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A QUANTITATIVE ESTIMATION OF THE EFFECT OF RUTIN ON THE BIOLOGICAL POTENCY OF VITAMIN C [1]

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ONE FIGURE

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INTRODUCTION

The nature and importance of vitamin P-active materials have been reported extensively in the literature since the middle of the last decade. The early findings were varied and sometimes contradictory, and as a result the concept of the function and mode of action of these materials was at first obscure. However, their role has become considerably clarified and recent work has brought forth a theory as to the mode of action of the many substances possessing such activity. That capillary walls become weakened under certain conditions has been known for some time. The decreased strength of the capillaries leads to hemorrhage, either through rupture of the walls or because of increased permeability. Prior to 1936, the belief existed that weakened capillaries were due wholly to a deficiency of vitamin C. In fact, capillary fragility was considered to be a part of the scurvy syndrome.

In 1936, Szent-Gyorgyi (Bentsath et al., '36) isolated a substance from both lemons and fresh paprika peppers that would restore weakened capillaries to normal. This effect was not noted when ascorbic acid alone was administered.

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Szent-Gyorgyi called his substance "citrin," and the active principle thereof became known as vitamin P. Bruckner and Szent-Gyorgyi ('36) stated that the extract was composed of the flavonones hesperidin and eriodictin. When the biological activity of these compounds was tested by Bentsath et al. ('37), both were found to be active; while a related compound, a flavonol named quercitrin, was inactive.

In England, the work of Scarborough is of considerable significance. Scarborough ('40) stated that vitamin P did not control in any way the clinical symptoms of gross scurvy, but that it did produce an increased capillary strength in the scorbutic subject. He found that the capillary strength of such subjects was not controlled by the administration of ascorbic acid, or of vitamins A, B_1 or D. He (Scarborough, '41) has also thrown doubt on the finding of Bruckner and Szent-Gyorgyi that citrin consists of a mixture of hesperidin and eriodictin. Taking an increase in capillary resistance in man as a criterion of vitamin P activity, he found that both hesperidin and its aglycone, hesperitin, were active. However, they can only be given orally because of their relative insolubility in an aqueous solution of suitable pH. Their activity, classed as "moderate," was greatly exceeded by that of a preparation obtained from orange peel, according to the Bentsath et al. ('36) method. Scarborough therefore stated that this latter preparation contained at least two substances which have not as yet been accurately identified. St. Rusznyak and Benko ('41) confirmed Scarborough's work by feeding a scorbutogenic diet to rats and observing the beneficial effect of citrin on the resultant weakened capillaries.

As a result of these findings, a distinct P-avitaminosis has been established, it being invariably associated with a much decreased capillary strength; it may be characterized by the development of spontaneous petechial hemorrhages, especially in areas exposed to stress. Thus, any material capable of increasing the strength of the capillary walls is said to have vitamin P activity. The chemical nature of many active extracts is still unknown but vitamin P activity has been at-

tributed variously to flavones, flavonones, flavonols and to many of their derivations. One of these, rutin, has become prominent in the literature because of its commercial availability. Griffith, Couch and Lindauer ('44) fed rutin to 14 patients with hypertension, all of whom subsequently showed increased strength in the capillary walls. This work was confirmed by Shanno ('46). Ambrose and DeEds ('47) administered rutin to rabbits and found that it decreased cutaneous capillary permeability.

The mode of action of vitamin P substances as antioxidants is stressed in the literature. The French workers Parrot et al. ('44), using epi-catechin, have postulated that vitamin P exerts its various antihemorrhagic effects by inhibiting the oxidative destruction of circulation epinephrine. This hormone (epinephrine) then appears to decrease capillary fragility, capillary permeability and bleeding time through a vasoconstrictive or tonic action on precapillary blood vessels. Somogyi ('45) added vitamin C-free extracts of lemon juice filtrate residues, known to have a high vitamin P content, to solutions of crystalline synthetic ascorbic acid. He showed that this addition served to protect against the *in vitro* oxidation of vitamin C by the ascorbic acid oxidase of white cabbage and other recognized oxidants of ascorbic acid, such as copper, the polyphenol oxidase of apples, and the peroxidase of horseradish. Richardson et al. ('47) have shown that flavonols, for example quercetin, quercitrin and rutin, are effective antioxidants for milk and lard. Furthermore, Wilson, Mortarotti and DeEds ('47) showed that rutin prolongs the action of epinephrine on intestinal strips, the prolongation presumably being due, they believe, to a protection of the epinephrine from oxidative destruction.

