THE BIOLOGICAL ACTIVITY OF A FLAVONOID (VITAMIN "P") COMPOUND [1, 2]

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The elaboration of a practical and reliable biological test for the estimation of vitamin "P" activity is the most urgent and important problem with which every investigator in this field is confronted. The complexity of the physiological activity of flavonoid compounds was responsible for the obstacles in finding a satisfactory solution of this problem. The attempts to induce experimental avitaminosis "P" gave contradictory observations making this test unreliable for any practical use (1–8). A method was suggested for the estimation of vitamin "P" activity by using the capillary bed in the rat-mesoappendix, with a compound topically applied. It was found, however, that most of the flavonoid compounds, except catechins, did not produce any vasoconstrictor effect (9, 10). Considerable work was done with the determination of capillary permeability by using chloroform wheals applied to the skin and simultaneous injections of trypan blue. This test pertaining rather to permeability changes than to increased capillary fragility has demonstrated a great variation in the response of experimental animals to the same flavonoid compound (11, 12). More recently it was suggested that radiation injury to the capillary wall might serve as a basis for the estimation of vitamin "P" activity. Purpura, for which ascorbic acid has proved ineffective, has been a constant feature of radiation illness. It has been shown that some flavonoid compounds exert protective action against radiation injury to the capillary system (13–18).

In our present report which covers both experimental and clinical data particular attention was paid to the determination of vitamin "P" activity in the condition where increased capillary fragility rather than permeability changes were evidenced.

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MATERIAL

A flavonoid compound isolated from citrus fruit was used for our experimental and clinical work. It contained four identified factors; namely, hesperidin-chalcone, glucose-hesperidin chalcone, eriodictin and quercitrin-like substance, which seemed to form a complex molecule as U.V. readings have shown. It was a yellow powder slightly hygroscopic, with impurity of 0.6%, mostly calcium and phosphorus, soluble in water at the pH 7.0 or in alkaline medium. It was tested by the technique of Ambrose-DeEds (11) by the application of chloroform to the skin of rabbits by means of a cotton-tipped applicator. We used a dose of 10 mg. per kilogram of weight of the flavonoid compound injected subcutaneously 20 minutes before the injection of trypan blue (10 cc.) in the marginal ear vein and the application of chloroform wheal to the skin. The average time of the appearance of the dye for 20 rabbits was 42 minutes 54 seconds as against one minute 57 seconds in the control batch of 10 rabbits. These figures when compared with those obtained by Ambrose-DeEds with rutin (three minutes for control and 15.7 minutes for rutin treated rabbits) seem to indicate a higher biological activity of the citrus flavonoid compound than that of rutin.

EXPERIMENTAL

1. Increased capillary fragility induced by leukotaxine

Menkin (19, 20) found that an inflammatory exudate induces an increase in capillary permeability. This was manifested by the accumulation of trypan blue from the circulation in a cutaneous area previously injected with an exudate. This effect was likewise duplicated by leukotaxine, a nitrogenous substance extracted from inflammatory exudation. The effect of leukotaxine on increased capillary permeability is wholly or in part inhibited by the presence of an extract of the adrenal cortex. Hyman and Chambers (21) also found that adrenal cortical extracts are extremely efficient in reducing capillary permeability in perfused isolated limbs. According to them, the cortical extracts have no dilator or constrictor action and presumably affect the capillary wall directly by decreasing the pore size of the intercellular cement.

TABLE I

Inhibitory effect of the flavonoid compound on induced capillary permeability

Rabbits Nos. 37–46

Rabbits	Treatment	Appearance of dye after injection in		
		15 min.	20 min.	30 min.
R. 37, f.	Flavonoid plus 3 mg. leukotaxine	0	0	traces
R. 38, m.		0	0	0
R. 39, m.		0	0	+
R. 40, m.		0	0	0
R. 41, m.		0	traces	traces
R. 42, m.		0	traces	+
R. 43, m.		0	0	0
R. 44, m.		0	traces	+
Controls				
R. 45, m.	No flavonoid and 3 mg. leukotaxine	+	++	++
R. 46, m.		+	++	+++

N.B. Diet of rabbits was Purina rabbit chow checkers. Ingredients of Purina rabbit chow: Ground oats, corn meal, soybean oil meal, linseed oil meal, corn germ meal, dehydrated alfalfa meal, wheat gray middlings, animal protein factor supplement, riboflavin supplement, D activated plant sterol, 2% defluorinated phosphate, .5% iodized salt.

