Effects of Flavonoids on Response of Rats Fed Massive and Suboptimal Doses of Vit. A.* (23320)

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Prolonged ingestion of massive doses of vit. A results in growth retardation, spontaneous leg fractures, paralysis and other toxic manifestations including death in the immature rat, the severity of symptoms and their rapidity of onset being proportional to dosage of vit. A administered(1-3). A vit. A intake of 10,000 to 12,500 U.S.P. units/day results in a significant retardation in growth; the dosage required to produce more severe symptoms (including leg fractures) in immature rats has generally been found to range from 25,000 to 50,000 U.S.P. units of vit. A/day. In the present communication data are presented indicating that administration of bioflavonoids significantly affects the physiologic response to massive but relatively non-toxic doses of vit. A in the immature rat.

Procedure. The basal flavonoid-free diet employed consisted of sucrose, 66%; casein,† 24%; salt mixture,‡ 5%; and corn oil, 5%. To each kg of above diet were added the following crystalline vitamins: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; vit. B_{12}, 150 μg; 2-methylnaphthoquinone, 5 mg; choline chloride, 2 g; vit. A, 5000 U.S.P. units; vit. D_2, 500 U.S.P. units; and alpha-tocopherol acetate, 100 mg. The vitamins and the various test additives were incorporated in the basal diet in place of an equal amount of sucrose. Diets were made up weekly and stored under refrigeration when not in use. The animals were fed daily and all food not consumed 24 hours after feeding was discarded. The rats were housed in metal cages with raised screen bottoms (2 or 3 animals/cage) and were provided with food and water *ad libitum*. Feeding was continued for 6 weeks or until death, whichever occurred sooner.

Results. Exp. 1. Two hundred and forty-seven female rats of the Holtzman strain were selected at an average weight of 49.2 g (range 44 to 52 g). Animals were divided into 26 comparable groups. One group was fed the basal diet; the remaining groups were fed the basal diet with supplements listed in Table I. The ingestion of a purified ration containing 3.75 million U.S.P. units of vit. A palmitate/kg of diet resulted in a significant retardation in growth and occurrence of spontaneous leg fractures in the immature rat. These symptoms of hypervitaminosis A were accentuated by the concurrent feeding of flavonoids (calcium flavonate glycoside, naringen, hesperidin complex, hesperidin methyl chalcone, lemon bioflavonoid complex or rutin) at levels of 0.5, 1 or 2% of diet. This was evidenced by the following criteria: (a) weight increment of rats fed the above flavonoids in conjunction with the vit. A supplement was significantly less than that of rats fed the vit. A supplement alone, (b) 90 to 100% of the rats fed both the flavonoid and vit. A supplements developed leg fractures in contrast to an incidence of 39% in animals fed the vit. A supplement alone, and (c) 50 to 100% of rats fed flavonoids in conjunction with the vit. A supplement succumbed during the 6 week experimental period in contrast to 0% mortality in animals fed the vit. A supplement alone. In general the effects of the flavonoids in accentuating symptoms of hypervitaminosis A were as marked when fed at a 0.5% level in the diet as when they were fed at higher levels. When the flavonoids were incorporated at 2% level in the basal diet (without the vit. A supple-

* Communication No. 432 from Department of Biochemistry and Nutrition, Univ. of So. California. This investigation was supported in part by grant-in-aid from Sunkist Growers, Ontario, Calif.

† Vitamin-free Test Casein, General Biochemicals, Chagrin Falls, O.

‡ Hubbel, Mendel and Wakeman Salt Mixtures, General Biochemicals, Chagrin Falls, O.

