			Plasma (per ml)	Urine (24 hrs)
	min	*mg/100 ml*	*% dose*	
TC (11)[b]		111.1 ± 11.3[c]	0.15 ± 0.02	9.6 ± 2.62
TC then C[d] (9)	5 to 10	59.1 ± 11.2[e]	0.09 ± 0.014[f]	22.4 ± 5.10[f]
TC and C (13)	0	17.8 ± 3.4[e]	0.02 ± 0.01[e]	41.2 ± 1.00[e]
C then TC (12)	5 to 10	10.0 ± 0.8[e]	0.02 ± 0.004[e]	51.7 ± 2.75[e]

[a] Tetracycline at 50 mg/kg; ascorbic acid at 150 mg/kg; 10 μc of tritiated TC were added to the TC solution.
[b] Number in parentheses indicates number of animals.
[c] $\bar{X}$ ± S.E. at 24 hours.
[d] When injections were given separately, there was an interval of 5 to 10 minutes.
[e] $P < .01$ when compared with group given TC only.
[f] $P < .05$ when compared with group given TC only.

TABLE 4

Effect of ascorbic acid (C) and d-isoascorbic acid (iso-C) on plasma urea nitrogen and tetracycline (TC) excretion

Treatment[a]	Urea Nitrogen	^{3}H Radioactivity		TC by Assay[b]		
		Plasma (per ml)	Urine (24 hrs)	Plasma (per ml)	Urine (24 hrs)	% excreted ($\bar{X}$)
		% dose		*μg/ml*	*mg*	
TC (4)[c]	99.6 ± 8.0[d]	0.06 ± 0.007	5.6 ± 1.67	5.70 ± 0.067	0.56 ± 0.20	5.2
TC and C (5)[e]	9.9 ± 1.1	0.01 ± 0.001	46.9 ± 1.42	0.29 ± 0.02	1.54 ± 0.21	27.1
TC and iso-C (5)[e]	10.5 ± 1.3	0.01 ± 0.001	47.9 ± 1.16	0.28 ± 0.02	1.80 ± 0.33	33.2

[a] Nine microcuries of ^{3}H-tetracycline were added to the tetracycline (TC) solution (50 mg/kg); ascorbic acid (C) and isoascorbic (iso-C) at 150 mg/kg.
[b] Microbiologic, see text.
[c] Number in parentheses indicates number of animals.
[d] $\bar{X}$ ± S.E. at 24 hours.
[e] All results in these groups are significantly different ($P < .01$) from those in TC group.

oses. Dimercaprol (BAL) was given i.m. at 25 mg/kg half an hour before i.v. injection of tetracycline·HCl at 50 mg/kg. In a typical experiment, the urea nitrogen was 71.0 ± 9.4 mg/100 ml in four rats treated with dimercaprol plus tetracycline as compared with 83.7 ± 26.2 mg/100 ml in three rats with tetracycline only and 11.0 ± 0.9 mg/100 ml in six rats given dimercaprol only. When dimercaprol was given i.m. at 10 mg/kg one-half hour before tetracycline and again at 3½ and 7½ hours afterward, no protective effect was noted.

Mannitol studies. To determine whether the protective effect of ascorbic acid is related to an osmotic effect, mannitol was mixed with tetracycline·HCl at 50 mg/kg in a 3:1 and in a 6:1 ratio by weight and given i.v. to rats (table 5). Neither of these concentrations altered consistently the azotemic effect of tetracycline when given with the antibiotic or in separate injections one to two minutes earlier. However, there was considerable variability in the urea nitrogen and urinary excretion of labeled tetracycline in the mannitol-treated animals, and a few rats appeared to have been partially protected.

A simple experiment was performed to test for rapid diuretic action of mannitol and ascorbic acid as used in the above experiments. Previ-

ously fed rats had their bladders emptied by pressure and were rapidly injected *via* tail vein with mannitol in saline (75 mg/ml; 585 mOsmol/l) or ascorbic acid in saline at the same concentration (695 mOsmol/l) with each animal receiving 50 mg/kg b.wt. of the test substance. A third group was given saline (282 mOsmol/l). Urine was collected hourly in individual metabolism cages while the rats were without food or water. During the first hour, five of the seven mannitol-injected rats and six of the seven ascorbic acid-injected rats excreted urine, whereas none of the saline controls urinated. All rats excreted urine in widely varying amounts over the next three hours.

The role of the kidneys in the protective effect. After bilateral ligation of renal pedicles of rats under light ether anesthesia, i.v. injections of antibiotic with or without ascorbic acid and of ascorbic acid only were made immediately thereafter and blood samples were taken 24 hours later. Those given ascorbic acid only at 150 mg/kg had urea nitrogen levels of 162 ± 2.9 mg/100 ml (nine rats); those given tetracycline only at 50 mg/kg had levels of 198 ± 3.2 mg/100 ml (11 rats) and those given tetracycline in solution with ascorbic acid had values of 208 ± 3.7 mg/100 ml (10 rats).

Metabolic effects of the antibiotic and ascorbic acid. When leucine incorporation into tissue proteins was measured, the label in the proteins of liver and kidney was significantly decreased in intact rats given tetracycline (72 and 80%, respectively, of the value in ascorbic acid-injected controls). Injection of ascorbic acid with the antibiotic resulted in similar depression of amino acid incorporation (72 and 82%) into these two tissues.

TABLE 5

Effect of mannitol (M) on plasma urea nitrogen and tetracycline (TC) excretion in rats

Treatment[a]	Urea Nitrogen	^{3}H Radioactivity	
		Plasma (per ml)	Urine (24 hrs)
	mg/100 ml	*% dose*	
TC[a] (22)[b]	64.6 ± 7.9[c]	0.06 ± 0.01	16.5 ± 2.1
M-150 (10)	10.7 ± 0.9[d]		
TC and M-150 (24)	53.4 ± 6.5	0.05 ± 0.01	21.8 ± 3.4
TC and M-300 (5)	71.0 ± 20.6	0.06 ± 0.01	14.1 ± 6.0
M then TC (14)[e]	60.0 ± 8.6	0.05 ± 0.004	19.7 ± 3.5
TC then M (4)[e]	52.3 ± 20.0	0.04 ± 0.01	27.9 ± 8.5
TC and C (5)	19.8 ± 9.1[d]	0.02 ± 0.01[d]	46.2 ± 5.0[d]

[a] Nine microcuries of ^{3}H-tetracycline were added to the tetracycline (TC) solution (50 mg/kg); ascorbic acid (C) at 150 mg/kg; mannitol was given in two dosages, 150 (M-150) and 300 mg/kg (M-300).

[b] Number in parentheses indicates number of animals.

[c] $\bar{X}$ ± S.E. at 24 hours.

[d] $P < .01$ when compared with group given TC only.

[e] Separate injections of M (150 mg/kg) and TC (50 mg/kg) were given in the order indicated with an interval of one to two minutes.

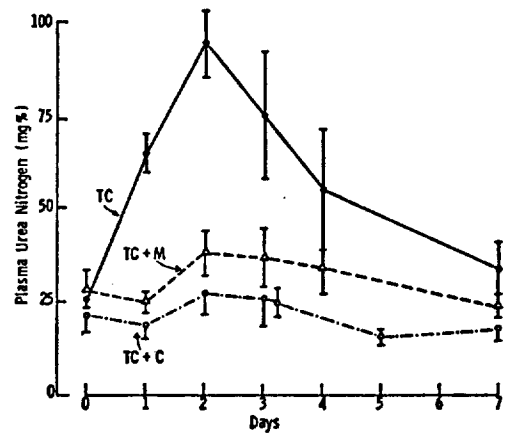

FIG. 1. Plasma urea nitrogen levels ($\bar{X}$ ± S.E.) in dogs in the week after i.v. administration of tetracycline (TC) (50 mg/kg) alone or in solution with ascorbic acid (C) at 300 mg/kg or mannitol (M) at 300 mg/kg. Each point represents data from five to eight animals.

STUDIES IN DOGS. *Effects of ascorbic acid and of mannitol.* Tetracycline·HCl (50 mg/kg) infused over 10 to 15 minutes caused a significant azotemia in association with increases in plasma phosphate and creatinine; these returned to base-line values within approximately eight days (fig. 1). Ascorbic acid at 150 mg/kg given with the antibiotic had no significant protective effect. However, when the concentration of the latter was doubled, a marked protection was apparent. Mannitol at 300 mg/kg exerted a similar beneficial effect when given with tetracycline.

At 48 hours, when plasma urea nitrogen was maximal in the antibiotic-treated animals, the creatinine values in the tetracycline- and tetracycline and ascorbic acid-treated dogs were 5.03 ± 1.05 and 1.06 ± 0.08 mg/100 ml, respectively, whereas the inorganic phosphate levels in tetracycline-, tetracycline and ascorbic acid-and tetracycline and mannitol-treated animals were 7.7 ± 0.8, 4.3 ± 0.4 and 4.9 ± 0.4 mg/100 ml, respectively. There were small but significant rises in plasma magnesium and cal-

TABLE 6

Effect of ascorbic acid and mannitol on plasma tetracycline levels in dogs

Interval[a]	Plasma Tetracycline[b]		
	Tetracycline	Tetracycline + ascorbic acid	Tetracycline + mannitol
hrs	*μg/ml*		
0	<0.1	<0.1	
8	123 ± 21[c] (2)[d]	20.4 ± 9.9 (3)[e]	
24	42 ± 11 (4)	17.2 ± 6.5 (4)	14 ± 5.0 (3)[f]
48	21 ± 8 (4)	2.5 ± 1.0 (4)[f]	1.1 ± 0.4 (3)[f]
80	23 ± 13 (2)	0.3 ± 0.1 (3)	
120	<0.1	<0.1	

[a] Interval between i.v. injections of solutions indicated and blood drawing.
[b] Microbiologic assay, see text.
[c] $\bar{X}$ ± S.E. at 24 hours.
[d] Number in parentheses indicates number of animals.
[e] $P < .01$ compared with tetracycline group at the same interval.
[f] $P < .05$ compared with tetracycline group at the same interval.

cium at one to three days in the dogs receiving tetracycline; when ascorbic acid was given with tetracycline there was a transient lowering of the magnesium. Plasma tetracycline levels were appreciably lowered when ascorbic acid or mannitol was given with the antibiotic (table 6).

In vitro studies. Tracer amounts of tritiated tetracycline with carrier antibiotic at 1 mg/ml in various media with or without ascorbic acid (3 mg/ml) were dialyzed for 22 hours; the tritium remaining in the bags was measured periodically (fig. 2). Ascorbic acid did not alter the rate of disappearance of radioactivity from saline. However, when plasma (rat or human) was used, the rate of disappearance of labeled tetracycline was appreciably increased when ascorbic acid was present; the values approached those of the protein-free medium. The observations with isoascorbic acid were similar. The sequence of addition of ascorbic acid and tetracycline to the plasma did not influence the rate of loss of radioactivity. When glucose (3 mg/ml) or mannitol (3 and 15 mg/ml) was tested in place of ascorbic acid, tetracycline dialyzability was no greater than with the antibiotic alone. The effect of ascorbic acid on the dialyzability of tetracycline from plasma was demonstrable when tetracycline was present at 1 mg/ml, but not at concentrations of 0.1 mg/ml or less. Ascorbic acid had no effect when purified bovine albumin or homogenates of rat liver or kidney were tested at approximately the same protein concentration as in plasma. Human serum with tritiated tetracycline (1 mg/ml) was passed through a Diaflo UM-2 membrane ultrafilter. When ascorbic acid (3 mg/ml) was added to the serum, the radioactivity in the ultrafiltrate was 28%; this was in contrast to 6.7% when ascorbic acid was omitted.

DISCUSSION. When tetracycline is maintained at abnormally elevated levels in blood and other tissues, a significant antianabolic effect may occur in man (Shils, 1963) and in experimental animals (Yeh and Shils, 1966). There is also evidence of a direct nephrotoxic action of this class of antibiotics when administered parenterally in large amounts to experimental animals (P'an *et al.*, 1950; Farhat *et al.*, 1958) or man (Lew and French, 1966).

Our observation of a marked difference in apparent nephrotoxicity between commercial i.v. preparations and purified tetracycline·HCl led to examination of the effect of ascorbic acid. The latter is added in relatively large amounts to i.v. tetracycline formulations in order to solubilize the antibiotic and to prevent undesirable chemical changes.

The ability of ascorbic acid to protect against the nephrotoxic action of injected tetracycline is not related to its vitamin action since a similar protection is afforded by isoascorbic acid, which is reported to have little or no antiscorbutic potency (Rivers *et al.*, 1963). Both are antioxidants with ascorbic acid being somewhat more potent under certain conditions (Borenstein, 1965). A protective action based on a general antioxidant effect is made unlikely by the finding that the relatively large dose of 150 mg/kg of ascorbic acid afforded no protection to dogs. A specific action on sulfhydryl groups was minimized by the fact that dimercaprol was ineffective. An action within the kidneys is indicated by the finding that ascorbic acid was without effect in rats when blood flow to those organs was excluded by pedicle ligation.

Since large doses of ascorbic acid and isoascorbic acid are excreted fairly rapidly and efficiently (Rivers *et al.*, 1963), attention was directed to an osmotic diuretic action as a partial or total explanation. The finding that mannitol was also effective in dogs gives stronger evidence

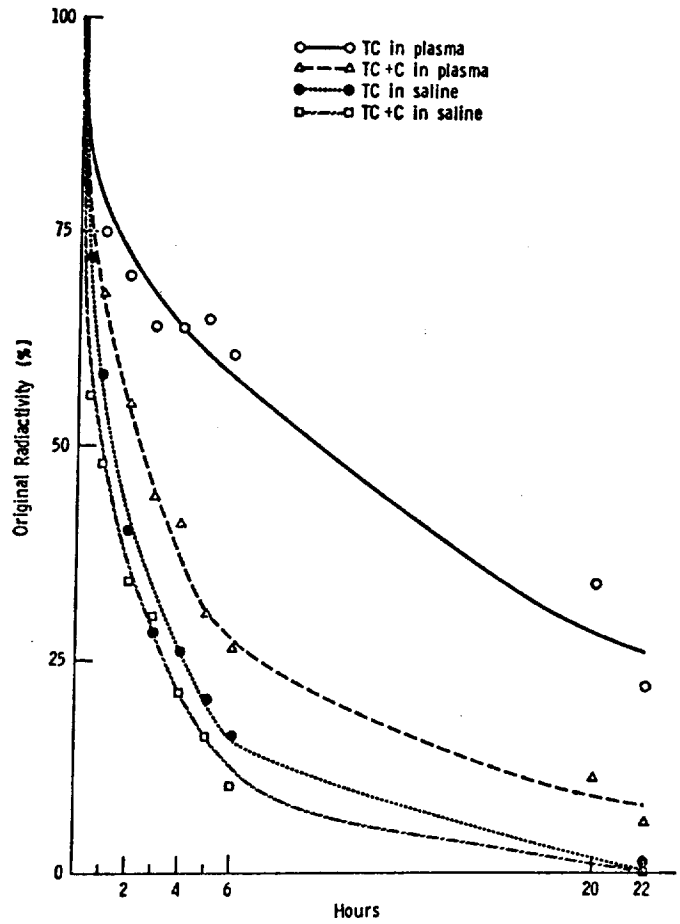

FIG. 2. Effect of ascorbic acid on the rates of dialysis of tritiated tetracycline from saline and plasma *in vitro*. Tetracycline·HCl (TC) at 1 mg/ml with ^{3}H-tetracycline in saline or pooled human plasma in the presence or absence of added ascorbic acid (C) at 3 mg/ml was dialyzed for 22 hours at 4°C. At intervals, dialysis bags were removed and the remaining radioactivity was measured and plotted as percentage of original activity. Initial pH values in bags were: TC in saline, 2.2; TC + C in saline, 2.5; TC in plasma, 5.7; and TC + C in plasma, 6.0. Final pH values in bags with plasma at 22 hours were all 5.7.

for the belief that these compounds in effective concentrations act by interfering with tetracycline reabsorption in the renal tubules by an osmotic effect, thus reducing plasma levels more rapidly and minimizing high cellular concentrations.