The concept that the protection of epinephrine from oxidative destruction is the sole mode of action of vitamin P-active materials was not accepted by Clark and Geissman ('49). Using two methods, these workers examined some 70 compounds to study the relationship between their reported vitamin P activity and their ability to potentiate the effects of

epinephrine. One method determined the extent to which those substances enhanced the effects of epinephrine on isolated intestinal segments, while the second estimated spectrographically the protection they afforded epinephrine from copper-catalyzed autoxidation *in vitro*. The authors reported that the activity series obtained by the isolated smooth muscle assay method coincided neither with that obtained by the spectrophotometric methods nor with capillary fragility-decreasing (vitamin P) activity as reported in the literature.

It is worth recalling that immediately after his isolation of citrin, Szent-Gyorgyi (Bentsath et al., '36) fed this material to guinea pigs on a scorbutogenic diet, and stated that the life of these animals was not only prolonged but that there was as well a marked decrease in the intensity of hemorrhages. He and his co-workers then suggested that experimental scurvy was caused by a combined lack of vitamins C and P. However, Zilva ('37) questioned this suggestion when he found that upon the administration of citrin the onset of scurvy was not delayed, nor was the fatal termination of the disease retarded in guinea pigs on a scorbutogenic diet. He also showed that sub-minimum prophylactic doses of ascorbic acid administered to such guinea pigs produced a pathological condition resembling that obtained by Szent-Gyorgyi and his colleagues by the administration of a daily dose of 1 mg of citrin. Zilva explained the results of the Hungarian workers by postulating that their crude extract of citrin contained traces of ascorbic acid. When Szent-Gyorgyi et al. failed to reproduce their earlier findings, the suggested role of vitamin P as a modifying factor in the scurvy syndrome was abandoned.

Recent work, however, indicates that there is a relationship between vitamin P-active materials and ascorbic acid. Cotereau et al. ('48) showed that catechin, when fed to guinea pigs, permitted a higher than average tissue storage of ascorbic acid, which was not the result when the latter was supplied alone, even at a level of 10 mg per day. Of more significance is the work of Ambrose and DeEds ('49), in which it was

found that in guinea pigs receiving sub-minimum amounts of ascorbic acid, ascorbic acid plus rutin, and ascorbic acid plus quercetin, the post mortem findings were essentially the same, except that the last two groups showed fewer fresh hemorrhages than the animals receiving ascorbic acid alone. They also stated that the combined supplements of ascorbic acid and rutin apparently prolonged the life of scorbutic guinea pigs.

The results of both groups of workers may be feasibly accounted for by the assumption that the two vitamin P-like materials were protecting the ascorbic acid from oxidative destruction. The work of Ambrose and DeEds shows the modifying effect of vitamin P-active materials on the scurvy syndrome; not directly, as originally assumed by Szent-Gyorgyi, but indirectly through their sparing action on ascorbic acid. Thus, Szent-Gyorgyi's earlier work would be explained if his crude extract did contain traces of ascorbic acid, as suggested by Zilva; while Zilva's findings would also be valid, since he fed purified hesperidin and eriodictin, with no vitamin C present.

EXPERIMENTAL

The specific object of this experiment was to test the extent to which rutin affected the apparent biological potency of synthetic ascorbic acid and of vitamin C from a natural source (commercial orange-grapefruit juice).

The odontoblast method of bioassay was used and each animal was maintained on test for a 42-day period. As a basis for dosing, the orange-grapefruit juice was analyzed chemically for ascorbic acid, using both the dichlorophenol indophenol method of Hochberg, Melnick and Oser ('43) and the dinitrophenylhydrazine method of Roe and Oesterling ('44).

Healthy 28-day-old guinea pigs, weighing approximately 300 gm at the beginning of the assay period, were fed syn-

thetic ascorbic acid or orange-grapefruit juice as the sole source of vitamin C. Rutin was fed to half the animals on each of these treatments.

Design of the assay

The general design of this assay is shown in the allotment plan set forth in table 1. The experiment was carried out in two replicates of 64 animals each. A total of 8 guinea pigs, 4 males and 4 females on each treatment, was involved for the complete test.

TABLE 1

Allotment plan

Showing distribution of 128 animals to treatment groups (8 in each group) and mean height of odontoblast cells (microns) for each treatment

RUTIN TREATMENT	ASCORBIC ACID LEVEL	MEAN HEIGHT OF ODONTOBLASTIC CELLS WITH ASSAY MATERIALS	
		Ascorbic acid	Orange-grapefruit juice
	mg	*microns*	*microns*
None	0.50	27.1 [1]	26.4
	0.79	31.8	33.0
	1.26	37.7	37.8
	2.00	42.4	41.5
100 mg rutin	0.50	31.7	31.5
	0.79	36.1	36.5
	1.26	39.8	40.1
	2.00	41.9	42.3

[1] Averages of 4 males and 4 females in each group.