TABLE I. Effects of Flavonoids on Symptoms of Hypervitaminosis A in the Immature Rat.*

Supplements fed with basal diet	No. of animals	Initial wt (g)	Body wt (g) after following days of feeding† 21st	42nd	% of rats with fractures	% surviving	Avg survival time of decedents (days)
None	7	49.0	133 (7)	173 (7)	0	100	
2% calcium flavonate glycoside	7	49.2	115 (7)	160 (7)	0	100	
2% naringen	7	49.2	122 (7)	162 (7)	0	100	
2% hesperidin complex	7	49.2	131 (7)	172 (7)	0	100	
2% hesperidin methyl chalcone	7	49.2	123 (7)	167 (7)	0	100	
2% lemon bioflavonoid complex	7	49.0	132 (7)	167 (7)	0	100	
2% rutin	7	49.2	130 (7)	171 (7)	0	100	
3.75 million units vit. A palmitate/kg of diet	18	49.1	106 (18)	131 (18)	39	100	
Idem + following supplements:							
.5% calcium flavonate glycoside	10	49.2	76 (10)	83 (4)	100	40	31
1 % *Idem*	10	49.1	73 (10)	61 (2)	100	20	34
2 % "	10	49.0	78 (8)	72 (1)	100	10	30
.5% naringen	10	49.3	68 (7)		100	0	26
1 % "	10	49.4	73 (6)	81 (1)	100	10	25
2 % "	10	49.4	72 (5)	76 (1)	100	10	22
.5% hesperidin complex	10	49.3	77 (6)	84 (2)	90	20	26
1 % *Idem*	10	49.2	75 (8)	79 (2)	100	20	28
2 % "	10	49.2	78 (9)	81 (5)	100	50	32
.5% hesperidin methyl chalcone	10	49.2	67 (7)		100	0	27
1 % *Idem*	10	49.2	61 (8)		100	0	27
2 % "	10	49.3	61 (6)		100	0	22
.5% lemon bioflavonoid complex	10	49.3	64 (8)	52 (2)	100	20	25
1 % *Idem*	10	49.4	61 (8)		100	0	26
2 % "	10	49.3	67 (9)		100	0	25
.5% rutin	10	49.4	57 (7)		100	0	22
1 % "	10	49.4	49 (2)		100	0	19
2 % "	10	49.3	57 (9)		100	0	25

* The calcium flavonate glycoside, naringen, hesperidin complex, hesperidin methyl chalcone and lemon bioflavonoid complex employed were kindly provided by Mr. W. E. Baier of Sunkist Growers, Ontario, Calif. Vit. A supplement was Synthetic Vit. A Palmitate in Corn Oil (1 million U.S.P. units/g), obtained from Hoffmann-La Roche, Nutley, N. J.

† Values in parentheses indicate No. of animals which survived and on which averages are based.

ment), no deleterious effects were observed on any of the rations in respect to weight increment, gross appearance, incidence of fractures or per cent survival.

Exp. 2. In Exp. 1 data were obtained on the effects of flavonoids on the response of rats administered excessive amounts of vit. A. The following experiment was undertaken to determine the effects of flavonoids on vit. A-depleted rats fed suboptimal amounts of this vitamin. The animals employed were female rats of the Univ. of S. California strain in which the dietary regime of mother rats was such as to make the young rats satisfactory for bioassay studies for vit. A. The mother rats were maintained for at least 2 months previous to breeding and for 7 days after birth of the litter on Sherman diet B(4) without addition of supplementary lettuce or meat. Litters were reduced to 7 at 3 days of age, and on the 7th day mothers and litters were placed on vit. A-free, flavonoid-free diet identical to basal diet employed in Exp. 1 except that the vit. A was omitted from this ration. The young rats were weaned at 21 days of age and were continued on the above

diet until they were depleted of vit. A. The end of depletion period was set as 5th day on which rats on the vit. A-free, flavonoid-free diet showed stationary or decreasing weight. The average time of depletion was 21 days after weaning (range 18 to 23 days); and average body weight at time of depletion was 91 g (range 68 to 115 g). On the day of depletion animals were divided into 8 comparable groups of 10 rats each. Four groups were fed vit. A-free, flavonoid-free diet indicated above supplemented with (1) 0, (2) 100, (3) 250, and (4) 1000 U.S.P. units of vit. A palmitate kg of diet. The remaining 4 groups were fed diets identical with the 4 rations indicated above but containing in addition 5 g of hesperidin complex and 5 g of lemon bioflavonoid complex kg of diet. The flavonoids were added in place of an equal amount of sucrose. Animals were placed in metal cages with raised screen bottoms and were fed the above diets *ad libitum* for 28 days or until death, whichever occurred sooner.