The infusions given to dogs were more dilute and administered much more slowly than were those injected into the rats. Hence, it is not unexpected (on the basis of the hypothesis of an osmotic diuretic action) to find that larger amounts of ascorbic acid were required to afford protection. An explanation for the differences in behavior of mannitol between the rat and the dog is not at hand.

The data in the dialysis and ultrafiltration experiments indicate that ascorbic acid and isoascorbic acid decrease plasma protein binding of the antibiotic. In these *in vitro* experiments, tetracycline concentration was critical with the deconjugating effect developing somewhere between 0.1 and 1.0 mg/ml. It is of interest to consider whether blood antibiotic levels achieved the upper range of concentration in the *in vivo* experiments. Assuming a rat blood volume of 20 ml with an antibiotic injection of 10 mg, the

concentration changed from the initial value of 25 mg/ml in the injection solution to 0.5 mg/ml when uniform blood distribution occurred with further decline occurring as excretion proceeded. Inasmuch as ascorbic acid was protective only when given within approximately 10 minutes of tetracycline injection, it is reasonable to assume that concentrations of the antibiotic are relatively high at the period of protective action. As noted in table 6, antibiotic levels in dogs eight hours after being given tetracycline were 0.123 mg/ml and were presumably much higher when ascorbic acid was acting. If ascorbic acid does decrease *in vivo* protein binding of tetracycline, the filtered load would be increased and the postulated accompanying osmotic diuresis would make excretion more efficient; thus, the two actions of the protective agent would supplement each other.

It is possible that neither of these explanations is the true one. An alternate hypothesis of the action of the protective agents involves an intracellular mechanism preventing tubular damage and thus resulting in an increased antibiotic excretion. Our experiments provide no data supporting this type of mechanism.

The results with renal ligation are of double interest. First, they indicate that ascorbic acid is without protective effect against tetracycline in the absence of renal perfusion. Secondly, the azotemia which developed in the pedicle-ligated groups given tetracycline is significantly greater than that developing in the ligated group given ascorbic acid only. Since the latter value (162 ± 2.9 mg/100 ml) may be taken as a measure of the effect of cessation of renal function, the additional rise of approximately 40 mg/100 ml of urea nitrogen observed in the tetracycline groups would appear to be caused by an effect of the antibiotic unresponsive to ascorbic acid. It is known that tetracycline has a metabolic action which inhibits protein synthesis in the intact animal (Shils, 1963; Yeh and Shils, 1966); the rise in urea nitrogen in the ligated rats may be related to that action.

The rapid initiation of renal damage by tetracycline is indicated by the finding that a delay of approximately 10 minutes in administering ascorbic acid to rats greatly increased the azotemia.

The commercial i.v. tetracycline-ascorbic acid preparations given to patients are not necessarily more dilute than that used in our dog experiments[a]; hopefully, they are not given as rapidly. Several questions of possible clinical interest are presented by this study. Does the ascorbic acid in commercial preparations increase the rate of urinary excretion of the antibiotic in normal clinical usage? Will administration of large amounts of p.o. or i.v. ascorbic acid result in increased excretion of tetracycline previously administered in amounts which are excessive in relation to the renal excretory capability of the patient? If significantly improved excretion does occur, ascorbic acid may be useful in helping prevent the untoward effects of antibiotic overdosage.

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[a] Product information statements from various drug houses indicate that 100 to 500 mg of the i.v. preparation may be given in 100 ml in approximately five minutes.

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Tolerance of Chickens to Thiabendazole

THIABENDAZOLE, a broad-spectrum anthelmintic[1,2], is well tolerated by mice, rats and rabbits, with LD_{50} values exceeding 3·1 g/kg in oral acute toxicity experiments[3]. Furthermore, rats and dogs tolerated the drug quite well in chronic toxicity experiments[3].

Recent unpublished experiments with adult chickens fed thiabendazole continuously for 3 weeks revealed that a level of 0·1 per cent in the diet has no effect on reproduction, egg production or feed intake, but that levels exceeding 0·2 per cent produced lower values for all three criteria. In addition, the birds fed these high levels of thiabendazole appeared anaemic and about 7 per cent mortality occurred at the 0·2 per cent level with progressively higher mortality at higher drug levels. Post-mortem examination of the hens revealed haemorrhaging throughout most internal organs, but particularly at the ovary. Haematological data were obtained in a series of experiments in which thiabendazole was fed for 21 days to hens. The data show that haemoglobin and haematocrit values were markedly below normal in hens treated with 0·2 or 0·4 per cent thiabendazole, and this correlated with their pale appearance (Table 1). Sedimentation rates were elevated, but whole-blood clotting time and prothrombin clotting time showed no consistent change from the norm. However, it should be noted that whole-blood clotting time assays are subject to wide variability and many values are required to show a trend. Also, it should be pointed out that two birds sampled at the end of the second week on 0·4 per cent thiabendazole had clotting times exceeding 60 min, the cut-off time for the assay, and that one of the four birds sampled at the end of the third week had a whole-blood clotting time exceeding 60 min. When these data are considered with the mortality values and the post-mortem findings, there is an indication that blood clotting time may be influenced provided the drug level is 0·4 per cent or higher.

Table 1. HAEMATOLOGICAL DATA ON ADULT FEMALE CHICKENS FED THIABENDAZOLE FOR THREE WEEKS OR THIABENDAZOLE WITH B_{12}, ASCORBIC ACID AND MENADIONE

	Control	0·2% Thiabendazole	0·4% Thiabendazole	0·4% Thiabendazole + 1 mg B_{12}/kg + 100 mg ascorbic acid/kg + 20 mg menadione/kg
No. birds	8	4	3	4
Haemoglobin (g %)	8·4 ± 0·3*	5·4 ± 0·7	4·4 ± 0·4	8·1 ± 0·2
Haematocrit (vol. %)	30·1 ± 0·8	20·9 ± 2·8	17·8 ± 1·4	27·5 ± 0·8
Sedimentation rate (mm/h)	0·5 ± 0·0	1·3 ± 0·3	1·3 ± 0·3	0·6 ± 0·2
Prothrombin clotting time (sec)	15·9 ± 0·9	17·4 ± 1·6	15·2 ± 0·9	14·0 ± 0·7
Whole-blood clotting time (min)	30 ± 3	24 ± 5	39 ± 11	29 ± 6
General appearance of birds	Normal	Pale skin Pale combs Partial moult	Pale skin Pale combs Partial moult	Normal

* Mean ± standard error of the mean.

Table 2. HAEMATOLOGICAL DATA ON ADULT FEMALE CHICKENS FED THIABENDAZOLE FOR THREE WEEKS OR THIABENDAZOLE WITH B_{12}, ASCORBIC ACID OR MENADIONE

	Control	0·2% Thiabendazole	0·2% Thiabendazole + 1 mg B_{12}/kg	0·2% Thiabendazole + 100 mg C/kg	0·2% Thiabendazole + 20 mg K/kg	0·2% Thiabendazole + B_{12} + C + K
No. birds	4	4	4	3	4	4
Haemoglobin (g %)	9·0 ± 0·2*	5·2 ± 1·2	7·0 ± 1·0	3·0 ± 0·4	6·3 ± 1·1	9·0 ± 0·3
Haematocrit (vol. %)	33·8 ± 1·6	19·1 ± 1·6	26·8 ± 4·7	12·8 ± 2·3	23·0 ± 3·5	33·0 ± 1·0
Sedimentation rate (mm/h)	0·3 ± 0·2	4·6 ± 3·4	0·6 ± 0·3	2·0 ± 0·6	1·4 ± 0·4	0·4 ± 0·1
Whole-blood clotting time (min)	20 ± 5	35 ± 5	20 ± 5	29 ± 7	17 ± 5	42 ± 8

* Mean ± standard error of the mean.

Most interesting was that the addition of 1 mg vitamin B_{12} per kg, 100 mg ascorbic acid per kg, and 20 mg menadione per kg diet containing 0·4 per cent thiabendazole counteracted the adverse effect of the drug on the haematological values (Table 1) of the hens. These birds at necropsy did not have the haemorrhages that were observed in those receiving the drug alone. As shown in Table 2, B_{12} and menadione, and not ascorbic acid, counteracted the adverse effect of thiabendazole on haemoglobin and haematocrit levels. Each vitamin alone, partially reversed the effect at the levels fed, and the addition of both vitamins to the diet completely overcame the adverse effect of 0·2 per cent (Table 2), as well as 0·4 per cent thiabendazole (Table 1).

In comparison to other species of animals in which thiabendazole is well tolerated, the adult female chicken appears to be more sensitive to the drug. Hens fed a diet containing 0·2 per cent thiabendazole consumed 80 mg thiabendazole per kg body-weight daily. This dose is about 0·5–0·33 of the amount required to produce a moderate anaemia in dogs[3]. Single oral doses of 50 mg/kg have proved highly efficacious in roundworm infections in those mammals for which the drug is recommended, so there is an adequate safety margin for thiabendazole. We have investigated the effect of thiabendazole when fed to chickens to 6 weeks of age and find that they tolerate (for growth) about 0·1 per cent of the drug in the diet and have normal haemoglobin levels.

Investigations in progress are designed to reveal the maximum tolerated level of thiabendazole in the hens' diet without effect on their haematology, which is estimated, from the data in Tables 1 and 2, to be in the range of 0·05–0·1 per cent thiabendazole in the diet. In addition, investigations are at present in progress to reveal the mode of action between thiabendazole and vitamins B_{12} and K, and the possible interaction of the endocrines on the drug–vitamin relationship.

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Ascorbic Acid & Colour Changes in Citrus Fruit Juices During Processing

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CITRUS fruit juices are a good source of vitamin C, and its retention in the juice during processing and storage is important from the standpoint of their nutritive value. The retention of the natural colour of the juice after processing is also important.

Changes in vitamin C content and colour changes in citrus and other fruit juices during processing have been investigated by several workers[1-14] in U.S.A. and U.K. Work on similar lines in India appears to be scanty. The retention of ascorbic acid and colour changes in indigenous citrus fruit juices during processing have been investigated in this Institute.

Experimental Procedure

The experimental procedure adopted in these experiments was the same as that reported before[15].

Analysis — The yield of juice, pulp, peels, etc., from fresh fruit was determined and the fresh juice analysed for sp. gr., *p*H and ascorbic acid content[16,17] (TABLE 1). After processing, the juices were analysed for ascorbic acid. The results are recorded in Figs. 1-4. The colour of the juices was measured in a Lumetron photo-electric colorimeter (model 400 A) at a wavelength range of 370-650 mμ, according to Pruthi and Lal[18]. The results are recorded in Table 2.

Results & Discussion

Ascorbic Acid Retention During Processing — The maximum loss of ascorbic acid in acid citrus juices (lime) during processing was *c.* 3·5 per cent; in comparatively less acid fruit juices (*sangtra*) the loss was 7·1 per cent. The lower *p*H of lime juice appears to favour the retention of ascorbic acid.

Addition of cane sugar and deaeration of juices help the retention of ascorbic acid. There was no appreciable difference between sweetened and deaerated juices in the percentage retention of ascorbic acid. Deaeration helps retention of ascorbic acid during storage[15,19]. The percentage retention during packing of deaerated lime juice under different methods of processing varied from 97·9 to 99·9, while in the control and in the sweetened samples it varied from 96·9 to 99·5 and from 97·2 to 99·5 respectively. In sweetened samples, though the retention of ascorbic acid is slightly better, it is not significant.

Ascorbic acid retention is lowest in benzoated samples and in samples pasteurized in bottles (97·9 and 98·1 per cent respectively); it is highest in samples flash pasteurized in cans (99·9 per cent). Sodium benzoate seems to have some destructive effect on ascorbic acid; this is supported by Evenden and Marsh[20], and Lal[21]. The low retention of ascorbic acid in holding pasteurization in bottles is probably due to longer contact of the juice with air during processing. The reduction of head space in the case of overflow pasteurization gives a higher retention of ascorbic acid than in holding pasteurization in bottles.

In samples preserved with sulphur dioxide, the retention of ascorbic acid is high (99·82 per cent)[22,23]. A better retention of ascorbic acid in lime and *Nagpuri sangtra* juices is obtained when they are processed with sulphur dioxide than when processed by overflow pasteurization.

Colour Retention During Processing — The extent of colour changes in juices was measured by measuring the optical densities of juices in a colorimeter at 6 different wavelengths, before and after processing. The values obtained at 420 mμ were higher than at other wavelengths.

There was marked visual colour change in most juices after processing and the readings (TABLE 2) show that the value for control samples of *Nagpuri sangtra* juice preserved by ordinary pasteurization (bottles) is nearly twice that obtained for unprocessed juice. The colour changes in Coorg orange juice were comparatively low.

TABLE 1 — ANALYSES OF CITRUS FRUITS

	ORANGE (*mosambi*)	*Sangtra* (*Nagpuri*)	LIME	*Khatta*	SWEET LIME	GRAPE-FRUIT	*Galgal*
Juice, w/w, %	43·10	50·20	49·40	31·70	33·10	36·00	34·60
Peel and rag, w/w, %	49·20	36·30	—	64·00	64·50	49·20	59·90
Pulp (pomace), w/w, %	7·70	13·50	50·60	4·20	2·50	14·90	5·50
Sp. gr. of juice, °Brix.	10·00	10·00	8·60	8·50	8·50	10·10	8·50
*p*H	4·65	3·84	2·32	2·28	5·38	3·06	2·34
Acidity, w/w (citric acid), %	1·02	1·66	6·70	5·80	0·12	2·17	4·45
Proteins, %	0·67	0·47	0·28	0·22	0·24	0·52	0·21
Vitamin C, mg./100 g.	47·10	34·49	25·23	22·76	50·10	42·37	28·06
Time for cutting 100 fruits, min.	10	28·30 (peeling by hand)	—	15-16	10	15-16	15-16
Time for extraction of juice, min.	60	90	—	70-75	60	70	70-75

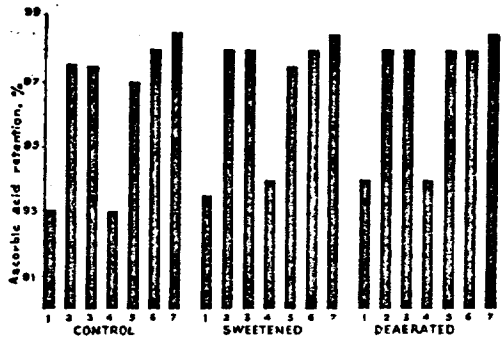

FIG. 1 — ASCORBIC ACID RETENTION IN GRAPE-FRUIT JUICE DURING PROCESSING. [(1) Ordinary pasteurization (bottles) at 175°F. for ½ hr., pre-heating to 175°F.; (2) ordinary pasteurization (cans) at 175°F. for ½ hr., pre-heating to 175°F.; (3) overflow pasteurization (bottles) at 175°F. for ½ hr., pre-heating to 180°F.; (4) sodium benzoate (bottles) @ 1,000 p.p.m.; (5) sulphur dioxide (bottles) @ 700 p.p.m.; (6) flash pasteurization (bottles) at 190°-95°F. for 1 min. and cooling to 175°F.; (7) flash pasteurization (cans) at 190°-95°F. for 1 min. and cooling to 175°F.; original ascorbic acid content in fresh juice, 42·37 mg./100 g.]

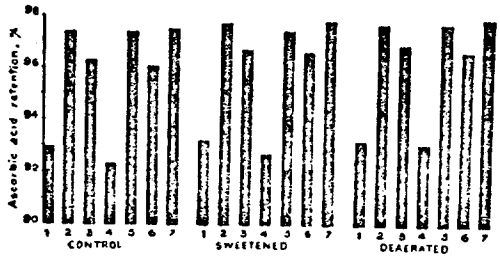

FIG. 2 — ASCORBIC ACID RETENTION IN *Nagpuri sangtra* JUICE DURING PROCESSING. [(1) Ordinary pasteurization (bottles) at 175°F. for ½ hr., pre-heating to 175°F.; (2) ordinary pasteurization (cans) at 175°F. for ½ hr., pre-heating to 175°F.; (3) overflow pasteurization (bottles) at 175°F. for ½ hr., pre-heating to 180°F.; (4) sodium benzoate (bottles) @ 1,000 p.p.m.; (5) sulphur dioxide (bottles) @ 700 p.p.m.; (6) flash pasteurization (bottles) at 190°-95°F. for 1 min. and cooling to 175°F.; (7) flash pasteurization (cans) at 190°-95°F. for 1 min. and cooling to 175°F.; original ascorbic acid content in juice before packing 34·49 mg./100 g.]

