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THE ANTI-OXIDANT ACTION OF RUTIN* ON TISSUE PEROXIDIZATION AND RELEASE OF LYSOSOMAL ACID PHOSPHATASE

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Abstract—Rutin, when administered intraperitoneally or orally, does not affect the *in vitro* formation of lipid peroxides by rat brain and kidney homogenates incubated aerobically. The drug, however, inhibits lipid peroxide formation when added to homogenates or tissue fractions rich in lysosomes but does not prevent the release of acid phosphatase and protein from the latter.

RAPID formation of lipid peroxide occurs when tissue homogenates are incubated in air.[1] Rutin, the rhamnoside of hesperidin, inhibits such accumulation of lipid peroxide by rat tissues.[2] As low a concentration as 5 μg/ml of rutin brings about 10–60 per cent inhibition of peroxide formation in rat liver and brain homogenates. Under identical experimental conditions, 1 mg/ml of α-tocopheryl acetate produces only 15–20 per cent inhibition. It was of interest, therefore, to ascertain whether prior administration of Rutin to rats would exert any inhibitory effect on the formation of lipid peroxides during subsequent aerobic incubation of their tissue homogenates.

There are reports in the literature on the toxic action of lipid peroxides on tissue enzymes.[3, 4] It has also been suggested that lipid peroxides are one of the agents that disturb the integrity of lysosomal particles leading to the release of hydrolytic enzymes.[5–7] The action of Rutin on the release of acid phosphatase (E.C. 3.1.3.2. orthophosphoric monoester phosphohydrolase) a typical lysosomal enzyme was also, therefore, studied in some detail.

MATERIALS AND METHODS

Animals

Adult male or female albino rats of the CDRI Animal House Colony were used in these experiments.

Protein

Protein content of tissues was estimated colorimetrically according to Lowry *et al.*[8]

Acid phosphatase

Acid phosphatase was estimated essentially according to Hawk *et al.*[9] using β-glycero-phosphate as the substrate.

* Rutin was supplied by E. Merck, Darmstead, A.G.

Preparation of subcellular fractions

Ten per cent homogenates in 0·25 M sucrose of freshly excised rat liver were freed of cell debris and unbroken cells by low speed centrifugation in the cold and then centrifuged in the Spinco Model L. The differential centrifugation procedure employed was similar to the one used by Shibko and Tappel for the preparation of lysosomes.[10]

Estimation of lipid peroxides

Lipid peroxides were estimated by the thiobarbituric acid method as described in an earlier paper.[2] While the present investigation was in progress, Wills[11, 12] reported that the thiobarbituric acid method was not reliable for the quantitative determination of peroxides since iron, if present in the medium, could extensively affect the colour value. In contrast to the behaviour of pure lipid emulsions studied by Wills, suspensions of liver mitochondria were, however, shown by McKnight and Hunter[13] not to exhibit any increase in thiobarbituric acid colour value on the addition of 1–500 μM Fe^{2+} or Fe^{3+}.

Under well-defined conditions, the thiobarbituric acid method was found in the course of the present study to give a reproducible measure of the extent of lipid peroxidation. The ferrous thiocyanate method as described by Wagner *et al.*[14] gave inconsistent results. Unsaturated fatty acids undergoing auto-oxidation exhibit absorption changes in the 230–240 mμ region of u.v. light.[15] The absorbancy at 232 mμ or 235 mμ of the alcohol–ether extracts of tissue homogenates or 5% trichloroacetic acid supernates of tissues adjusted to pH 10 was not found to increase with time of incubation of the tissue whereas there was a time-dependent increase in TBA value.

RESULTS

Effect of administration of Rutin on in vitro *lipid peroxides formation in rat tissues*

Twelve adult male rats were divided into three batches of four each keeping equal number of weight and litter mates in the three groups.

Animals from two of these batches were starved overnight and given intraperitoneally 5 mg or 10 mg Rutin suspended in 1·0 ml 0·1 M phosphate buffer, pH 7·4. The animals were killed 5 hr later, brain and kidneys removed and homogenized in 0·1 M phosphate buffer. In a parallel set, 6 mg of Rutin suspended in 1 ml of 0·1 M phosphate buffer, pH 7·4 were fed orally to each rat by a mouth tube for 5 days during which they received a diet of the following per cent composition: casein, 18; starch, 42, D-glucose, 10; groundnut oil, 10; cane sugar, 5; Hubbell's[16] salt mixture, 4, and vitamin mixture, 1. (The vitamin mixture was made as follows: thiamine hydrochloride, 500 mg; riboflavin, 500 mg; pyridoxin hydrochloride, 500 mg; niacin, 1500 mg; biotin, 10 mg; Inositol, 10 mg; folic acid, 20 mg; cyanocobalomin, 4 mg; vitamin A acetate, 500 mg; calciferol, 20 mg; α-Tocopheryl acetate, 10 mg; menadione, 50 mg; calcium pantothenate, 2 g; methionine, 250 g; choline chloride, 100 g; ascorbic acid, 6 g. Final weight made up to 1000 g with D-glucose.) On the sixth day, they were killed by decapitation, their tissues removed and homogenized. Only brain and kidney were used for this study since preliminary investigations had shown that these tissues were very active in producing lipid peroxides.