All rats fed the vit. A-free diet either with or without the flavonoids succumbed after an average survival of 7 to 8 days (range 4 to 16 days). All rats fed vit. A-free diet supplemented with 100 U.S.P. units of vit. A palmitate/kg of diet either with or without the flavonoids also succumbed within 28 days. Average survival time on the non-flavonoid diet was 14 days, whereas average length of survival on the flavonoid-containing ration was 12 days. At higher levels of vit. A intake, however, the flavonoids exhibited a highly significant effect. Only 2 of the 10 rats fed the non-flavonoid ration supplemented with 250 U.S.P. units of vit. A/kg of ration survived the experimental period of 28 days (average weight increment of the 2 survivors was 29 g). In contrast 8 of the 10 rats fed the flavonoid-containing ration at the same level of vit. A supplementation survived (average weight increment of the 8 survivors was 25 g). Of the rats fed 1000 U.S.P. units of vit. A/kg of ration, 8 of the 10 rats in the non-flavonoid group survived with average weight increment of 47 $\pm$ 2.4 g.§ Of rats fed the same level of vit. A in presence of flavonoids, 9 out of 10 survived with average weight increment of 69.4 $\pm$ 5.0 g.§ It is apparent from these findings that supplements of hesperidin complex and lemon bioflavonoid complex when fed at 0.5% level in the diet were without significant effect when fed with rations containing 100 U.S.P. units of vit. A or less/kg of ration but significantly increased both the number of animals surviving and the average increment in body weight of vit. A-depleted rats fed diets containing 250 and 1000 U.S.P. units of vit. A respectively/kg of diet. It would seem from these findings that the above flavonoids were active in increasing absorption and/or utilization of vit. A not only when this vitamin was present in excess (Exp. 1) but under conditions of suboptimal intake as well.

Discussion. Present findings indicate that flavonoids can significantly affect the response of immature rats fed purified rations containing either excessive or suboptimal amounts of vit. A. When immature rats were fed flavonoid-free diets containing massive but relatively non-toxic doses of vit. A, supplements of calcium flavonate glycoside, naringen, hesperidin complex, hesperidin methyl chalcone, lemon bioflavonoid complex or rutin resulted in a striking accentuation of the symptoms of hypervitaminosis A. This was evidenced by a more pronounced retardation in growth, a higher incidence of spontaneous leg fractures and a significantly greater mortality in rats fed the flavonoid-containing rations than in those fed similar diets with the flavonoids omitted. These symptoms were identical in appearance, time of onset, severity and incidence to those of rats fed the flavonoid-free diet supplemented with amounts of vit. A considerably in excess of the amounts present in above rations. Supplements of hesperidin complex and lemon bioflavonoid complex also significantly increased both the number of animals surviving and the average increment in body weight of vit. A-depleted rats fed diets containing 250 and 1000 U.S.P. units of vit. A respectively/kg of diet. These find-

§ Including standard error of mean calculated as follows: $\sqrt{\frac{\Sigma d^2}{n}} / \sqrt{n}$ where "d" is deviation from the mean and "n" is number of observations.

ings suggest that the above flavonoids may have promoted an increased absorption and/or utilization of vit. A. That unidentified factors exist in natural foodstuffs which promote utilization of vit. A has been reported by a number of investigators. Palm kernel meal(5,6), coconut cake(5,6), acetone-extracted herring roe(5,6), soybean lecithin (7,8), yeast(9), fish solubles(10,11), alfalfa and other succulent plants(12), liver(12) and other substances including the tocopherols(13-15), xanthophyll(16), aureomycin(17,18) and DPPD(19,20) have all been shown to improve vit. A utilization as judged by various indices in experimental animals. Present findings indicate that the flavonoids also have significant activity in this regard and suggest that the activity of at least some of the natural foodstuffs indicated above may have been due, at least in part, to their flavonoid content.