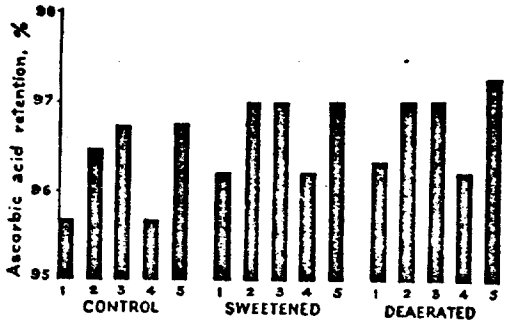

FIG. 3 — ASCORBIC ACID RETENTION IN *mosambi* ORANGE JUICE DURING PROCESSING. [(1) Ordinary pasteurization (bottles) at 175°F. for ½ hr., pre-heating to 175°F.; (2) ordinary pasteurization (cans) at 175°F. for ½ hr., pre-heating to 175°F.; (3) overflow pasteurization (bottles) at 175°F. for ½ hr., pre-heating to 180°F.; (4) sodium benzoate (bottles) @ 1,000 p.p.m.; (5) sulphur dioxide (bottles) @ 700 p.p.m.; original ascorbic acid content in juice before packing, 47·10 mg./100 g.]

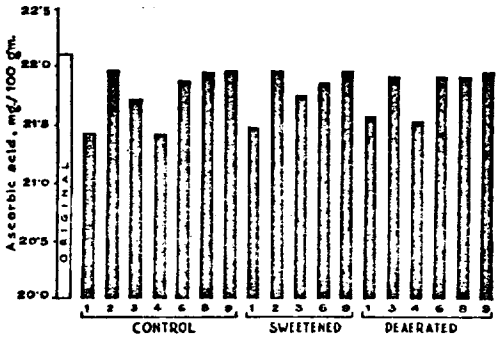

FIG. 4 — EFFECT OF DIFFERENT METHODS OF PROCESSING ON VITAMIN C IN LIME JUICE. [(1) Ordinary pasteurization (bottles) at 175°F. for ½ hr., pre-heating to 175°F.; (2) ordinary pasteurization (cans) at 175°F. for ½ hr., pre-heating to 175°F.; (3) overflow pasteurization (bottles), pre-heating to 180°-85°F.; (4) sodium benzoate (bottles), 0·1 per cent; (6) sulphur dioxide (bottles), @ 350 p.p.m.; (8) flash pasteurization (bottles) at 190°-95°F. for 1 min. and cooling to 175°-80°F.; (9) flash pasteurization (cans) at 190°-95°F. for 1 min. and cooling to 175°-80°F.]

Sweetened samples (under the same method of processing) suffer greater changes in optical density than unprocessed samples. The changes are comparatively less in deaerated samples irrespective of the method of processing.

Flash pasteurization brings about less change in colour than holding pasteurization. The change in colour is lowest in samples preserved with sulphur dioxide.

The optical density of samples preserved by overflow pasteurization was slightly higher than the value for samples preserved by flash pasteurization in cans and less than that for samples preserved by ordinary pasteurization (bottles). Overflow pasteurization method creates a partial vacuum in the container and the change in colour is thereby minimized.

The colour changes in benzoated samples under all treatments were high.

The colour changes in citrus fruit juices during processing may be attributed to the combined effect of temperature, and oxygen (present both in the

TABLE 2 — OVERALL COLOUR CHANGES IN COORG ORANGE JUICE & *NAGPURI SANGTRA* JUICE DURING PROCESSING

Method of processing	Colour index (optical density at 420 mμ)			
	Coorg orange, Control	*Nagpuri sangtra*		
		Control	Sweetened	Deaerated
Unprocessed (before packing)	0·0108	0·0170	0·0170	0·0170
Ordinary pasteurization (bottles)	0·0146	0·0358	0·0382	0·0204
Ordinary pasteurization (cans)	0·0128	0·0226	0·0257	0·0224
Overflow pasteurization (bottles)	0·0136	0·0245	0·0318	0·0243
Sulphur dioxide, @ 700 p.p.m.	0·0112	0·0200	0·0220	0·0200
Flash pasteurization (bottles)	...	0·0311	0·0317	0·0267
Flash pasteurization (cans)	...	0·0237	0·0239	0·0225

head space and in the juice), on the natural colouring matter.

Summary

1. The loss of ascorbic acid during processing in acid citrus juices (lime, *khatta* and *galgal*) is *c.* 3 per cent; in less acid juices (orange, *Nagpuri sangtra* and sweet lime), it is *c.* 7·0 per cent.

2. Colour changes in citrus fruit juices are maximum in samples preserved with sodium benzoate and by ordinary pasteurization (bottles); it is minimum in samples preserved with SO_2.

3. Processing temperature, oxygen, and added sugar in the juices are responsible for the colour changes in the juices during processing.

4. Deaeration and flash pasteurization, and preservation with SO_2 were found to be satisfactory processing methods.

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A STUDY OF NORMAL HUMAN REQUIREMENTS FOR ASCORBIC ACID AND CERTAIN OF ITS METABOLIC RELATIONSHIPS [1]

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The necessity for further research to determine specific nutrient needs for individuals was emphasized in the recommendations made to the President by the National Nutrition Conference for Defense ('41). Kruse ('42) discussed our need for determining optimal intakes of ascorbic acid since it is evident that a large proportion of the population show a past dietary regimen inadequate with respect to this nutrient. As recently as September, 1942, Gordon ('42) stated "the question of the daily human requirement of ascorbic acid must be considered still unsettled". Our need for more knowledge concerning optimal intake of food nutrients was recently summarized in Nutrition Reviews ('43) in the statement that a "good" diet must furnish an optimal quantity of all essential food factors. A number of investigators (Göthlin, '33, Hawley, Stephens and Anderson, '36, Widenbauer, '37, and Abbasy, Harris, Ray and Marrack, '35) who have sought to determine human requirements for ascorbic acid have obtained figures which are now recognized as the minimal intake necessary to prevent the manifestation of a

[1] Material based on a doctoral thesis submitted by Helen J. Purinton to the Graduate School, April, 1943. Aided by a grant from the Purdue Research Foundation. Present address of H.J.P., Agricultural Experiment Station, University of New Hampshire, Durham, N. H.

deficiency of this substance. It was also noted that several investigators (Gaehtgens and Werner, '37, Baumann and Rappolt, '37, Todhunter, Robbins and McIntosh, '42 and Crandon, Lund and Dill, '40) have used the term "at least" in connection with some standard daily requirement figures for ascorbic acid. Studies attempting to develop a means for determining optimal intakes of ascorbic acid are very few. The requirement figures for ascorbic acid occurring in the literature vary from 20 to 150 mg. per day, and from 0.4 mg. to 2.0 mg. per kilogram of body weight.

The principal factor contributing to our lack of knowledge concerning an optimal intake of ascorbic acid has been the disagreement among various laboratories as to the best method for determining human requirements. The use of the saturation test dose method for determining these requirements has met with success in the laboratories of several workers (Goldsmith and Ellinger, '39, Ludden and Wright, '40, Wright, Lilienfeld and McLenathan, '37, and Glazebrook and Thomson, '42). The main reason for variations in results obtained by this method seems to be due to the manner in which the test dose is administered. Several investigators have compared the validity of results obtained with oral versus intravenous administration of the test dose. Ludden and Wright ('40), Wright, Lilienfeld and McLenathan ('37), Faulkner and Taylor ('38) and Hawley and Stephens ('36) have compared the oral and intravenous technics as means of measuring absorption and utilization of a test dose. These investigators obtained more consistent results in any one individual when the test dose was administered intravenously. Their work gave evidence that, in response to a specific dose administered orally, great variations could be expected due to physiological differences in the rate of absorption and utilization.

Ludden and Wright ('40), Harris and Ray ('35), Levcowich and Batchelder ('42) and Hawley, Stephens and Anderson ('36) have determined that long time balance studies of

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ascorbic acid are unnecessary for the investigation of normal requirements.

Since our knowledge as to the manner in which ascorbic acid functions is far from complete, there are no specific means at present by which optimal as contrasted to adequate intakes of ascorbic acid can be measured. The authors of this study proposed to determine an optimal utilization figure by measuring the blood concentration and urinary elimination of a relatively large number of apparently normal individuals in response to a test dose administered intravenously.

EXPERIMENTAL PROCEDURE

College students including both men and women and a few women staff members served as subjects for this study. The men were from the Department of Biochemistry and the women represented majors and instructors in the Department of Foods and Nutrition.

The subject to be tested was given a definite diet with an ascorbic acid content of 15 mg. for the males and 14 mg. for the females. For 24 hours prior to the administration of the test dose of ascorbic acid the subject was requested to continue his normal activities. The amount of water consumed throughout the diet period was not restricted. A "fasting" 24-hour urine sample was collected from the subjects upon this diet. A "fasting" sample of blood was taken before breakfast on the day of administration of the test dose. This sample was taken from the finger and was obtained in order to determine whether or not the subject being studied was to be classed in the normal or subnormal group as regards the fasting content of ascorbic acid in the body. In these studies, using the evidence of Wright, Lilienfeld and McLenathan ('37), Ludden and Wright ('40), and Thysell ('39), all subjects whose ascorbic acid content of the "fasting" plasma was 0.8 mg. per 100 ml. or above were classed as normal and those whose "fasting" plasma values were below this figure were classed as subnormal. (In the evaluation of the results obtained, these two classifications will be con-

sidered separately as well as combined, to determine the percentage of an apparently normal group of individuals who actually showed past nutritional inadequacy with respect to ascorbic acid, and to determine the requirements of individuals who gave proof of an adequate past dietary regimen.)

Although the original plans for this study called for the administration of the test dose intravenously, due to circumstances beyond our control, it was necessary in some cases to give the test dose by mouth. After the "fasting" blood sample was taken, the 500 mg. test dose of ascorbic acid was given, either orally or intravenously (500 mg. in 5 ml. physiological saline [2]). The diet for the 24 hours following administration of the test dose was exactly the same as that of the previous day. The response of the subject to the test dose was followed in the blood stream by measuring the ascorbic acid of the plasma at regular intervals until the absorption of the dose (either from the intestine or from the blood into the tissues) was indicated by a return of the plasma value to the fasting level. The response to the test dose was followed simultaneously in the urinary excretion values. The urine for the entire 24 hours following administration of the test dose was analyzed, at definite intervals, and as soon as possible after voiding. It was necessary to know the actual quantity of ascorbic acid of the test dose excreted in the 24 hours after administration in order to determine the quantity metabolized by the body during this period.

After determining the quantity of ascorbic acid excreted in the 24 hours following administration of this test dose, it was possible to determine the quantity retained by the particular individual being studied. To do this, the quantity of ascorbic acid present in the diet was taken into account, and the level of ascorbic acid in the "fasting" urine sample was taken into consideration. By subtracting the quantity of

[2] We wish to express our gratitude to Hofmann-La Roche for supplying the sterile ampules, and for the crystalline ascorbic acid used in this study.

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ascorbic acid excreted by the individual in the test period from the quantity administered (both in the diet and in the test dose), a retention figure was obtained. By subtracting the quantity of ascorbic acid excreted by this individual under "fasting" conditions from this retention figure, a value was obtained which has been considered in this study the quantity of ascorbic acid metabolized (and therefore, probably required) by the subject in question.

In the course of this study, certain metabolic relationships of ascorbic acid were investigated. Hemoglobin values and the urinary output of citric acid were determined before and after the administration of the test dose of ascorbic acid. Observations were also made on the relation of the ascorbic acid requirement to the basal metabolic rate.

The methods described by the authors in a previous paper (Purinton and Schuck, '43) were used for the determination of ascorbic and citric acids.

For the determination of hemoglobin, the method of Breuer and Militizer ('38) was used, the only modification being the use of ammonium persulfate in place of potassium persulfate suggested by these workers.

The method used for determining the human requirement values for ascorbic acid was checked for validity by means of repeated tests on one person. Altogether, five determinations were completed on this individual. The determinations were made at different seasons, to offset the possibility of the influence of fresh fruits and vegetables in the past dietary history. The greatest variation noted in the different determinations for this individual was only 6.5 mg. per day. The fact that this variation was so small was interpreted to be a good indication of the applicability and validity of this method for the determination of human requirements. The plasma content of ascorbic acid for this subject under fasting conditions was at all times well above the standard normal value used in this study (0.8 mg. ascorbic acid per 100 ml. plasma).

RESULTS

Sixty-three apparently normal individuals, 52 women, aged 16 to 49 years, and 11 men, aged 20 to 25 years, were studied by means of the saturation test dose method for determining human requirements for ascorbic acid. Forty-six per cent of these had "fasting" plasma ascorbic acid levels of 0.8 mg. or more per 100 ml. of plasma. Citric acid excretion was determined for 42 of these subjects. The test dose of ascorbic acid was administered by injection to 47 subjects and by mouth to 16 subjects. The results are summarized in table 1.

TABLE 1

Ascorbic acid requirements[1] and citric acid excretion.

CATEGORY OF INTEREST	AGE IN YEARS AND SEX			
	15 – 20 women	20 – 25 women	20 – 25 men	25 – 50 women
No. subjects	8	29	11	15
Aver. "fasting" plasma asc. acid, mg./100 ml.	0.72	0.71	0.76	0.88
Aver. "fasting" urine asc. acid, mg./day	20.1	25.6	19.7	28.2
Asc. acid administered in test period (diet + test dose) mg.	514	514	515	514
Aver. urinary excretion in test period, mg./day	381.4	382.8	370.6	404.9
Aver. requirement, mg./day	112.5	105.6	124.7	80.9
Aver. requirement, mg./Kg.	1.95	1.86	1.71	1.50
Aver. citric acid excreted prior to test dose, gm./day	0.55	0.38	0.54	0.69
Aver. citric acid excreted after test dose, gm./day	0.80	0.66	0.90	1.24

[1] Results obtained from orally administered doses and with subjects showing very low "fasting" plasma levels accompanied by unusually high retentions were not used in computing average asc. acid requirements.

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It is of interest to note that the younger women subjects showed a higher ascorbic acid requirement than the older ones. Also it was found that the average requirement figure for those with low "fasting" plasma ascorbic acid levels was higher than that for subjects with "fasting" plasma levels of 0.8 mg. or more per 100 ml. plasma.

Hemoglobin studies were made upon 19 of the subjects. It was found that 17 or 89.5% showed a definite correlation of hemoglobin in the blood with ascorbic acid content of the plasma. Only 2 individuals or 10.5% failed to show this correlation. In general, these individuals whose concentration of ascorbic acid in the fasting blood sample was above the figure used in this study as a normal fasting level (0.8 mg. per 100 ml. plasma) also had concentrations of hemoglobin above normal (15.6 gm.-Haden). Those subjects with low concentrations of ascorbic acid in the fasting sample were found to have low levels of hemoglobin. The incidence of low hemoglobin content of the blood was greater in the women subjects than in the men.

Of 16 subjects reporting a low basal metabolic rate, 68.8% had an ascorbic acid requirement below the average.

In table 2 are found figures on the relationship between ascorbic acid retention and citric acid excretion which appear

TABLE 2

Ascorbic acid retention and citric acid excretion.

	NO. OF SUBJECTS	TOTAL CITRIC ACID EXCRETED AFTER ADM. TEST DOSE ASC. ACID AVG. GM./DAY	INCREASE IN CITRIC ACID OUTPUT AFTER ADM. TEST DOSE ASC. ACID AVG. GM./DAY
Total	39		
Retention asc. acid below avg.[1]	22	0.93 (Range 0.41–2.30)	0.41 (Range 0.08–1.08)
Retention asc. acid above avg.	17	0.75 (Range 0.25–1.54)	0.35 (Range 0.05–0.94)

[1] The average retention figure for this group following administration of test dose of ascorbic acid was 138.8.