In our experiments we have studied the effect of the flavonoid compound upon the capillary changes induced by leukotaxine using Menkin's technique for indicating these changes. Rabbits of an average weight of 2 kilograms were given 10 mg./kilo/weight of the flavonoid compound 20 minutes before subcutaneous injection of 3 mg. of leukotaxine. Immediately afterwards 12 cc. of 1% trypan blue in saline solution were introduced in the marginal ear vein. The change in capillary permeability was gauged by the degree of dye accumulation in the various skin areas. Altogether 10 rabbits were used for these experiments. The flavonoid compound was injected subcutaneously 10 mg./kilo/weight, shortly before the injections of trypan blue. In Table I the results are summarized.

It appears that the citrus flavonoid compound decreases capillary permeability induced by leukotaxine. Its effect is similar in this respect to that of adrenal cortical extracts, as compared with the data reported by Menkin.

2. *Increased capillary fragility induced by bacterial polysaccharide*

The polysaccharide isolated by M. J. Shear from *Serratia marcescens* produces an extensive hemorrhage in the tumors of animals and, when the dose is sufficiently large, can even cause death (22, 23). The striking fact is that normal rats and mice are much less susceptible to the toxic effect of this compound and can survive a much larger dose than can the cancerous animals. Four or five hours after the bacterial polysaccharide is injected into the cancerous animals, profuse hemorrhages are also observed in the adrenal gland. When adrenal cortical extract is employed in combination with polysaccharide, tumor breakdown is delayed (instead of in six to seven hours it might occur in 24 hours) and the effect on the adrenal gland is minimized (24).

For our experiments we used August Rat Carcinoma (Crocker Laboratory, Columbia University), a fast growing tumor. As a rule only tumors averaging in size 8–9 cm^3. were used for experimentation. When a rat bearing such a tumor is given 0.5 mg./100 g./weight of a bacterial polysaccharide (P–25) which we received from Dr. Shear, death occurred in about seven hours preceded by profuse hemorrhage in the tumor. Histological examination of the tumor, performed every

TABLE II

Inhibitory effect of the flavonoid compound on induced capillary fragility of tumors (bacterial polysaccharide)

Rat Nos.	Treatment		Result: death or survival
	P-25*	Flavonoid	
171-A, m.	0.5 mg.	3 mg.	Death in 17 hrs.
171-B, f.	0.5 mg.	3 mg.	Death in 22 hrs. 25 min.
171-C, m.	0.5 mg.	3 mg.	Death in 18 hrs.
171-D, m.	0.5 mg.	3 mg.	Death in 19 hrs. 10 min.
171-E, m.	0.5 mg.	3 mg.	Death in 20 hrs. 40 min.
174-A, f.	0.5 mg.	3 mg.	Death in 19 hrs.
174-B, m.	0.45 mg.	3 mg.	Death in 24 hrs. 30 min.
174-C, m.	0.5 mg.	10 mg.	Death in 36 hrs.
174-D, m.	0.5 mg.	10 mg.	Survived
174-E, m.	0.5 mg.	10 mg.	Death in 52 hrs.
174-F, m.	0.5 mg.	10 mg.	Survived
177-A, f.	0.5 mg.	10 mg.	Survived
177-B, m.	0.5 mg.	10 mg.	Death in 66 hrs.
177-C, f.	0.5 mg.	10 mg.	Survived
177-D, m.	0.4 mg.	10 mg.	Survived
177-E, m.	0.45 mg.	10 mg.	Survived
177-F, m.	0.5 mg.	10 mg.	Survived
177-H, m.	0.5 mg.	10 mg.	Death in 26 hrs.
178-A, m.	0.5 mg.	10 mg.	Survived
Controls			
178-B, m.	0.5 mg.	None	Death in 6 hrs. 25 min.
178-C, f.	0.5 mg.	None	Death in 7 hrs. 35 min.
178-D, m.	0.5 mg.	None	Death in 9 hrs.
178-E, m.	0.5 mg.	None	Death in 7 hrs. 30 min.
178-F, f.	0.5 mg.	None	Death in 8 hrs. 20 min.
178-H, m.	0.5 mg.	None	Death in 7 hrs. 15 min.