No data are available to indicate the mechanism(s) whereby the flavonoids indicated above exert their physiologic effects. The possibility that the flavonoids are functioning as antioxidants that prevent destruction of vit. A in the diet and hence leave more vitamin present for absorption appears to be ruled out by chemical tests which indicate that no detectable destruction of vit. A occurred in the purified basal diet supplemented with 2.5 million U.S.P. units of vit. A palmitate/kg of ration after 30 days of refrigeration. Under conditions of our experiment, diets were made up at weekly intervals and were consumed within 10 days of the time they were made. In addition no significant difference in response occurred between rats fed the above ration and animals fed an identical diet which was made up daily immediately before feeding. Flavonoids, however, can function not only as *in vitro* antioxidants (21) but in the intact animal as well(22,23). Hence further studies are indicated to determine to what extent the effects of flavonoids in modifying the response to vit. A feeding under conditions of our experiment were due to their function as biological antioxidants which protected vit. A from destruction in the intestinal tract and/or the animals' own tissues. It must be emphasized that the effects of the flavonoids in accentuating symptoms of hypervitaminosis A in the rat occurred with diets containing approximately 2000 to 3000 times the amount of vit. A required for optimal growth, breeding and longevity as determined by Sherman and co-workers(24,25). Preliminary findings indicate that with diets containing smaller amounts of this nutrient (up to 1000 times the optimal vit. A intake) the flavonoids did not accentuate symptoms of hypervitaminosis A. In view of these findings the possibility that flavonoids might induce symptoms of hypervitaminosis A in man even when fed with large therapeutic doses of this nutrient would appear to be extremely remote. On the contrary present findings indicate that flavonoids may play an important role in the treatment of vit. A deficiency states, not only those arising from the ingestion of diets deficient in this vitamin but those resulting from impaired absorption or utilization of this nutrient as well.

Summary. Supplements of calcium flavonate glycoside, naringen, hesperidin complex, hesperidin methyl chalcone, lemon bioflavonoid complex or rutin at levels of 0.5 to 2% of the diet significantly increased the vit. A utilization of immature rats fed massive doses of this vitamin. Flavonoids also increased the apparent utilization of this nutrient in vitamin A-depleted rats fed suboptimal amounts of vit. A.

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Received May 3, 1957. P.S.E.B.M., 1957, v95.

Failure of Hypothalamic Tissue to Reinitiate Thyrotropin Production By Mouse Pituitaries in Tissue Culture.* (23321)

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Guillemin and Rosenberg(1) reported that pituitary explants, which normally lost their ability to produce corticotropin rapidly in tissue culture, could be stimulated to reinitiate corticotropin production by the addition of hypothalamic explants. The stimulating potency resided in the median eminence, a region which previously had been shown to be involved in control of corticotropin production in the intact animal(2).

The importance of hypothalamic centers in regulating production of thyrotropin by the pituitary has been established(3,4,5), but the mechanism of control has not. Greer(3) has postulated the existence of 2 thyrotropins, a "metabolic" and a "growth" factor, and the production of the latter appears to depend upon both pituitary and hypothalamic centers. We therefore thought it of interest to utilize Guillemin and Rosenberg's technic to see whether evidence for a direct stimulation by hypothalamic tissue of hypophyseal thyrotropin production could be obtained.

Materials and methods. Pituitaries were taken from adult male Swiss albino mice under sterile conditions. The tissues were washed in Hanks' balanced salt solution and quartered with a sharp scalpel. Four quartered pituitaries were placed in the lower third of 16 x 150 mm test tubes which had been coated with 3 drops of chicken plasma. Following coagulation of the plasma, 1.0 ml of nutrient fluid was added, the tubes stoppered and incubated in a Roller Drum at 36°. Thirty-two such tubes were prepared in this study. The tissues were observed daily for growth and the nutrient medium was changed twice a week. The removed medium was kept at −35° prior to analysis for thyrotropin. Hypothalamic explants were added to 20 of the tubes on the twelfth day of *in vitro* growth and imbedded in a fresh plasma clot. Two entire mouse hypothalami, extending from the optic chiasm to the mammillary bodies were minced into 1 mm^3 fragments and added to each tube containing 4 pituitaries. The nutrient fluid consisted of 20% Beef Serum, 10% Chicken Embryo Extract, 20% Hank's

* Aided in part by grants from U. S. Public Health Service.

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