The differences were considered as having some meaning because they represent quantities greater than the total citric acid output of some of these subjects under fasting conditions.

to confirm the findings in animal studies reported in an earlier paper (Purinton and Schuck, '43). It was observed with our human subjects that lowered retention of ascorbic acid was accompanied by an increase in the total quantity of citric acid excreted. Conversely, high retention of ascorbic acid was accompanied by a lower excretion of citric acid than was noted when retention of ascorbic acid was low.

DISCUSSION

The daily human requirements for ascorbic acid, determined in this study, were found to be higher than most figures occurring in the literature.

Since slightly more than half of the subjects had "fasting" plasma ascorbic acid levels of less than 0.8 mg. per 100 ml. plasma, the high requirement figures may reflect in part the need for ascorbic acid for tissue saturation.

An optimal intake for the young adult appears to be in excess of 100 mg. per day. For adults between 25 and 50 years of age, less than 100 mg. per day appears to represent an optimal intake. This lowered requirement for older subjects is what might be expected since bodily requirements in general tend to decrease with maturity.

The orally administered test dose for determining human requirements for ascorbic acid by the saturation test dose method did not in this study give results comparable to the injected dose. In most cases the proportion of the oral test dose excreted during the 24 hour test period was small and retention figures averaged much higher than was the case with the injected test dose.

The findings of this study seem to indicate that requirements for ascorbic acid are influenced by certain factors, such as basal metabolic rate, and hemoglobin levels.

The relationship noted between ascorbic acid retention and citric acid excretion might be interpreted as signifying that citric acid plays a definite role in the metabolism of ascorbic acid, or that ascorbic acid functions in the metabolism of citric acid.

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SUMMARY

1. The daily requirements for ascorbic acid of a group of 63 individuals, 52 women and 11 men, were determined by use of the saturation test dose method administered intravenously.

2. A requirement greater than 100 mg. daily was obtained for the subjects under 25 years of age. The requirement for a small number of older women between 25 and 50 years of age was well below 100 mg.

3. A correlation between the ascorbic acid of the plasma and the hemoglobin level was found in 17 out of 19 individuals.

4. The basal metabolic rate also appeared to be related to the ascorbic acid requirement, this requirement being less for subjects with low basal metabolism.

5. Confirmation of earlier findings with animal studies on the existence of a relation between ascorbic acid retention and citric acid excretion was obtained.

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VITAMIN C STUDIES IN THE AGED.

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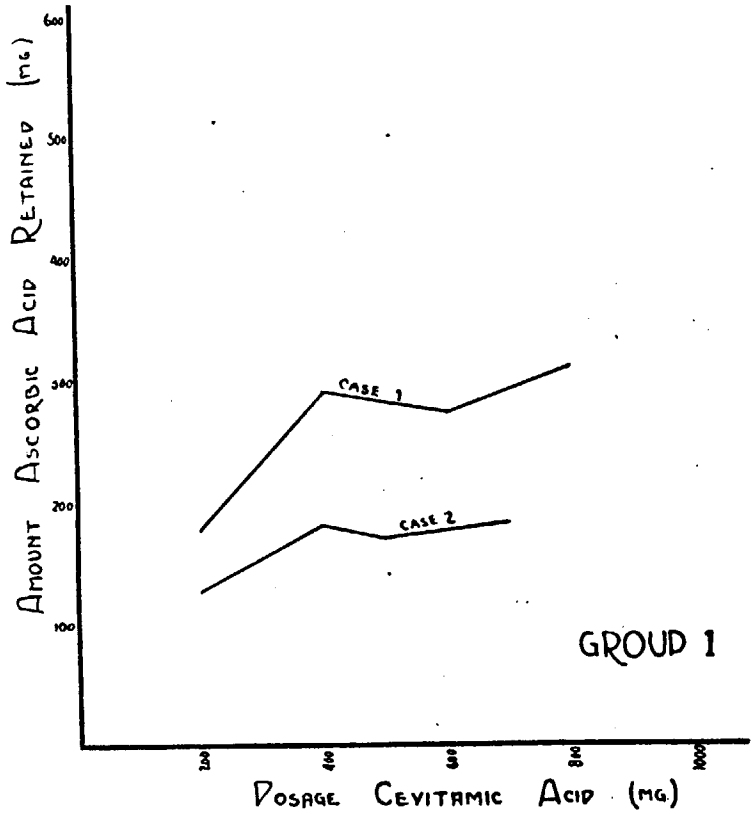
Vitamin C studies have been carried on extensively in infants and children and numerous articles have been written on the clinical significance of vitamin C deficiency in adults. Scant attention, however, has been paid to vitamin C studies in the aged. The only reports thus far have been those of Gander and Niederberger[1] and Kirchmann.[3] Gander and Niederberger administered cevitamic acid to a series of 14 patients (average age, 73 years) in daily doses of 200 mg. for a period of 4 days, followed by daily doses of 400 mg. In most of their cases urinary excretion of cevitamic acid did not increase until a total of 2000 mg. had been received. Kirchmann determined the cevitamic acid levels in a series of 50 patients, ranging in age from 50 to 87 years. Only 1 patient who had been living on an exclusively vegetarian diet for years was found to have the so-called normal cevitamic acid level of 1 mg. per 100 cc. No American investigators, to the best of our knowledge, have reported on vitamin C studies in the aged. It was therefore thought of interest to investigate vitamin C retention and excretion in aged individuals.

This study is based on a series of 25 so-called "normal" aged men and women. Their ages ranged from 66 to 83 years; 14 were men and 11 were women. The subjects who were chosen for this study were individuals who were not suffering from any active disease, were housed in the "Home" division of the institution and were ambulatory. In 10 of the cases, oranges, tomatoes and lemons were part of their dietetic intake.



Method. The amount of cevitamic acid* in the blood 1 hour after the noon-day meal and in the urine at the end of 24 hours was determined. These readings were regarded as our controls. Then, varying amounts of cevitamic acid were administered by mouth, starting with a dosage of 100 mg. and increasing this by 100 to 200 mg. doses over a period of time. The cevitamic acid was gradually increased until the saturation point was reached as evidenced by a sharp drop in the curves showing the amount of cevitamic acid retained. This was computed by subtracting the number of milligrams excreted from the amount given, as plotted against the

* The cevitamic acid, oral and injectable, was supplied through the courtesy of Hoffmann-La Roche, Inc., Nutley, N. J.

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TABLE 1.

Case No.	Age.	Sex.	Date.	Dose of cevitamic acid (mg.).	Cevitamic acid in blood (mg. per 100 cc.).	Urinary excretion of cevitamic acid (mg. per diem).	Cevitamic acid retention (mg.).
1	76	♂	4- 5-40	Control	0.2	9	
			4-17-40	200	1.1	21	179
			4-26-40	400	2.4	110	290
			5- 5-40	600	2.8	327	273
			5-17-40	800	3.1	490	310
2	79	♂	4-11-40	Control	1.7	82	
			4-26-40	200	2.9	73	127
			5- 4-40	400	3.1	219	181
			5- 9-40	500	Not determined	329	171
			5-18-40	700	3.8	517	183
3	77	♀	7- 9-39	Control	0.4	10.8	
			7-15-39	100	0.4	18.8	81
			7-23-39	200	1.0	14.3	186
			8-27-39	400	0.92	97	303
			9-19-39	600	Not determined	143	257
			10-27-39	700	Not determined	340	360
4	77	♂	4-10-40	Control	0.8	38.8	
			4-16-40	200	1.4	59.8	140
			4-25-40	400	2.1	179	221
			5- 5-40	600	3.1	312	288
			5-12-40	800	3.8	490	310
5	72	♀	5- 1-40	Control	0.6	11.8	
			5- 8-40	200	1.3	29.9	170
			5-16-40	400	2.9	210	190
			5-24-40	500	3.2	380	120
			6- 2-40	700	3.6	430	270
6	72	♂	5- 5-40	Control	0.6	7	
			5-16-40	200	1.0	26	174
			5-24-40	400	2.1	89	311
			6- 1-40	600	0.9	133	467
			6-17-40	900	3.2	390	510
7	73	♀	5- 6-40	Control	0.3	7.2	
			5-12-40	200	0.6	19	181
			5-18-40	400	1.1	67	333
			5-26-40	600	1.8	104	496
			6- 2-40	800	3.6	214	586
			6-10-40	1000	3.8	397	603
8	69	♀	5- 6-40	Control	0.7	11	
			5-12-40	200	1.8	30	170
			5-18-40	400	2.4	103	297
			5-25-40	600	Not determined	194	406
			6- 2-40	800	Not determined	340	460
9	73	♀	8-27-39	Control	0.42	22	
			9-21-39	200	0.78	19	181
			10-18-39	400	1.2	77	323
			11- 4-39	500	Not determined	90	410
			11-29-39	600	1.9	122	478
			12-25-39	800	2.7	480	320
			1-14-40	1000	3.1	540	460
10	79	♂	3-29-40	Control	0.7	17.9	
			4- 6-40	200	Not determined	86.8	112
			4-17-40	300	1.9	129	171
			4-28-40	500	2.9	380	110
			5- 3-40	700	Not determined	410	290
			5-12-40	900	Not determined	650	250

TABLE 1—*Continued.*

Case No.	Age.	Sex.	Date.	Dose of cevitamic acid (mg.).	Cevitamic acid in blood (mg. per 100 cc.).	Urinary excretion of cevitamic acid (mg. per diem).	Cevitamic acid retention (mg.).
11	73	♂	7-17-39	Control	1.7	22.6	
			8-24-39	200	1.6	67	133
			9-14-39	400	1.4	107	293
			10- 7-39	500	1.6	129	371
			11-10-39	600	1.9	390	210
			11-27-39	700	2.4	520	180
12	73	♂	7-14-39	Control	0.4	23	
			9-21-39	200	1.0	11	189
			10- 7-39	400	Not determined	97	303
			11-10-39	500	2.3	390	110
13	66	♀	7- 5-39	Control	0.9	0	
			7-16-39	100	1.0	7	93
			7-23-39	200	1.2	13	167
			7-30-39	300	1.6	82	218
			8-22-39	400	1.4	17	383
			8-30-39	500	1.0	17	483
			9-14-39	600	1.1	38	562
			10-20-39	800	2.1	173	627
			11-27-39	1000	2.7	830	170
14	68	♀	1- 2-40	Control	0.38	11.3	
			1-27-40	200	1.7	29	171
			2-29-40	400	2.7	53	347
			3-16-40	500	2.8	99	401
			3-29-40	700	2.6	177	523
			4-10-40	900	3.7	410	490
15	66	♀	5- 1-40	Control	0.73	16	
			5- 9-40	200	1.3	39	161
			5-20-40	400	1.9	98	302
			6- 2-40	600	2.6	167	443
			6- 7-40	800	3.4	314	486
			6-12-40	1000	Not determined	611	389
16	73	♀	4-25-40	Control	0.57	12	
			5- 5-40	200	1.0	34	176
			5-18-40	400	1.4	81	329
			6- 2-40	600	Not determined	136	464
			6- 7-40	800	2.9	380	420
			6-16-40	1000	3.8	642	358
17	80	♂	7-14-39	Control	1.2	13	
			7-25-39	100	0.8	30	70
			9- 3-39	200	1.3	33.5	164
			9-30-39	400	1.8	58	342
			10-17-39	600	2.0	110	490
			11-24-39	800	2.3	290	510
			12- 7-39	900	2.6	580	320
18	81	♂	11-19-39	Control	0.5	10.1	
			12-19-39	200	1.9	29.7	170
			1- 7-40	300	2.8	40.2	260
			2-19-40	500	2.6	106	394
			3- 3-40	600	3.5	212	388
			3-11-40	800	3.9	479	321
19	71	♀	8-15-39	Control	0.92	4.1	
			9-19-39	200	0.9	41	159
			10-16-39	400	1.3	120	280
			11-29-39	500	1.5	122	378
			12- 5-39	700	2.6	350	350

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TABLE 1—*Continued.*

Case No.	Age.	Sex.	Date.	Dose of cevitamic acid (mg.).	Cevitamic acid in blood (mg. per 100 cc.)	Urinary excretion of cevitamic acid (mg. per diem).	Cevitamic acid retention (mg.).
20	68	♂	4- 4-40	Control	0.74	32	
			4-10-40	200	1.4	32	168
			4-17-40	300	1.8	97	203
			4-21-40	500	2.8	126	374
			4-29-40	700	3.3	410	290
			5- 7-40	900	Not determined	698	202
21	68	♀	7-23-39	Control	1.6	76	
			8-24-39	100	1.4	84	16
			8-31-39	300	1.8	116	184
			9-14-39	500	3.1	219	281
			9-24-39	700	Not determined	419	281
			10-17-39	900	3.9	710	190
22	83	♂	8-17-39	Control	0.6	12.2	
			8-30-39	200	1.4	19.9	180
			9-11-39	400	2.0	37	363
			9-30-39	500	2.7	113	387
			10-31-39	600	1.9	260	340
			11-19-39	800	3.1	620	180
23	61	♂	4- 1-40	Control	0.39	14.8	
			4- 9-40	200	0.9	34	166
			4-18-40	400	1.4	126	274
			4-29-40	600	2.9	239	361
			5- 5-40	800	3.3	608	192
24	79	♂	7- 3-39	Control	0.7	13.6	
			7-16-39	100	1.5	22.3	78
			9-14-39	200	1.3	19.8	180
			9-29-39	400	Not determined	77	323
			10- 9-39	600	0.95	190	410
			10-30-39	800	2.2	610	190
25	81	♂	5- 3-40	Control	0.4	2	
			5- 9-40	200	2.4	19.7	180
			5-16-40	400	2.3	97	303
			6-24-40	600	3.3	219	381
			6- 2-40	800	3.8	288	512
			6-16-40	1000	3.6	496	504

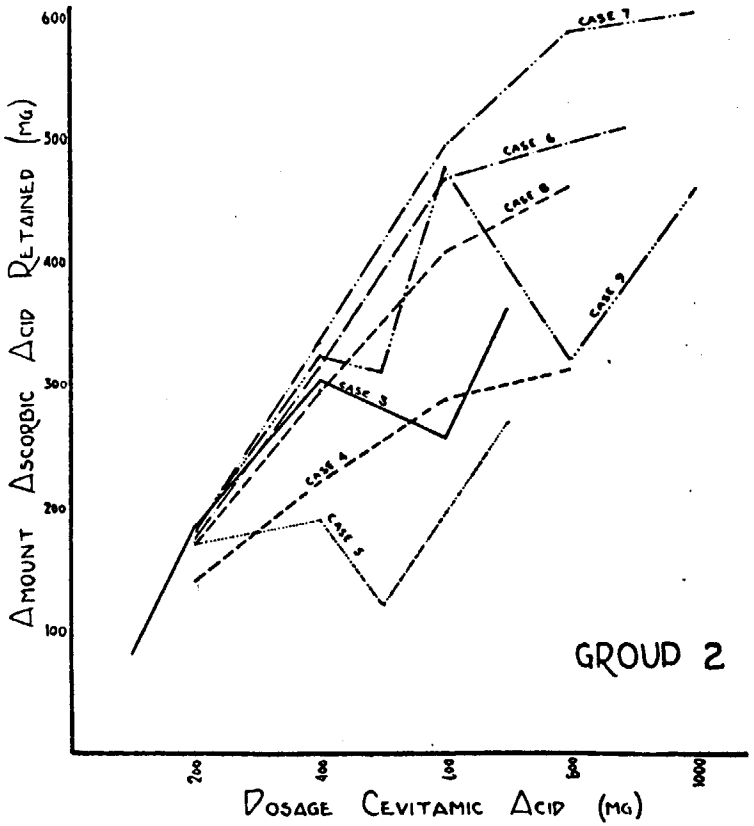
amount of cevitamic acid administered. No more than 1000 mg. of cevitamic acid were given as a single dose to any one subject because diarrhea was experienced by some with this dose. The periods chosen for the administration and determination of vitamin C were selected far enough apart to ensure the complete excretion of the previous dose.