* P-25 is a preparation of Shear bacterial polysaccharide.

TABLE III

Effect of flavonoid compound on survival of irradiated rats

A. Control group of 40 rats (no flavonoid) *Initial average weight: 183 g.*														
Number of days of survival:	11	12	13	14	15	16	17	18	19	20	21	22	23	
Number of rats succumbed:	1	3	3	2	3	3	3	3	2	4	2	1	2	= 32
Mortality rate:	80%													
B. 20 rats given 4 mg. flavonoid for 10 days *Initial average weight: 186 g.*														
Number of days of survival:	17	18	19	20	21	22	23	24	25					
Number of rats succumbed:	1	1	2	0	0	1	1	1	1	= 8				
Mortality rate:	40%													
C. 40 rats given 5 mg. flavonoid for 30 days *Initial average weight: 175 g.*														
Number of days of survival:	18	19	20	21	22	23	24	25	26					
Number of rats succumbed:	1	0	0	0	1	0	0	1	1	= 4				
Mortality rate:	10%													

N.B. Basal diet of rats was Purina laboratory chow. Ingredients of basal diet: Meat meal, dried skim milk, wheat germ, fish meal, liver meal, dried beet pulp, corn grits, oat middlings, soybean meal, dehydrated alfalfa meal, molasses, animal protein factor supplement, riboflavin supplement, brewer's dried yeast, thiamin, niacin, vitamin A and D feeding oils, D activated plant sterol, 1% steamed bone meal, 0.5% iodized salt, 0.02% manganese sulfate.

hour after the injection of the compound, revealed that the capillary system of the tumor was first to respond to the toxic effect of polysaccharide by steadily increasing bleeding. There seemingly was present a gradual increase in capillary fragility. When a small dose of 3 mg./100 g./weight of the flavonoid compound was given subcutaneously, one or two hours before the injection of polysaccharide, the life of the animal was prolonged up to 18–24 hours and the capillary hemorrhages considerably delayed and less pronounced. A larger dose of the flavonoid, 5 mg./100 g./weight given twice, before and shortly after the injection of P–25, prevented in many instances the death of the animal. The hemorrhages were still present but in a relatively small area and there was a considerable delay in their appearance, in some instances 48 hours after the injection of P–25. In the group of rats receiving 3 mg./100 g./weight there was a partial breakdown of the tumor which was absent, however, in the majority of the cases receiving 10 mg./100 g./weight of the flavonoid compound.

The following Table II summarizes the results of the experiments on 25 rat bearers of tumor (19 males and six females).

There seems to be some similarity in the protective action of the flavonoid against the hemorrhage-producing effect of bacterial polysaccharide in tumor-bearing rats with that of adrenal cortical extracts. The flavonoid compound gives protection not only to the capillary system of the tumor but to the capillary system of the adrenal gland as well. Both cortex and medulla in the animals treated with polysaccharide and the flavonoid appear relatively normal with few residual hemorrhages in zone X of the cortex.

3. Increased capillary fragility induced by ionizing radiation

In our next series of experiments, radiation injury to the capillary wall was investigated. Field and Rekers (16) in their extensive investigation on dogs found that certain flavonoids gave a considerable protection against radiation, and expressed the opinion that vitamin "P" factors affect the vascular system directly. They concluded that: "previous misunderstanding of the nature of vitamin 'P' has arisen from both the failures to recognize that several flavonone analogues possess very similar anti-hemorrhagic activity and that ascorbic acid has the capacity to potentiate activity in other flavonones."