Animals

Guinea pigs from the stock colony at Macdonald College were weaned at 21 days of age, and after 7 ± 2 days from the weaning date, were randomized within sexes to each of the 16 feeding groups.

Diets

All animals received ad libitum the Macdonald College guinea pig diet no. 6, plus water, throughout the 42-day test

period. The percentage composition of this diet is as follows: oats, 15.0; wheat, 13.0; beet pulp, 20.0; linseed oilmeal, 12.5; skim milk powder, 15.0; fish meal, 5.0; brewers' dried yeast, 10.0; feeding molasses, 5.0; bone char, 4.0; and salt (0.1% KI), 0.5.

TABLE 2

Analysis of variance

SOURCE OF VARIATION	D.F.	$S(x-\bar{x})^2$	VARIANCE s^2	VARIANCE RATIO	
				Observed	Necessary minimum (P = 0.05)
Total	95	2001.95			
Sub groups	23	1950.02			
Levels	2	1505.41	752.71	990.1	3.14
Rutin	1	316.49	316.49	416.5	3.99
Sex	1	0.97	0.97	1.3	3.99
Materials	1	1.05	1.05	1.4	3.99
Levels × rutin	2	25.53	12.76	16.8	3.14
Levels × sex	2	0.69	0.35	0.5	3.14
Levels × materials	2	3.83	1.92	2.5	3.14
Rutin × sex	1	0.29	0.29	0.4	3.99
Rutin × materials	1	0.001	0.001	0.09	3.99
Sex × materials	1	0.36	0.36	0.5	3.99
Second order interactions	7	95.40	10.50	13.8	2.08
Third order interactions	2				
Calculated values	4				
Error	68	51.93	0.76		
		S.D. = 0.87			

The diet was prepared by grinding, mixing, and pressing the mixture into pellets approximately one-eighth of an inch in diameter. Previous work in this laboratory has shown that the minor variations in consumption under ad libitum feeding have no direct influence on this method of bioassay. Thus no feed records were kept.

Supplements

Vitamins A, D and E were administered weekly by a calibrated syringe. The dose was such that every animal was

given the equivalent of a daily intake of 3 mg of alpha-tocopherol, 425 I.U. of vitamin A, and 48 I.U. of vitamin D_2.

The ascorbic acid was dissolved in distilled water immediately before dosing, while a fresh tin of orange-grapefruit juice was opened daily. Both these sources of vitamin C were administered directly by calibrated syringe.

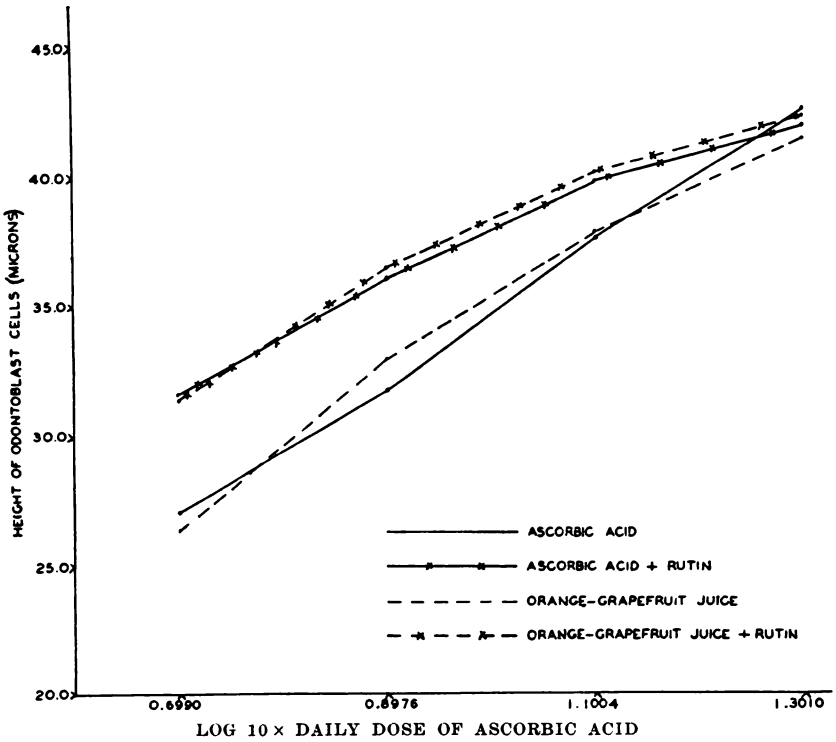

Fig. 1 Regression of height of odontoblast cells on daily intake of vitamin C from ascorbic acid and from orange-grapefruit juice, showing the effect of added rutin.