The blood levels of cevitamic acid were determined by Tillmans' method as described by Pijoan and Klemperer[1] and the determination of cevitamic acid in the urine was made by a modification of Tillmans' method as employed by Harris and Ray.[2]

Clinical Studies. The dosage of cevitamic acid, the days on which it was given, the blood levels, the urinary excretion and the amount retained in the system are given in Table 1. In the accompanying graphs the number of milligrams of cevitamic acid which was retained has been plotted against the number of milligrams of cevitamic acid administered.

The levels of the retained cevitamic acid seemed to indicate three types of curves:

1. A more or less constant level (Cases 1 and 2).
2. A constant upward trend during the period these experiments were carried out (Cases 3 to 9).
3. A rise until a peak was reached and from there on a downward trend (Cases 10 to 25).



When the cevitamic acid was given in large amounts, the blood levels of cevitamic acid were likewise found to be appreciably high. The maximum levels were reached at the end of ½ to 1 hour, whether administration was oral or intravenous. The highest level reached in the blood after 1000 mg. of cevitamic acid given by mouth was 3.8 mg. at the end of 1 hour and when given intravenously the maximum level varied in 1 case from 4.5 mg. in 1 hour with a dose of 400 mg. to 4.2 mg. in ½ hour in another case with a dose of 1000 mg.

Comment. When cevitamic acid was given in comparatively large amounts to 25 so-called "normal" aged individuals, cevitamic acid retention was noted in most of the subjects and the blood

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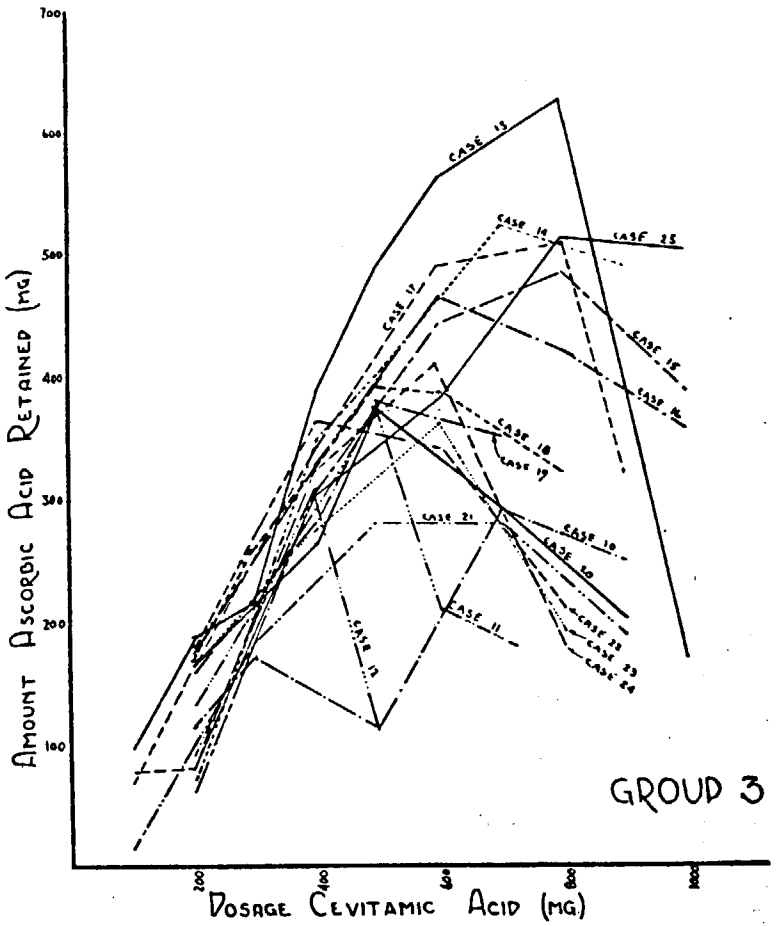
levels of cevitamic acid were likewise appreciably high. The cevitamic acid retention curves were grouped as follows:

GROUP 1. In this group a more or less steady level of retained cevitamic acid was noted. Irrespective of the amounts given, the excretion and retention closely followed the same pattern. These levels would seem to indicate that the saturation point was reached with relatively smaller doses as compared with most of the subjects under study.



GROUP 2. In this group, the cevitamic acid retention continued to increase notwithstanding the fact that constantly larger doses were administered. No saturation point was observed during the time these experiments were carried out. It would be logical to assume from these curves that the subjects in this group required increasingly larger doses of cevitamic acid.

GROUP 3. In this group comparatively large amounts of cevitamic acid seemed to be required before the levels of cevitamic acid retention showed a drop. It is difficult to explain just why there should be less retention with maximum doses of cevitamic acid, unless a reserve of sufficient magnitude had been stored in the system from the previous doses that further cevitamic acid was not required. In this group, the cevitamic acid retention level, after reaching the peak, showed a variable drop from a slight to a sharp descent.

The maximum blood levels of cevitamic acid did not show any appreciable changes which could be related to the avenue of administration, whether oral or intravenous. This was of special interest in view of the rôle which the factors of assimilation, absorption and utilization play in vitamin studies.

The relationship of cevitamic acid retention to arteriosclerosis, which more or less accompanies old age, was of interest to us. This phase of the study is still under investigation and no definite conclusions have been found.

Conclusions. In a series of 25 so-called normal aged individuals, who received comparatively large doses of cevitamic acid, only 2 showed a more or less constant saturation point. In the rest, high retention values were observed; in fact, in 7 cases the amount of retained cevitamic acid continued to increase throughout the experiment.

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EFFECT OF TRANQUILIZERS ON REGIONAL DISTRIBUTION OF ASCORBIC ACID IN THE RAT BRAIN

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Abstract—Studies were made of the effects of fluphenazine, chlorpromazine and triflupromazine on tissue concentration, liver synthesis of ascorbic acid and its distribution in different areas of the brain. All the three drugs were found to increase liver concentration and synthesis of the vitamin at 24 hr after administration of a single oral dose of the vitamin, but only fluphenazine was found to increase its concentration in the adrenals and brain; the increase in the latter case was found to vary in different regions of the brain, the olfactory lobes, hypothalamus and residual brain showing maximum increases, and the basal ganglia, visual cortex and remaining dorsal cortex showing minimum increases. The effects were found to be reversed 72 hr after drug treatment.

THE URINARY excretion of ascorbic acid has been found to be markedly stimulated by the administration of various drugs possessing completely unrelated chemical and pharmacological properties. These drugs include drugs which affect the central nervous system such as hypnotics, chloretone, barbitone, analgesics such as aminopyrine and antipyrine, muscle relaxants such as orphenadrine and meprobamate, antirheumatics such as diphenhydramine and chlorcyclizine and carcinogenic hydrocarbons such as 3,4-benzpyrene and 3-methylcholanthrene (LONGENECKER, FRICKE and KING, 1940; CONNEY, BRAY, EVANS and BURNS, 1961; CONNEY and BURNS, 1959; EVANS, CONNEY, TROUSOF and BURNS, 1960; BURNS, CONNEY, DAYTON, EVANS, MARTIN and TALLER, 1960; BEYLAND and GROVER, 1961 and TOUSTER and HOLLMANN, 1961).

The brain has the highest concentration of ascorbic acid except for the adrenals but in experiments on the ascorbic acid status of animals, the brain ascorbic acid is seldom determined, possibly because of the belief widely held till recently that the chemical composition of the brain is relatively stable. However, previous studies in this laboratory showed the ascorbic acid concentration of brain tissue to decrease in protein deficiency, and to vary in different regions of the brain (RAJALAKSHMI, MALATHY and RAMAKRISHNAN, 1967). Protein deficiency was also found to affect enzymes of glutamic acid metabolism in the brain (RAJALAKSHMI, GOVINDARAJAN and RAMAKRISHNAN, 1965). Since tranquilizers are drugs which act on the central nervous system and can be expected to stimulate ascorbic acid synthesis in the liver on the basis of evidence cited earlier, studies were made of the effects of selected tranquilizers in albino rats on tissue concentration and liver synthesis of the vitamin *in vitro* and the distribution of ascorbic acid in different areas of the brain. Additional studies were made of the reversibility of the changes observed. The tranquilizers used were: fluphenazine (anatensol), chlorpromazine (largactil) and triflupromazine (siquil).

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EXPERIMENTAL

Four groups of ten adult albino rats each matched for age, sex and weight (weight given in tables) were used. The drug was prepared in the form of a pellet by mixing with 1 g of wheat flour and water if necessary. A single dose of the drug consistent with therapeutic practice in man and the body weight of the animal was given orally before feeding. This was 0·02 mg/day/kg body wt. in the case of fluphenazine, 0·6 mg/day/kg body wt. in that of chlorpromazine and 0·6 mg/day/kg. body wt. in that of triflupromazine. The animals were killed 24 hr after administration of the drug. Five animals from each group were used for the determination of ascorbic acid in the brain and the other five for studies on different regions of the brain. Eight animals from each group were used for studies on liver synthesis of the vitamin *in vitro* and its concentration in the liver and adrenals. For studies on the reversibility of the changes observed, two groups of five animals each were used. In this experiment, the experimental groups were given a single dose of the fluphenazine and both the experimentals and controls were killed 3 days after drug treatment. This period was believed to be sufficient for the drug effect to disappear.

Determination of ascorbic acid. The method of ROE and KUETHER (1943) was used for the determination of ascorbic acid.

The animals were sacrificed by decapitation in a cold room maintained at 4°. Immediately after sacrifice, the brain was excised by opening the skull followed by removal of the liver and adrenals. The organs were pressed between filter papers to free them from adhering blood and minced; a known amount of minced tissue was homogenized in 6% trichloroacetic acid and the ascorbic acid content determined. For studying the regional distribution, the different regions were separated by the procedure described by RAJALAKSHMI *et al.* (1967).

In vitro *synthesis of ascorbic acid by liver homogenate:* A 25 per cent homogenate was prepared from liver by homogenizing a known amount of minced liver tissue in 0·25 M-sucrose for 2 min at 0° in a Potter–Elvahjem homogenizer. The homogenate was centrifuged at 9000 *g* at 0° for 1 hr. The supernatant was used for studying the synthesis of ascorbic acid by the method of CHATTERJEE, GHOSH, GHOSH and GUHA (1958).

RESULTS AND DISCUSSION

The data on the ascorbic acid concentration of different organs and the synthesis of ascorbic acid by liver homogenate after treatment are presented in Table 1. As expected, all the three drugs were found to increase liver concentration and synthesis of the vitamin. What was not expected was the difference between fluphenazine and the other two drugs in their effect on the concentration of the vitamin in the adrenals and brain. Similar differences have been found in the effects of these drugs on brain glutamate–NAD oxidoreductase (RAMAKRISHNAN, unpublished).

The data on the regional distribution of ascorbic acid in the brain are shown in Table 2, from which it will be seen that the concentration of ascorbic acid in the fluphenazine treated rats increased in nearly all regions, the greatest increases being found in the olfactory lobes, the hypothalamus and the residual brain, and the least increases in the basal ganglia, the visual cortex and the remaining dorsal cortex. A comparison of the pattern of distribution for the control group in this study with that of the low protein diet in the previous study (RAJALAKSHMI *et al.*, 1967) shows the distribution to be essentially similar except for the remaining dorsal cortex although the values were higher, probably because of differences in diet. In the present study, the value for the remaining dorsal cortex was relatively high as compared to other regions whereas in the previous study it was low. With the omission of the value for this region as atypical, a rank order correlation of 0·88 ($P < 0{\cdot}01$) was obtained between the two sets of values.

In the previous study the increase in the concentration of ascorbic acid with high protein diet was maximum in the case of the hypothalamus, the visual cortex and the residual brain and minimum in the case of the olfactory lobes, the remaining dorsal cortex and the basal ganglia. In the present studies, the increases obtained with fluphenazine treatment were similar in the case of the hypothalamus and the residual brain (greatest increases) and the basal ganglia and the remaining dorsal cortex

TABLE 1.—EFFECT OF TRANQUILIZER DRUGS IN RATS ON ASCORBIC ACID CONTENT OF TISSUES AND ITS SYNTHESIS BY LIVER HOMOGENATE

	Control I	Chlorpromazine (Largactil) II	Fluphenazine (Anatensol) III	Triflupromazine (Siquil) IV	*t* test of significance of difference between means I–II	I–III	I–IV
No. of animals	8	8	8	8			
Whole body weight (g)	231 ± 12	230 ± 12	230 ± 12	231 ± 11			
Weight (g) of:							
Liver	8·4 ± 0·4	8·8 ± 0·3	8·7 ± 0·3	8·2 ± 0·2			
Adrenals	0·026 ± 0·002	0·028 ± 0·003	0·027 ± 0·002	0·028 ± 0·003			
Brain*	1·6 ± 0·1	1·6 ± 0·1	1·5 ± 0·2	1·6 ± 0·1			
Ascorbic acid content (mg/100 g) of:							
Liver	31·8 ± 1·1	36·3 ± 1·0	39·8 ± 1·6	35·7 ± 1·4	$P < 0·01$	$P < 0·005$	$P < 0·05$
Adrenals	365 ± 16	395 ± 17	450 ± 17	376 ± 17	ns	$P < 0·005$	ns
Brain*	35·0 ± 1·3	37·7 ± 1·1	44·6 ± 0·8	37·2 ± 0·7	ns	$P < 0·001$	ns
Ascorbic acid (μg) synthesized in 2 hr by homogenate of 250 mg of liver tissue	66 ± 2·0	88 ± 2·7	98 ± 2·4	76 ± 3·7	$P < 0·001$	$P < 0·001$	$P < 0·05$

Values are means ±S.E.
* Values based on five animals.
ns—not significant.

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TABLE 2.—CHANGES IN THE DISTRIBUTION OF ASCORBIC ACID IN THE RAT BRAIN WITH TRANQUILIZER DRUGS

Region	Weight of tissue* (mg)	Ascorbic acid content (mg/100 g)			
		Control	Chlorpromazine (Largactil)	Fluphenazine (Anatensol)	Triflupromazine (Siquil)
Cerebellum	204 ± 8·2	41·4 ± 0·2	43·2 ± 1·4	45·9 ± 0·6	43·0 ± 0·5
Brainstem	174 ± 4·5	20·0 ± 0·1	20·3 ± 0·3	22·7 ± 0·1	20·8 ± 0·4
Olfactory lobes	60 ± 2·3	50·5 ± 1·3	52·5 ± 1·1	65·0 ± 0·2	54·4 ± 1·6
Visual cortex	75 ± 2·1	46·0 ± 1·2	48·5 ± 1·8	48·7 ± 1·6	47·4 ± 1·5
Hypothalamus	34 ± 0·4	35·7 ± 1·8	34·4 ± 2·7	41·4 ± 1·0	38·1 ± 0·9
Basal ganglia	73 ± 2·1	40·0 ± 0·3	41·0 ± 0·3	43·0 ± 0·8	41·4 ± 1·0
Hippocampus	93 ± 3·1	50·3 ± 1·2	54·4 ± 1·9	57·6 ± 1·3	56·2 ± 1·4
Remaining dorsal cortex	172 ± 3·9	49·4 ± 2·4	51·6 ± 2·3	51·5 ± 2·7	51·0 ± 1·5
Residual brain	617 ± 9·1	35·4 ± 2·5	37·9 ± 1·5	43·1 ± 0·7	37·6 ± 1·5

Five animals in each group; values are means ± S.E.
* All the groups are of almost equivalent weight.

(least increases) but the olfactory lobes showed a relatively big increase and the visual cortex a relatively small increase in the present study, the reverse being true of the previous study. It must be mentioned in this context that the micromethod of BESSEY, LOWRY and BROCK (1947) was used in the previous study whereas the method of ROE and KUETHER (1943) was used in the present study. Although the validity of the micromethod was ascertained, because of the small amount of tissue used, an occasional error in weight may result in a magnified error in the value calculated. This might account for the differences in the relative values obtained for the remaining dorsal cortex. The other differences could have been due to the differential effects of a high protein diet and tranquilizer treatment.