In our investigation 100 rats, 63 males and 37 females, were submitted to a total body, near-lethal dose of radiation. The average weight of the rats was 180 g., ranging from 160 to 205 g. The radiation factors were: 250 kv, with 0.5 mm. Cu and 3.0 mm. Bakelite Filters. Target distance was 27.5 cm. and 210 r/min. dose rate. All rats received 800 r total body X-radiation in a single exposure. Forty rats served as control. The mortality rate in the control group was 80%. Only eight rats survived at the end of three weeks. Of the treated animals 20 were given 4 mg. of the flavonoid compound for 10 days, three days prior

TABLE IV

The protective action of the flavonoid compound against radiation erythema

14 cases

Case no.	Diagnosis	X-ray dosage	Flav. cpd.	Skin reaction
1. F—38	Carcinoma of cervix	3,100 r skin dose to 4 pelvic fields	600 mg.	Minimal skin reaction. Nausea and vomiting.
2. M—44	Carcinoma of gingiva	3,000 r skin dose to 3 fields	300 mg.	No erythema. No nausea or vomiting.
3. F—61	Carcinoma of pleura	2,900 r to 3 thoracic fields 4,000 r to cerv. f.	300 mg.	No erythema. No nausea. Slight tanning.
4. F—32	Carcinoma of gingiva	2,600 r to 2 skin fields; 10,000 gamma	300 mg.	No skin erythema, dry erythema of mucous.
5. F—44	Carcinoma of cervix	4,000 r skin dose to each of 4 pelvic fields	600 mg.	No erythema. Bleeding arrested.
6. F—50	Hepatoma	3,000 r skin dose to each of 4 abd. portals	300 mg.	Mild erythema. No nausea or vomiting.
7. F—70	Carcinoma of cervix	3,000 r to each of 4 skin portals	300 mg.	Slight nausea. No erythema.
8. F—38	Adenocarcinoma of rectum	5,000 r to each of 4 pelvic fields	600 mg.	No nausea. Slight skin reaction.
9. M—56	Metastatic carcinoma cervical node	4,000 r skin dose delivered in 10 days	300 mg.	Slight dry erythema.
10. F—65	Carcinoma of ovary	1,800 r to each of 4 abd. fields	300 mg.	No erythema.
11. F—45	Scirrhous carcinoma of breast	3,400 r skin dose to each of 4 skin portals	600 mg.	Slight tanning. Skin intact.
12. F—55	Carcinoma of cervix	3,000 r skin dose to each of 4 pelvic portals	600 mg.	No erythema.
13. F—70	Carcinoma of cervix	3,100 r skin dose to each of 4 pelvic portals	300 mg.	Mild skin reaction.
14. M—58	Lymphosarcoma of stomach	3,200 r skin dose to each of 3 abdom. portals	300 mg.	No erythema.

to radiation and seven days post radiation. This dose gave a partial protection. The mortality rate was reduced 40%, with 12 rats surviving the exposure. Forty rats were given 5 mg. of the compound for 30 days, five days prior to the exposure and 25 days post radiation, with a total amount of 150 mg. In this group the mortality rate was reduced to 10% with 36 rats surviving. (Table III.)

From these observations it appears that the citrus flavonoid compound gives considerable if not complete protection to rats against a total body, near-lethal dose of radiation.

CLINICAL

Roentgen sickness is a syndrome in which many factors seem to be involved. It was reported that vomiting and nausea can be alleviated by the use of some antibiotics (aureomycin) and the opinion was expressed that these manifestations are partially due to bacterial infections controlled by antibiotic substances (25). In our clinical studies particular attention was paid to radiation erythema and the biological activity of the flavonoid compound was measured in terms of prevention of the erythematous response. The present report covers 92 patients submitted to large doses of radiation and receiving the flavonoid compound. The preparation was administered orally, 300–500 mg./day for five days prior to the exposure and during the whole course of radiotherapy. Table IV presents the results of the investigation conducted by Dr. Isidore Arons, Department of Radiotherapy, Harlem City Hospital, New York City.