Approximately 100 mg of crystalline rutin were fed daily to each guinea pig involved. They were given orally by means of a calibrated tube.

Analysis of experimental data

At the conclusion of the 42-day feeding period, each animal was sacrificed. The incisor teeth were prepared for micro-

scopic examination of the odontoblast cells according to the procedure developed in this laboratory (Crampton, '47). The odontoblast cell measurements (microns) were analyzed statistically, with the variance partitioned as shown in table 2. The method of Bliss and Marks ('39) was applied in calculating the relative potencies of the vitamin C sources.

TABLE 3

The vitamin C potencies of ascorbic acid and orange-grapefruit juice, showing the effect of rutin

MATERIAL	RELATIVE POTENCY [1]	LIMITS OF RELATIVE POTENCY P = 0.05	BIOLOGICAL POTENCY	PROBABLE LIMITS OF BIOLOGICAL POTENCY P = 0.05	MEAN VITAMIN C POTENCY — CHEMICAL (HOCHBERG ET AL., '43)
	%	%	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
Ascorbic acid	100.0	99.0–101.0	35.0	34.6–35.4	
Ascorbic acid + rutin	156.2	143.5–170.0	54.7	50.2–59.5	
Orange-grapefruit juice	101.8	97.7–106.0	35.7	34.3–37.1	35.3
Orange-grapefruit juice + rutin	152.9	141.6–165.0	53.1	49.5–57.7	35.3

[1] Differences between relative potencies greater than 1.5 percentage units are statistically significant.

RESULTS

Chemical

The average apparent vitamin C potencies, as determined by the two chemical methods on random samples of orange-grapefruit juice, were: (1) Method of Roe and Oesterling ('44), 42.9 mg/100 ml; (2) method of Hochberg et al. ('43), 35.3 mg/100 ml. The standard deviation of the individual determinations with the Roe method was $\pm$ 5.1% and with the Hochberg $\pm$ 3.5%.

Because of the smaller variability of results, figures obtained with the latter method were used in all calibrations as well as in comparisons between the biological and chemical values of vitamin C potency.

Biological

The mean heights of the odontoblast cells of the guinea pigs receiving synthetic ascorbic acid and orange-grapefruit juice, each with and without rutin and at the 4 dosage levels of 0.5 mg, 0.79 mg, 1.26 mg, and 2.00 mg, are summarized in table 1. These results are illustrated graphically in figure 1.

The inclusion of the 2.00-mg level of vitamin intake caused a deviation in the linear regression of odontoblast height on logarithm of dose, and this level was therefore excluded from the analysis of variance.

The potencies of ascorbic acid plus rutin, orange-grapefruit juice, and orange-grapefruit juice plus rutin were determined relative to synthetic ascorbic acid. Figures illustrating the effect of rutin and the values of the mean ascorbic acid potencies of the orange-grapefruit juice, as determined chemically and biologically, are given in table 3.

DISCUSSION

A very close agreement between the mean vitamin C potencies as determined biologically and chemically (Hochberg, Melnick and Oser, '43) was found for the orange-grapefruit juice. This agreement was in accordance with previous work in this laboratory.

When fed at ascorbic acid levels where it could exert its full effect, rutin produced striking results. Considering the apparent biological potency of synthetic ascorbic acid as 100%, it may be seen from table 3 that the effect of supplementing this material with rutin was to increase its apparent biological potency by 56.2%. Furthermore, when orange-grapefruit juice constituted the source of vitamin C rutin increased the apparent biological potency of the ascorbic acid therein by 48.8%.

At levels of vitamin C intake above these, rutin had progressively less effect. Presumably at these levels sufficient ascorbic acid was available to the animals and the administration of rutin had little or no effect on the height of the

odontoblast cells, which was at, or was approaching, a maximum.

These quantitative findings tend to support the concept that rutin, and other vitamin P-like substances, carry out their physiological function as antioxidants. Rutin either made more ascorbic acid available or delayed its "*in vivo*" oxidative destruction in the original source.

CONCLUSIONS

Rutin significantly increased the apparent biological value of ascorbic acid when the vitamin was supplied in sub-maximum amounts either in crystalline form or from a natural source.

The mechanism of action of rutin in this role is uncertain, but prevention of the "*in vivo*" oxidative destruction of vitamin C would explain these results.

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