Table 3 shows the tissue concentration of ascorbic acid and its synthesis by liver homogenate in animals killed 72 hr after treatment. The high value for liver synthesis of the vitamin in this experiment as compared to the first experiment (Table 1) must be presumed to be due to the difference in environmental temperatures (108°F in the former case and 92°F in the latter). No significant difference is found between the

TABLE 3.—ASCORBIC ACID CONCENTRATION OF TISSUE AND ASCORBIC ACID SYNTHESIS BY LIVER HOMOGENATE IN RATS KILLED 72 hr AFTER ADMINISTRATION OF FLUPHENAZINE

	Control	Fluphenazine (anatensol)	*t* test of significance of difference between means
No. of animals	5	5	
Whole body weight (g)	187 ± 9	192 ± 15	
Weight (g) of:			
Liver	6·2 ± 0·3	6·2 ± 0·2	
Adrenals	0·026 ± 0·002	0·026 ± 0·002	
Brain	1·6 ± 0·1	1·6 ± 0·1	
Ascorbic acid content (mg/100 g) of:			
Liver	34·3 ± 2·9	34·6 ± 1·2	ns
Adrenals	369 ± 10	347 ± 15	ns
Brain	36·0 ± 1·0	36·6 ± 0·8	ns
Ascorbic acid (μg) synthesized in 2 hr by homogenate of 250 mg of liver tissue	81 ± 4·2	85 ± 4·1	ns

Values are means ± S.E. ns—not significant.

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experimental and control animals; this suggests that the ascorbic acid content of organs as well as its synthesis in liver increase during fluphenazine treatment and come down to normal levels with the clearance of the drug from the body.

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Potentiometric Determination of Vitamin C

Combined Use of 2,6-Dichlorophenolindophenol and Iodate

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THAT sodium 2,6-dichlorophenolindophenol is a specific oxidant for ascorbic acid in fruit extracts is generally accepted. Harris and Ray (*5*) have shown this to be true if titration is carried out in acid solution, but not so in the neutral, or nearly neutral, solutions used by Tillmans (*10*). Since the dye is red in the oxidized form in acid solution, it is often difficult or impossible to obtain the end point visually with precision in certain highly pigmented extracts. That the difficulties involved in the colorimetric method may be avoided by electrometric titration has been demonstrated by Kirk and Tressler (*7*).

A serious disadvantage, however, in the use of standard aqueous solutions of the dye is their instability. Frequent standardizations are necessary (*1*). A method which would eliminate this disadvantage and at the same time depend on the specificity of the dye for ascorbic acid seems desirable. Such a method involving the combined use of iodate and the dye has been developed.

Preliminary Experiments

In order to determine the effect of pH on the specificity of iodine as an oxidant for vitamin C, results were obtained with triiodide and with iodate (iodide previously added) on samples of various citrus juices over the pH range 0.3 to 3. The quantity of reducing substances found in these extracts with either of these oxidants was consistently higher, approximately 10 per cent, than that found with the indophenol dye. This is in agreement with the conclusions of previous investigators (*5, 8, 9*) so far as the nonspecificity of triiodide is concerned. That iodate, with iodide previously added, should be no more specific than triiodide is to be expected from the fact that in acid solutions iodate and iodide react practically instantaneously to form iodine. In fact, without previous addition of iodide, more iodate was reduced than triiodide by equal volumes of the same fruit juice. All titrations in this investigation were followed electrometrically (to be described later).

Other experiments were carried out to determine whether the dye might act as a selective alternate oxidation-reduction catalyst for the oxidation of ascorbic acid by iodine in citrus juices. Over the pH range 0.3 to 3 no catalytic effect was observed. However, it was noted that iodine did not oxidize the dye (reduced) at an appreciable rate even at those pH's where the oxidation of the dye is thermodynamically possible (*3*). It seemed probable, therefore, that the dye solution could be added to the extracts until the end points corre-

TABLE I. TYPICAL POTENTIOMETRIC DATA WITH GRAPEFRUIT JUICE

With Indophenol Dye			Followed by Iodate, Iodide Previously Added			With Iodate Alone, Iodide Previously Added		
Potentiometer Reading	Increment	Buret, Ml.	Potentiometer Reading	Increment	Buret, Ml.	Potentiometer Reading	Increment	Buret, Ml.
....	....	...	....	....	...	...	....	0.01
....	....	...	....	....	...	0.2317	....	4.01
....	....	0.00	....	....	0.00	0.2356	....	4.06
0.2212	....	8.83	0.2413	....	0.25	0.2373	0.0017	4.11
0.2298	0.0086	8.87	0.2413	....	0.28	0.2556	0.0183	4.16
0.2417	0.0119	8.92	0.2512	0.0099	0.33	0.2757	0.0201	4.20 e. p.
0.2573	0.0156	8.97	0.2752	0.0240	0.37 e. p.	0.2832	0.0075	4.25
0.2763	0.0190	9.02 e. p.	0.2851	0.0099	0.42	0.2912	0.0080	4.30
0.2903	0.0140	9.07	0.2907	0.0056	0.47	0.2953	0.0041	4.35

sponding to the oxidation of the vitamin C were reached, after which the titration could be completed with iodate in the presence of iodide. The total reducing capacity of another equal volume of the citrus juice could then be determined with the standard iodate solution alone. The difference between the equivalents of iodate reduced in the two titrations would thus yield the equivalents of vitamin C present. The determination would therefore be based solely on the standard iodate while making use of the specificity of the dye whose concentration need be known only very approximately.

Procedure and Apparatus

An ordinary student potentiometer, equipped with a reflecting walltype galvanometer with lamp and scale attachment (sensitivity, 0.014 microampere), was used. An agar bridge, saturated with potassium chloride, connected the 200-ml. lipless titration beaker with a saturated calomel half-cell. A platinum wire electrode completed the circuit with the potentiometer setup. Slow uniform stirring was maintained during each titration by means of a mercury-sealed stirrer. By placing the citrus juice in series with the titration beaker, one source of oxygen-free nitrogen served to blanket the juice during the analyses as well as to provide an inert atmosphere during titration, and to force portions of the juice into the pipet.

PROCEDURE ADOPTED. Ten milliliters of the citrus juice are transferred to the titration beaker and sufficient 2 *N* hydrochloric acid and water are added to make the initial acid concentration of the resulting solution (approximately 100 ml.) between 0.1 and 0.5 *N*. The indophenol dye (Eastman No. P3463) solution, containing 0.5 to 1 mg. per ml., is then added until one drop of the dye solution remains in the oxidized form. Approximately 10 ml. of 0.1 *N* potassium iodide are then added, and the titration is completed with standard 0.01 *N* potassium iodate. The iodate solution must be added dropwise, allowing at least 5 seconds between each drop. Another 10-ml. portion of the citrus juice is titrated with the standard iodate alone at approximately the same acidity and iodide concentration. The main portion of the iodate may be added, in this case, at any convenient rate. Ten-milliliter burets, graduated to 0.05 ml., are used in all iodate titrations.

A simulated fruit juice, containing known quantities of ascorbic acid and glutathione, was used in the development of the above procedure. Equally satisfactory results were obtained at any pH over the range 0.3 to 3. It is somewhat simpler, however, to work at the higher acid concentrations (0.1 to 0.5 *N*) since readily measured volumes (via graduated cylinder) of common acid reagents may be used.

When the main portion of the residual iodate was added too rapidly after the dye end point was attained, more iodate was reduced than that equivalent to the glutathione present. This is due to the fact that the dye (reduced) is oxidized by iodate to a measurable extent, even with iodide present, unless the iodate is added slowly.

The equivalence point is recognized, without plotting titration data, when the largest change in e. m. f. occurs per drop (0.05 ml.) of oxidant solution added. In the titration of pure ascorbic acid solution or citrus juice with the dye, location of the end point with certainty by this procedure necessitates the inert atmosphere.

As is usually the case in potentiometric titrations, it is necessary to go slightly past the end point to be sure that the largest change has been registered. If the approximate volume of the oxidant required is determined by a rapid titration until a color change is noted, then, in the potentiometric determination, oxidant may be added to within several tenths of a milliliter of the approximate end point before recording potentiometric readings (color change not readily observable with dye solution over a week old). When within a few tenths of a milliliter of the end point, an e. m. f. balance is established after each subsequent drop of oxidant added. From 25 to 40 seconds were required for attainment of the maximum e. m. f. reading after addition of each drop. During this time interval, the potentiometer was continually adjusted to maintain zero deflection of the galvanometer, in order to obtain the reading before the drift downward in potential occurred. By this procedure the maximum change in potential per drop added was readily observed. The titration should not be interrupted during the addition of the last few tenths of a milliliter of oxidant; otherwise the drift in potential will make the results unreliable.

The sharpness of the potentiometric end point is assured if the platinum wire electrode is dipped into chromic acid cleaning solution and rinsed with water before each titration.

To illustrate the precision with which the end point can be determined, results of a typical titration on a sample of citrus juice are given in Table I.

Analytical Results

All citrus juices were filtered through a double thickness of cheese cloth. A drop of capryl alcohol was added to each total sample to prevent foaming caused by nitrogen bubbling through. The nitrogen prevented oxidation of the juices during analysis and also kept them uniform.

The results obtained by the combined use of indophenol dye and standard iodate and by the ordinary method, based on standard dye solution, are given in Table II. The dye solution was standardized by the usual procedure, involving the use of a known solution of pure ascorbic acid (Eastman No. 4640), as determined with standard iodate (or triiodide). Standardization was carried out potentiometrically. In every case 10.00 ml. of the citrus juice were used.

Discussion

Certain investigators (*5*, *6*) have reported that the rate of addition of the dye to either pure ascorbic acid solution or to citrus juice is a determining factor. Presumably the titrations were performed in the presence of air. In the inert atmosphere of nitrogen used in this investigation, the volume of dye solution required was found to be independent of the rate of its addition to either of these solutions. This suggests the possibility that in the presence of air the oxidation of ascorbic acid by oxygen may be induced during its oxidation by the dye. This possibility is also indicated by the observations of Harris and Ray (*5*) and of Fujita and Ebihara (*4*) that greater precision is obtained in the ordinary determination with the standard dye solution if the ascorbic acid solution is added to a measured volume of the dye solution. The conditions under which variations in the method of mixing the reductant and oxidant may change the extent of an induced oxidation by oxygen have been described by Bray and Ramsey (*2*, p. 2284).

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TABLE II. VITAMIN C CONTENT OF CITRUS JUICES

(Comparison of direct dye titration with dye-iodate method)

Citrus Juice[a]	Orange I	Orange II	Orange III	Lemon I	Lemon II		Grapefruit I
HCl normality	0.25	0.25	0.60	0.25	0.25	0.25	0.50	0.25
Dye normality × 10^3	4.670	5.697	5.697	5.687	5.708	3.146	3.146	4.339
Volume, ml.	11.28	9.89	9.91	9.06	11.12	20.08	20.09	9.09
	11.25	9.90	...	9.08	...	...	...	9.07
Milliequivalents × 10^3, av.	52.60	56.37	56.46	51.58	63.47	63.17	63.20	39.40
Residual iodate, 0.01040 *N* Volume, ml.	0.51	0.55	0.55	0.56	0.40	0.42	0.43	0.39
	0.52	0.53	...	0.58	...	...	...	0.37
A. Milliequivalents × 10^3, av.	5.36	5.62	5.72	5.93	4.16	4.37	4.47	3.95
Iodate without dye Volume, ml.	5.65	6.00	6.01	5.54	6.56	6.50	6.50	4.18
	5.64	...	...	5.55	...	...	6.51	4.20
B. Milliequivalents × 10^3, av.	58.70	62.40	62.50	57.67	68.22	67.60	67.65	43.58
B minus A	53.34	56.78	56.78	51.74	64.06	63.23	63.18	39.63
Difference from dye titer, %	+1.40	+0.72	+0.57	+0.31	+0.93	+0.09	−0.03	+0.58

[a] Orange I, fresh navel; II, fresh Valencia; III, Valencia after refluxing in air for 0.5 hour. Lemon I, fresh; II, after standing one week out of contact with air (blanketed with nitrogen). Grapefruit, fresh. One milliequivalent of ascorbic acid = 88 mg.

Summary and Conclusions

This potentiometric method for the determination of vitamin C in citrus juices depends upon a stable potassium iodate solution as the only standard oxidant and, at the same time, makes use of the specific oxidation of ascorbic acid by 2,6-dichlorophenolindophenol. Standardization of the unstable dye solution is thereby eliminated.

The results obtained by this method (Table II) are in close agreement with those dependent on direct titration with an accurately standardized dye solution.

The method should be applicable to other biological fluids in which the indophenol dye is a specific oxidant for vitamin C.

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SPECIAL ARTICLE

The Administration of Large Doses of Ascorbic Acid in the Prevention and Treatment of the Common Cold

Part I

Edme Regnier, M.D.

Any physician whose interest lies in the treatment of what is usually referred to as the "common cold" soon learns that he is faced with a number of seemingly insurmountable obstacles. As will be described, a cold is not one particular disease but one of many, all caused by viruses with some due to a viral-bacterial invasion and others associated with allergic reactions of the mucous membranes or with responses to fumes, as cigarette smoke, smog and the like. Should he venture into the field of treatment as, for example with ascorbic acid, he finds himself faced with a new set of obstacles in that there is a wide variation in the normal need for the substance, with a range of 20-fold in some animals and presumably in man. It is known to prevent scurvy, but its other uses have, by no means, been defined either for the normal or the infected animal or human being.

The Common Cold

The textbook description of the common cold is particularly uninspiring to the investigator. It is irritatingly vague and omits any references or uncommonly mentions the misery of the disease.

We learn that the common cold is a syndrome characterized by a catarrhal inflammation of the upper respiratory tract, namely the nose, pharynx, accessory nasal sinuses and the larynx. Should extension occur, the disease becomes laryngotracheobronchitis in which there is usually a bacterial invasion, although this may be present in 35 per cent or more (K. P. Nichol and J. D. Cherry, New Eng J Med 277:667 [Sept 28] 1967) of the patients with ordinary colds. There is no doubt that the disease is caused by more than 30 of the 120 viruses which may invade the tissues of the respiratory tract, but there is some doubt as to whether the bacteria are primary or secondary invaders or perhaps quiescent commensuals which become pathogenic when a viral infection develops. This subject will be discussed again in another context.

The so-called rhinoviruses are many and include those formerly labelled coryzaviruses, muriviruses, Salisbury viruses and the enterovirus-like viruses (the Picorna-like viruses or picornaviruses). Although colds are sometimes differentially diagnosed from influenza a syndrome which cannot otherwise be distinguished from a cold may be caused by the presence of the influenza and parainfluenza viruses as well as ECHO II and 28 and Coxsackie A-21 (myxoviruses and syncytial viruses). Medical World News (8:26 1967) carried a report of the isolation and identification of six newly discovered viruses by Dr. Robert M. Channock and his associates at the National Institute of Allergy and Infectious Diseases. An earlier study done in 1966 at the University of Chicago proved that 41 types of rhinoviruses (of the

80 known antigenically different types) had been isolated from 131 of 425 specimens taken during the respiratory tract illnesses of 55 medical students studied for a span of four years (JAMA 198:31 Nov 1966). Dr. D. A. J. Tyrrell, Director of the Common Cold Research Unit at Harvard Hospital, Salisbury, England, is quoted as saying that, "Very little is known of the epidemiology of colds.

"The 'cold season' apparently differs in many localities and varying populations. It must be established whether newly isolated serotypes are 'old' or variants which may occur with influenza viruses. It is possible to produce rhinovirus variants in the laboratory by bringing them into contact with various amino acid bases." According to Te-Wen Chang (Medical Science, Feb:23, 1967), "In addition, a small fraction of respiratory disease results from simultaneous invasion by several agents."