From this preliminary clinical report it appears that the flavonoid compound prevents to a considerable degree the appearance of radiation erythema, while nausea and vomiting might persist in spite of flavonoid therapy.

DISCUSSION

All the available evidence indicates that the capillary wall in normal tissue is comparatively impermeable both to serum albumin and to serum globulin. Consequently the average effective pore size of the capillary wall must be less than six millimicrons. It has been suggested that pore size is confined by the intercellular cement lying between the endothelial cells. It is known from the work of Chambers and Zweifach and others (26, 27) "that the intercellular cement dissolves under certain physical conditions such as the absence of calcium or at slightly acid pH." J. F. Danielli

pointed out that: "It is quite certain that anything which affects the physical condition of the intercellular cement affects the pore size" (28). This in turn will affect the effectiveness of the pores as "perfect sieves" and consequently induce what we know as increased capillary fragility. According to Danielli "under normal conditions associated with increased capillary permeability the walls of the pores through this cement are coated with a layer of adsorbed serum protein which effectively reduces the area of pore through which filtration may occur." Displacement of this adsorbed serum protein by other molecules of small diameter may result in an increase in pore size and in a consequent injury to the capillary wall.

This basic concept of capillary pathology indicates the necessity of making a clear distinction between increased capillary permeability and capillary fragility. The latter term should be used only in those cases where chemical lesions in the capillary wall, or more specifically in the intercellular cement, are present.

As Haden, Schneider and Underwood have shown (29) capillary injury "is by far the most frequent cause of clinical hemorrhagic disease" and this injury "may be due to infection, drugs, toxemia, allergy, or nutritional disturbances." They pointed out that increased capillary fragility is due to changes in the intercellular cement substance or to primary damage to the endothelial cells.

In our present work we have demonstrated that the flavonoid compound gave considerable protection against the toxic effect of leukotaxine, bacterial polysaccharide and ionizing radiation. In all these instances an increase in capillary fragility was prevented by the administration of this compound. Although the mechanism of action of the flavonoid compound is not as yet explained we are inclined to agree with Field and Rekers that vitamin "P" factors affect the capillary system directly, perhaps participating as a principal in the "wear and tear" of a part or all of the capillary system, inhibiting its degeneration and taking part in its regeneration, specifically as far as the intercellular cement is concerned. Seemingly vitamin "P" activity is most evident when chemical lesions in the capillary wall are present and increased capillary fragility exists.

SUMMARY

1. A flavonoid preparation from citrus fruit and containing four identified factors was tested, experimentally and clinically, on its biological activity.

2. Ten mg./kilo/weight of this mixture administered to rabbits 20 minutes before subcutaneous injection of 3 mg. of leukotaxine wholly or in part inhibited the effect of this inflammatory exudate factor on capillary permeability.

3. Ten mg./100 g./weight of this material protected to a considerable degree the capillary system of August Rat Carcinoma and of the adrenal gland of cancerous rat against the toxic effect of 0.5 mg. of bacterial polysaccharide.

4. Five mg. of this preparation administered orally for 30 days, five days prior to the exposure and during 25 days post radiation, gave a considerable but not complete protection against a total body, near-lethal dose of X-ray radiation. The mortality rate in the group of 40 irradiated rats was reduced to 10% as against 80% of mortality in the control batch of 40 irradiated rats.

5. Ninety-two patients submitted to deep X-ray therapy and given the mixed flavonoids for five days prior to the exposure and during the whole period of radiotherapy, 300–600 mg./day, orally, manifested considerable diminution in radiation erythema of the skin and in some cases a complete absence of it. Nausea and vomiting, however, persisted in some of the treated patients.

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