Not all of those who are exposed to the viruses become ill, and some of these, nevertheless, develop antibodies and become immune. The viruses may produce asymptomatic disorders demonstrated by increases in antibody production and serological tests. Dr. Chang notes that exposure to influenza virus A and B results in clinical illness in only 75 per cent of those exposed as compared to 50 to 75 per cent exposed to parainfluenza, 1, 2 and 3; 50 to 90 per cent on exposure to adenovirus types 1, 2, 3, 4 and 7; 45 per cent after exposure to RS virus; and only 2 to 3 per cent after exposure to Coxsackie A-21 virus. "Reinfections with respiratory viruses are, fortunately, mild and involve only the upper respiratory tract. They are not easily recognized because the presence of antibodies decreases the quantity of viral excretion and influences the degree of antibody response." Readers interested in more details as to the epidemiology, pathology, virology and clinical details of the "Common Cold and Related Diseases," should consult this text by D. A. J. Tyrrell, London, Arnold, 1965 and the chapter of the same title in the British Medical Bulletin 23:124 May 1967.

Dr. Chang also notes that during the winter months when the viral infections are most common, the incidence of nasopharyngeal carriage of group A *streptococcus pyogenes* and *D. pneumoniae* is also highest, but that the relationship between the two groups of pathogens is not clear. Of interest are the discoveries that the staphylococcal carrier state is twice as common in the patients infected with the parainfluenza 1 and 3 viruses and that these same patients do not carry streptococci, pneumococci and *H. influenzae*. The so-called "cloud babies" who are infected with viruses carry staphylococci in the nasopharynx. There is a high risk of secondary infection with staphylococci, pneumococci, streptococci and *H. influenzae* viral pnuemonia affects the patient. "This coincident infection, or 'dual epidemic' involved 89 per cent of a closed population of 200. Of those infected, 50 per cent had both agents, 36 per cent had influenza virus alone and 14 per cent had streptococcus alone. The nature of the interaction is unknown."

Symptoms, Signs and Clinical Course of Viral Infections

The textbooks agree that the incubation period is 18 to 48 hours, but in some epidemics the incubation period can be as long as one week. The onset of the infection may be abrupt or insidious, and when acute there is a scratchy sensation in the throat or palate associated with or soon followed by sneezing, rhinorrhea accompanied by varying degrees of malaise with a rise in temperature which is more often seen in infants and children (100 to 102°F.). There may be a partial or

complete loss of the sense of taste and particularly of smell. In the study of Gwaltney (J. M. Gwaltney, J. O. Hendley, G. Simon and W. S. Jordan, JAMA 202:158 Nov 6, 1967), 468 employees experienced 3,314 respiratory illnesses during a three-year period. In less than 10 per cent of the 139 patients in whom rhinovirus infections were demonstrated to be present, was there vomiting, diarrhea or the need to stay at home with an increase in the production of sputum noted in 15 per cent with 10 to 20 per cent of the patients complaining of malaise, feverishness, chilliness and myalgia. In almost 40 per cent there were headaches with 60 per cent sneezing and more than 60 per cent with a nasal discharge and less than 40 per cent, nasal obstruction. Twenty-five per cent of those infected complained of a scratchy throat, with 45 per cent of a sore throat, with 25 per cent hoarse and 40 per cent coughing. The nasal discharge, obstruction, cough and hoarseness were more pronounced in the rhinovirus infected patients for ten to 14 days of illness. A sore or scratchy throat was a more frequent component of the illness in nonsmokers, and a cough was more common and persisted for a longer time in those who smoked.

The texts seem to agree on a duration of 7 to 14 days, but the study of Gwaltney, *et al* shows that the rhinovirus infected patients were ill for periods which varied from one to 33 days with a median of 7.4 days, a mean of 8.9 days and a mode of four days. The patients in whom no rhinoviruses were isolated were ill for periods of one to 42 days with a median of 5.9 and a mean of 7.4 and a mode of five days. Only one-fourth of the rhinovirus-positive group ". . . had illnesses lasting as long as two weeks." Headache occurred more often in the female patients and sputum production in the males. There are no comments as to diet or to nutritional factors as vitamin ingestion.

The Epidemiology of Respiratory Virus Infections

At first glance it would appear that the epidemiology of "colds" would be easy to understand. There is an infection of the local mucous membranes which react with inflammation and an increase in the production of mucus with coughing and sneezing and the infection of others. The complex epidemiology of respiratory virus infections has been reviewed by Sir Christopher Howard Andrews, the former deputy-director of the National Institute for Medical Research, London, who was also in charge of the World Influenza Centre (WHO) and the Common Cold Research Unit, Salisbury, England (Science 146:1274 Dec 4, 1964).

He notes that the viruses responsible for the infections grouped together are different from the point of view of chemical composition, size and other properties which determine their taxonomic position. The myxoviruses contain RNA with lipid-containing outer membranes. Influenza virus A causes the disease with the all-too-familiar symptoms with widespread outbreaks of short duration; influenza virus B causes similar attacks but not as widespread, and often only in closed communities and influenza virus C is not as yet implicated as a reason for serious disease. The four parainfluenza viruses do not cause epidemics in adults but attack children causing croup and other serious diseases. "Immunity to such viruses is at the most partial; re-infection in adults may appear as the common cold. The syncytial virus is a major cause of bronchiolitis and bronchopneumonia among small children. Like the parainfluenza viruses it may strike more than once, causing in adults nothing worse than colds." In ten per cent of those afflicted, the Eaton agent which is not a virus but a pleuropneumonia-like organism susceptible to the tetracyclines is responsible for the in-

fection which in some epidemics may cause up to 80 per cent of all viral pneumonia.

Although the rhinoviruses were first recognized as a group in 1960, there are now (1964) 40 or 50 serotypes known. They are small RNA-containing viruses (picornaviruses) and may become adapted to living in the upper respiratory tract. Others of the same family, the Coxsackie B and some ECHO viruses, normally inhabit the gut but may be associated with respiratory tract infections. Coxsackie A-21 prefers to live in the nose rather than in the gut and is a cause of typical colds in closed populations as service recruits. There are 30 types of DNA-containing viruses which are the causes of pharyngitis associated with conjunctivitis rather than colds affecting closed populations. "There remain a large number of common colds and other minor infections from which no causative agent has been isolated. Some of these may prove to be myxoviruses which we do not yet know how to cultivate." It is obvious ". . . that the symptoms produced by these different viruses are not clearcut but overlap most confusingly; so diagnosis on a purely clinical basis is very difficult."

In winter, resistance is presumably lower and crowding is greater and spread would appear to be due to these two factors but, ". . . there are doubts about the extent to which these infections are catching." According to Lovelock, about ten per cent of adults exposed to those infected with colds become infected. In families, O. M. Lidwell and T. Somerville (J Hygiene 49:365, 1951) discovered that the chance of catching cold from an infected member of the household was only one in five. Analysis of the colds infecting office workers failed to prove that the infection was due to another employee with a cold. Epidemics have been shown to occur simultaneously with no possible patient-to-patient transmission. Changes in the weather are important, at least in the similar infections of animals, especially swine. Sir Christopher draws attention to the fact that adenovirus and Coxsackie virus A-21 affects troops but not the population-at-large, and that rhinoviruses are the causes of colds during the early winter with the myxoviruses, including influenza "usually quiet until the New Year." He poses the question as to why the parainfluenza viruses chiefly affect children, while the closely related influenza A is no respector of age.

It is commonly thought that the viruses which inhabit the surface of the mucous membranes ". . . are less apt to engender a solid immunity than those which enter the bloodstream and cause generalized infections. . . . There is perhaps some truth in this but perhaps less than was once thought. Influenza A virus gives rise to potent antibodies and useful immunity; it surmounts the obstacle of herd-immunity by changing its antigenic structure between one outbreak and the next; prevalent antibodies cannot therefore stop it. . . . Children and even adults may have repeated infections, although the viruses have not been shown to change antigenically. The same is true of the respiratory syncytial virus." D. Hamre (unpublished data) ". . . has isolated five different rhinoviruses from five colds occurring in one individual during the course of 18 months. . . ."

The frequency of colds is inversely related to the temperature and ". . . influenza virus persisted in artifical mists when the relative humidity was lower, as it commonly is indoors in winter." Chilling of the body surface is not an epidemiological factor except perhaps in women during the middle of the menstrual cycle. It may, however, tilt the balance in favor of the virus. Women appear to have a transient immunity during the three days before the period is due.

Stressors of various types are important factors in the epidemiology of virus infections (M. Dietz, Jr. Industr Med 26:229 May 1957). In animals, the introduction of susceptible mice into a herd ". . . stirs up outbreaks of infectious disease." Sir Christopher notes that something similar may occur when young people are suddenly brought into close and continuous contact. It has frequently been observed that there is an outbreak of colds when schools first open in the fall of the year. The Great Lakes Medical Research Unit uncovered a different type of stress effect. Recruits at the station received their inoculations during their first five weeks of training. When this program took place during the winter there was a high incidence of febrile respiratory infections which did not affect seasoned personnel. When half of the group received the same inoculations during the second five weeks of basic training there was a 20 per cent decrease in respiratory tract illnesses as compared with the control group. There was a similar reduction in the incidence of rubella. It was concluded that the stress of inoculations in addition to that of the new, highly disciplined life were responsible for the increased susceptibility to the viral infections.

Sir Christopher discusses three methods of controlling the viral infections; interference with the spread of viruses; raising the resistance of the body or by buffering and protecting against the more dangerous stresses. At the moment none of these is practical.

Predisposition and Susceptibility

There is no clear picture of the predisposing factors, although a number have been suspected. Chilling does not, of itself, cause colds. Drafts may act because of the carriage to the patient of more of the viruses or bacteria. It has been noted that children with an allergic background show a greater tendency to wheeze when their infections are parainfluenza 1 and 3, RS or adenovirus types 1-5. Infection appears to be facilitated by the inhalation of fumes and of polluted air whether from cigarette smoke, primarily that of the patient, or secondarily when others smoke, or by smog, smaze or industrial pollutants of the atmosphere, especially during temperature inversions. Women seem to catch colds more often during their periods. Folk lore and some nutritional studies point to avitaminosis, especially for ascorbic acid and general malnutrition as important factors; but, as will be discussed, this is an area in which there are controversial and contradictory points of view.

There is a seasonal factor with the great majority of the virus infections occurring during the fall, winter and spring, with epidemics noted during September and early October, and the middle and latter parts of December or early January and again in late February or early March. Influenza and Coxsackie A-21 viral infections are more frequent from autumn to spring with influenza viruses taking over during the middle winter months. The infections caused by the adenoviruses and parainfluenza viruses may be present throughout the year with a lower incidence during mid-summer.

The age of the patient is an important factor in that children seem to be most often affected by the parainfluenza viruses with the rhinovirus and influenza viruses attacking human beings regardless of age. The RS virus is responsible for lower respiratory tract infections of children. One-fourth to one-third of the acute upper respiratory illnesses of a large group of adults was associated with rhinovirus invasions (J. M. Gwaltney, *et al.* JAMA 202:158 Nov. 6, 1967).

The effects of certain host factors upon susceptibility will be discussed elsewhere (H. S. Dowling, G. G. Jack-

son and T. Inouye. J Lab Clin Med 50:516 1967).

The Magnitude of the Problem

The common cold is the most prevalent of all diseases with adults suffering from two to three colds each winter and children from eight to ten or more for a total of more than 80 of 660 million colds each year, of which more than 30 million occur during February or early March requiring medical attention. Seventy per cent of all of the colds are experienced between September and the end of March, with more than 285/1,000 people affected during the first three months of the year. The urban infection rate is almost 20 per cent higher than it is in rural areas, and in the northeast states the rate of infection is almost 20 per cent higher than it is in the south. It has been estimated that the cost of the common cold is two billion or more days of misery with a cost to the economy of 750 million dollars and 70 million days of absence from school. The amount of money spent on medicines is estimated at $250 million each year. The costs of all research in the fields of diagnosis, treatment, vaccines and the possibility of enhancing resistance is estimated at $20 billion annually. There are no accurate statistics regarding the bacterial invasions and their permanent ill effects or of the sensitization reactions to antibiotic agents or antimicrobial drugs or of the total costs of hospitalizations because of extensions or complications of the disease.

Traditional Treatment of the Common Cold

Present-day treatment of the common cold is an expression of despair. Bed rest is recommended, although there are many patients who are more restless in bed than out of it, and those who are severely afflicted will need no urging to remain at rest. Should there be a temperature rise, the treatment consists of acetylsalicylic acid or its homologues as mixed with supposedly synergistically acting drugs, usually analgesics. A high fluid intake is recommended but not on the basis of any controlled studies. In that it is difficult to differentiate between an allergic reaction of the mucous membranes and a viral infection, antihistamines have been used but with indifferent success except as these drugs have an atropine-like effect which helps to lessen the flow of mucus so that, at present, belladonna in a delayed-release form is a popular form of treatment. Codeine or its homologues and other antitussive agents are used for the control of cough, if present, and expectorants if the mucus is either not easily raised from the bronchial tubes (in lower respiratory tract extensions of the disease) or is so thick that it is felt as a post-nasal drip. Antibiotic agents are used to control bacterial infections or those due to the Eaton agent. As a result of folklore and experience, many patients dose themselves with and physicians prescribe fruit juices. If fruit juices, other than their water content, are of any use it can only be because of their ascorbic acid.

Scurvy

As is generally known, scurvy affected seafaring peoples, especially those who went on long trips. It was the first deficiency disorder to be recognized. In 1536, Jacques Cartier, the French explorer, reported on the miraculous curative effects of infusions of pine bark and needles. Lime juice was made a part of the diet of the sailors of the British navy in 1795 and for those in the merchant marine in 1865. Infantile scurvy was first recognized by Smith and investigated by Sir Thomas Barlow (Barlow's Disease) in 1876. Scurvy is unique among the deficiency disorders in that it only affects man

and the primates and guinea pigs. All other species can synthesize ascorbic acid endogenously. Some substances, as the terpene-like cyclic ketones, stimulate rat tissues into the production of ascorbic acid. Animals otherwise synthesize the vitamins from glucose by way of glucuronic acid and 3-ketogulonate. In man there is a deficiency of the enzyme which converts the gulonate to ascorbate. In this country true scurvy is not often encountered. Since 1932 when hexuronic acid (now known as ascorbic acid) was first isolated from the adrenal cortex of animals and from cabbage leaves and characterized as a six-carbon-atom chain closely related to glucose, its primary role in the metabolism of animals has not been difficut to establish. There have been great difficulties in establishing the human requirements of ascorbic acid other than the fact that in its absence or deficiency, scurvy occurs.

In the experimental animal maintained on a scorbutic diet, there are defects in mesenchymal tissues and an impairment in the ability to synthesize collagen, dentine, osteoid and intercellular cement substance. In human infants there is extreme irritability, anorexia, weight loss, severe pain when touched or handled, with hemorrhages in the skin, mucous membranes and subperiosteal tissues near the ends of the long bones. There is a typical hemorrhagic swelling of the gingivae so that the teeth may be lost to sight. In adults, the chronically ill, indigent and insane, the alcoholic and food faddist may present the signs and symptoms observed in infants, and in addition, petechiae of the dependent parts of the body with hemorrhages into the muscles and viscera.

Ascorbic Acid

Ascorbic acid is a water soluble lactone widely distributed in nature and especially in vegetation. It occurs in high concentrations in rose hips, citrous fruits, tomatoes, berries and some of the leafy vegetables. It is easily destroyed by heat, light, alkalinity and the catalytic action of copper as in some types of cooking vessels or containers. Much of the vitamin is lost during food processing, storage and preparation.

Despite more than 30 years of intense research in the field, many of the secondary functions of ascorbic acid are not understood. For almost a year a group of British volunteers remained on a diet which contained 10 mg of ascorbic acid. Although the plasma levels of the vitamin were low, the volunteers remained in health and showed normal wound healing. In another study conducted by Crandon no ascorbic acid was ingested and the subject's plasma level fell to zero by the forty-first day with a depletion of leucocytes and platelets. Abnormal hair follicles and hair did not appear until the fourth month. Easy fatiguability and anorexia were experienced before objective signs were noted. Zero plasma concentrations of ascorbic acid may be present with no evidence of scurvy, since small quantities may be present in the buffy coat or leucocyte cream observed above packed red cells in centrifuged blood.

There is no completely satisfactory explanation for the high concentration of ascorbic acid in the adrenal cortex, the corpus luteum or the ovary, the pituitary or in the other centers of high metabolic activity which are (some of them) the first to respond to stress. When adrenal stimulation is intentional, there is a temporary depletion of both ascorbic acid and of cholesterol, and it has been suggested that the two compounds are both related to hormone synthesis. If infection is regarded as a stressor, then the depletion and the need for repletion would be logical assumptions.

Chemically speaking, ascorbic acid is a strong reducing agent, but this one of its attributes does not serve to fit it into

any one metabolic pathway, although Hopkins has proposed a relationship between ascorbic acid and the oxidation of glutathione. It has also been suggested that the vitamin may play a part in the oxidation and reduction of the sulfhydryl groups of cells. There is some evidence that ascorbic acid is a necessary part of the mucopolysaccharides of ground substance. There are a number of enzyme systems, as alkaline phosphatase, hepatic esterase and perhaps "dopa" oxidase which depend on its presence. Ascorbic acid is known to be a part of two metabolic pathways; the metabolism of phenylalanine and tyrosine and the preservation of the reduced form of folic acid, that is, folinic acid. Neither of these functions is associated with the development of scurvy. Advanced scorbutic patients demonstrate normocytic or macrocytic anemia and leucopenia. According to Drill (Pharmacology in Medicine, McGraw-Hill Book Company). ". . . there seems to be an obscure relationship between ascorbic acid and the capacity to produce antibodies, and a correlation between a deficiency state and diminished resistance to infection has been demonstrated both experimentally and clinically. The mechanism by which this occurs, however, is unknown, and it is possible that the effect is mediated through a complex endocrine disturbance related in some manner to the alarm and adaptation reactions."

Human Requirements of Ascorbic Acid

It must be remembered that the daily requirements of ascorbic acid are those for people whose diets are supposedly otherwise adequate and who are not ill. The National Research Council suggests a daily intake of 75 mg for an adult with 100 mg during the latter months of pregnancy, with 150 mg during lactation. To quote Drill; "Without any question, a very large segment of the American population does not actually consume as much as this, and the incidence of suboptimal nutrition as regards this component, at least, is quite large. Supplementation of suboptimal diets is accomplished with 25 to 75 mg daily, and most of the commercial multi-vitamin preparations contain at least the former level. . . . The usual therapeutic dose is 30 to 50 mg per day." Elsewhere Drill writes, ". . . the cooking methods and dietary plans of many housewives lead to the preparation of nearly scurvy-producing meals for their families."

The controversial aspects of vitamin supplements in general and of vitamin C in particular were made into an issue when the Food and Drug Administration proposed stringent new regulations which would establish the minimum and maximum amounts of up to seventeen specified vitamins and minerals that could be contained in food supplements. The FDA has used, as an endorsement of its views a statement ascribed to the Food and Nutrition Board of the National Research Council, but several of the members of the board disagreed vigorously with the conclusion of the FDA that normal persons could obtain all the vitamins and minerals they needed from a regular diet. It was their position that because of poverty, lack of knowledge and other reasons, many people did not get an adequate allowance of vitamins and minerals in their daily diets.

At the meeting in Chicago of the Federation of American Societies of Experimental Biology there were a number of reports on the role of vitamins and health. There were new studies of the importance of vitamins E, A and of the B complex. H. G. Hampton of the University of Alberta noted that a lack of vitamin C resulted in fibrous growths in the vascular system of animals and that vitamin C deficiency may lead to activity on the part of latent viruses.

At the meeting of the National Academy of Sciences at Washington, D. C., Dr. Roger J. Williams and his research assistant Gary Deason of the Clayton Foundation Biochemical Institute at the University of Texas, reported on vitamin C metabolism and demonstrated that there was a 20-fold range in the amounts needed by individual animals. To begin with, guinea pigs were maintained on an ascorbic acid-free diet. The supplements of vitamin C administered varied from amounts described as ranging from minute to 3.2 mg/100 gm body weight. The latter dose, if extrapolated to human beings would represent 2.24 gm/154 lbs. For comparative purposes it should be recalled that the FDA's current recommendations are for daily allowances of 18-70 mg for adults with 30 mg for infants and 100 mg for lactating women. The range of 18 to 100 mg is sixfold, whereas that of animals was 20-fold.

One of the guinea pigs which had received no ascorbic acid developed scurvy in ten days but, nevertheless, maintained its weight and lived for the duration of the experiment which lasted eight weeks. Others of the same group both lost weight and died by the twelfth day after the deficiency became apparent.

Of a second group of the animals the members of which received small quantities of the vitamin, again, one animal remained well and showed a steady weight gain for a total which was greater than that of the animals which had received from 16 to 32 multiples of the amount of the vitamin administered. Another animal of this group developed obvious signs of scurvy by the eighth day and died on the next day.

A third group of animals did not do well on high doses of vitamin C. When these received as much as 12.8 mg/100 gm, which is four times the maximum administered to the other test groups of guinea pigs, "The change was almost incredible." In every animal of the group there was weight gain.

Although perhaps not pertinent to this review, the authors cite a study of the chairman of neurosurgery of the Methodist Hospital of Houston, Dr. James Greenwood, Jr., who discovered that daily doses of ascorbic acid of the range of 500 to 1,000 mg were beneficial both to him and to his patients treated for back pain and for the "preservation of disk integrity." Dr. Williams notes that ". . . medical reports have persisted over many years indicating that vitamin C intake beyond that obtained in the usual diet is valuable in many pathologic conditions." These reports are concerned with dental disease including gingivitis, infections, wound healing, bleeding ulcers, allergic disorders and infections.

The reason why such reports are not readily accepted by physicians is because there is no wish to be associated with faddists, especially those who promote food supplements and because such ". . . acceptance opens the door to variation in the needs of all nutrients. . . . The desire of this agency (the FDA) to protect the public against charlatans and extravagant claims is laudable, but when it goes so far as to hold the food supplements are valueless for the average person, it is yielding to an unscientific taboo." It is the opinion of Dr. Williams that within a period of 30 years or less, studies of vitamins, minerals and amino acids will result in knowledge of the recognition of individual needs which vary widely.

As of August, 1966 there were numerous and weighty objections to the stand taken by the FDA with the objectors including leaders of the Department of Agriculture, the Committee on Recommended Allowances of the Food and Nutrition Board, National Academy of Sciences-National Research Council. As of December, 1966 the news media carried the announcement

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from the FDA that it had postponed, indefinitely, the effective date of regulations, proposed in June to regulate composition and labeling of vitamin-mineral dietary food supplements and fortified foods.

As evidence of the conflicting opinions in this field there is the recent report from the Common Cold Research Unit (Salisbury, England) (Georgina H. Walker, M. L. Bynoe and D. A. J. Tyrrell, Brit Med J 1:603 1967).

The authors' opening paragraphs are typical of much of the literature of the field and as such are quoted verbatim. "Ascorbic acid is widely used as a prophylactic and therapeutic agent against the common cold, and is, in fact, often recommended for this purpose by medical practitioners. It may be administered as a large dose as soon as possible after the first symptoms are felt and this dosage is then continued for a few days in order to 'abort' the cold. Though many people use this treatment on themselves and believe it is effective it is impossible to assess its efficacy. Untreated subjects may experience symptoms such as sneezing or a mild sore throat, which then disappear without going on to a full cold, and patients' assessments of their colds are much influenced by any treatment owing to the placebo effect (Diehl, *et al*, 1938). In these studies we attempted to show by in-vitro experiments that exposure of cells to ascorbic acid increased their resistance to infection with viruses. Though there was no activity in vitro, we also attempted to demonstrate a protective effect in animals or man, since it was thought that host resistance might be enhanced directly.

"The volunteers took 1 g of ascorbic acid three times a day as effervescent tablets supplied by Roche Laboratories. These were given for three days before virus inoculation, and for six days after. . . . Control subjects were given placebo tablets which were indistinguishable from the ascorbic acid tablets except by chemical analysis.

"Porton mice were used. They were given 4 mg daily of freshly prepared solution of ascorbic acid in saline or of plain saline by intraperitoneal injection for six days before and nine days after virus inoculation. Influenza A (Mel) virus was administered as intranasal drops under light ether anesthesia."

For the human beings, the diet was generous, but the dietary intake was expected to be low, and the effects of the supplement were expected to be maximal. The usual specimens of blood were taken before and after inoculation with rhinoviruses and influenza virus. The former were M and H types and the third, H.S., and the influenza, Type B.

It was concluded that the number of colds and their severity were not affected by ascorbic acid. The length of the incubation period and the duration of symptoms were variable, but the mean values of both these periods were the same in treated and untreated volunteers. Of the 18 volunteers who received non-infectious intranasal drops two developed a cold. Thirteen volunteers who developed colds while taking ascorbic acid complained of a sore throat, while 15 who were on placebo tablets did so. The comparable figures for mucopurulent nasal discharge were 10 and 8. It was concluded that there was no evidence for a general, antiviral or symptomatic prophylactic effect of ascorbic acid in these experiments.

In their discussion of their studies the authors make the following remarks. "Uncontrolled observations are, of course, impossible to evaluate, but even where placebos are used as a basis for comparison there have been many important differences between the studies reported; they have differed in the type of clinical illness occurring in the population, the assay and method of clinical assessment, the age and nutri-

tional state of the subjects, and the amount of vitamin given and whether it was administered prophylactically or therapeutically. One trial, for instance, suggested that there was a marked reduction in baseball players given prophylactic vitamin C (Barnes, 1961), but the control group observed were not players.

"In another controlled trial there was an apparent improvement two days after treatment in subjects given a mixture of bioflavonoids, ascorbic acid and aspirin rather than another analgesic capsule (Macon, 1956); it is impossible however to decide which of the differences in medication accounted for the results. In another large trial (Tebrock, *et al.*, 1956) a mixture of bioflavonoids and 200 mg of ascorbic acid daily was given after the onset of the cold, but the clinical observations, which were made only one and three days after treatment began, showed no evidence of improvement. Other smaller trials also showed no effect. Banks (1964, 1965) has drawn attention to the possibility that treatment with ascorbic acid may reduce the number of colds which are followed by long-continued purulent discharge. Table VI, which is explained below, summarizes some facts from what seem to us to be among the most critically evaluated of the earlier trials; this supports the view that small doses of ascorbic acid administered to those on a low intake or large doses administered to those on a normal intake may reduce the duration of pharyngitis or colds."

The table mentioned lists three studies of which one concerns 20 volunteers who took either 10 or 70 mg of ascorbic acid daily with the colds in the deprived group lasting 6.4 days and in the non-deprived, 3.3 days with the difference said to be statistically insignificant. In a second group of adolescents the dosage of ascorbic acid was 50 to 200 mg daily, and it was not noted in the results that the incidence of tonsillitis was diminished, but the duration of the illness was shorter in the treated group while ". . . colds were unaffected." Members of a ski camp took 1,000 mg of ascorbic acid daily and pharyngitis (colds apparently uncommon) showed a diminished incidence (Ritzel, 1961).

The authors ". . . thought it unlikely that ascorbic acid could influence the uptake or replication of viruses. Nevertheless, the 'uncoating' of viruses and the damage they produce may be mediated by the release of enzymes from lysosomes (Mallucci and Allison, 1965), and these organelles may be stabilized by ascorbic acid—at least in photosensitized epithelial cells (T. F. Slater, personal communication, 1966). It was therefore possible that the vitamin might influence multiplication and cytopathic effect of viruses. The results of the in vitro experiments were negative, but were not entirely convincing because of the rapid rate of inactivation of the vitamin in the cultures used. Since ascorbic acid can apparently improve the response to stress of the rat and influence the repair of skin, it might assist repair of the mucosa from the damage produced by viruses. . . . We conclude that there is no evidence that the administration of ascorbic acid has any value in the prevention or treatment of colds produced by five known viruses. The literature indicates that the administration of the vitamin to deficient subjects may reduce the incidence or severity of colds and possibly of pharyngitis due to streptococci, as well as the incidence of rheumatic fever and pneumonia (Glazebrook and Thompson, 1942). However, it is quite reasonable to conclude that administering very large doses of the vitamin to well-nourished people does not have the same effect." These conclusions do not all seem to be equally warranted, and there is the known precept that the laws that are generally true of the members of groups taken

not true of individuals.

An opposing point of view was published in the same journal within three months of the study quoted. In a letter to the editor (Brit Med J 2:698 June 10 1967), C. W. M. Wilson of the Department of Pharmacology, University of Dublin, wrote: "The consumption of large doses of ascorbic acid in the very early stages of a cold is popularly believed to have a therapeutic effect in suppressing cold symptoms. The effect of ascorbic acid was investigated in Dublin in large field surveys during two winter periods of six months each since 1965. Tablets were given daily to the subjects, and the ability of ascorbic acid to prevent the occurrence of colds in a population, i.e., its prophylactic effect was studied. Initial results in these trials do not support the negative view reported by others. Analysis is still in progress."

There is one point which must be made that is only in part pertinent to the subject of this communication. Deficiencies, although induced, are uncommonly as single as they appear to be. There is a relationship of the vitamins and minerals to each other so that for maximal or, perhaps for true effects all must be present in adequate amounts and, at the same time. There is no evidence in the studies mentioned or in our own, but not to the same extent that there were no other deficiencies, although it has been shown that the antigen immunity response for antibodies as remote from each other as those for tetanus and for typhoid are decreased when antagonists for pantothenic acid and pyridoxine are administered. The clinical pictures presented by the subjects used are those of a general vitamin deficiency. The subjects show a fall in gamma globulins and no significant alterations in beta or alpha-2 globulin levels.

NOTE: Part II of this Special Article will appear in the October issue.

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THE EFFECT OF HIGH DOSES OF ASCORBIC ACID ON THE FUNCTIONS OF THE ADRENAL GLANDS

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SUMMARY

Four grams ascorbic acid given daily by mouth for 4 days increase the urinary excretion of 11-oxysteroids and decrease the excretion of 17-ketosteroids. Ascorbic acid probably acts directly on the adrenal cortex.

The relations which exist between ascorbic acid and the adrenal glands have been the object of many studies. Stress, adrenaline and ACTH deplete ascorbic acid in the adrenal cortex, but the production of such an effect by stress or adrenaline requires an intact pituitary gland [Sayers & Sayers, 1948; Dury, 1948; Selye & Stone, 1950; Selye, 1950]. Selye notes that in subjects who have an intact pituitary gland, ascorbic acid decreases during the alarm reaction phase and increases during the resistance phase in the blood, that ACTH and cortical extracts increase the ascorbic acid content of the blood but have no effect on that of the tissues, and that ascorbic acid facilitates the production of the resistance phase.

The therapeutic activity of ACTH increases with the rise of the ascorbic acid content of the blood [Ferstl & Heppich, 1950].

The hypertrophy of the adrenal glands, which is produced in guinea-pigs and rats by exposure to cold, can be prevented, and resistance to cold be increased with high doses of ascorbic acid [Dugal & Thérien, 1949]. Schaffenburg, Masson & Corcoran [1950] have found that in guinea-pigs with scurvy, the adrenal glands are hypertrophic, that the ascorbic acid content is diminished and that cortisone and ACTH abolish the symptoms of scurvy, while DCA increases them.

The purpose of this paper is to investigate the influence of high doses of ascorbic acid on the functional activities of the adrenal glands.

METHODS

This investigation has been carried out on eleven normal adult human subjects. In order to investigate the effect of ascorbic acid on gluco-corticoid and androgen production of the adrenal cortex, the 17-ketosteroid and 11-oxysteroid excretion in urine were determined, before and after the administration of ascorbic acid. Four grams ascorbic acid *per os* were given daily for 4 days and the hormone determinations made on the day preceding and on the fourth day following the administration of ascorbic acid. 11-oxysteroids were determined by the method of Talbot, Saltzman, Wixom & Wolf [1945] and 17-ketosteroids by Zimmermann's method [1944, 1946]. The average normal value for 17-ketosteroid excretion in man was found to be 20 mg./24 hr. in our clinic.

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