The results summarized in Table 1 indicate that the administration of Rutin either by the i.p. or the oral route does not affect the output of lipid peroxides *in vitro* by

rat brain and kidney homogenates. The zero-hour TBA values, a measure of the lipid peroxide already formed *in vivo*, do not differ very significantly between the Rutin fed and unfed animals. Thus, Rutin does not appear to function as an anti-oxidant *in vivo*.

TABLE 1. EFFECT OF ADMINISTRATION OF RUTIN ON THE FORMATION OF LIPID PEROXIDES BY RAT TISSUES

Tissue	Route of administration of the drug	Quantity of drug given (mg/rat)	TBA value*	
			0 hr	3 hr
Brain	—	—	189 ± 49 (4)	2168 ± 699 (4)
	i.p.	5	217 ± 28 (2)	1603 ± 283 (2)
	i.p.	10	160 ± 9 (2)	2670 ± 30 (2)
Kidney	—	—	148 ± 56 (4)	823 ± 225 (4)
	i.p.	5	122 ± 38 (2)	506 ± 28 (2)
	i.p.	10	226 ± 0 (2)	820 ± 9 (2)
Brain	—	—	233 ± 63 (2)	2452 ± 283 (2)
	Oral	6	237 ± 18 (2)	2518 ± 160 (2)
Kidney	—	—	132 ± 9 (2)	853 ± 42 (2)
	Oral	6	268 ± 62 (2)	896 ± 122 (2)

Figures in brackets refer to the number of animals used.

* Thiobarbituric acid colour developed with 1 ml TCA supernatant and the absorbancy value at 535 mμ multiplied by 1886.

Addition of Rutin to the homogenates of both control and experimental animals while incubating aerobically promptly inhibited peroxide formation.

Effect of Rutin on the release of lysosomal acid phosphatase

Adult female rats were starved overnight and killed in batches of four by decapitation; livers were excised out and homogenized in 0·25 M sucrose and after removal of cell debris and unbroken cells by low speed centrifugation spun successively at 11,000 rev/min for 2 min (Fraction I), at 11,000 for 20 min (Fraction II) and at 39,000 rev/min for 1 hr (Fraction III). The sediments obtained in each case were dispensed in 0·25 M sucrose to correspond to a tissue concentration similar to the original homogenate. The suspensions were shaken in 50 ml Erlenmeyer flasks at 37°C in a Dubnoff metabolic shaker. The contents of each flask were centrifuged at 39,000 rev/min for 1 hr and aliquots of supernate used for estimation of peroxides, phosphatase and protein. The sediment obtained by centrifugation at 39,000 rev/min was dispersed in 10 ml 0·25 M sucrose and aliquots employed for acid phosphatase estimation. The results presented in Tables 2 and 3 show that Rutin inhibited lipid peroxide formation but did not affect the release of acid phosphatase on incubation of the heavier subcellular particles.

DISCUSSION

The feeding of Rutin does not have any effect on the *in vitro* production of lipid peroxides by rat brain and kidney homogeantes. The content of lipid peroxides in the tissues of animals receiving Rutin as also of the controls was of the same order. It would appear that Rutin does not presumably act as an antioxidant in the normal rats under *in vivo* conditions although it inhibits the *in vitro* formation of lipid peroxides

by their tissue homogenates. Rutin is known to be readily absorbed though slowly metabolized by animal tissues, from which one infers that a concentration of the drug adequate for exerting inhibitory action on lipid peroxide formation should be present in the kidney tissue under the condition of the present study. Yet the kidney tissue homogenates prepared from animals receiving Rutin produced significant

TABLE 2. EFFECT OF RUTIN ON RELEASE OF LIPID PEROXIDES AND ACID PHOSPHATASE FROM RAT LIVER SUB-CELLULAR FRACTION RICH IN LYSOSOMES

Fraction No.	Time (hr)	TBA value*		Protein (mg/ml)		Acid phosphatase† sp. act.	
		Control	Rutin	Control	Rutin	Control	Rutin
I	0	13·5	—	2·50	—	31·0	—
	1	22·0	4·7	2·96	2·72	—	—
	2	42·0	4·0	3·55	2·80	29·4	32·0
II	0	14·0	—	2·00	—	0·75	—
	1	—	6·4	—	2·02	—	0·91
	2	40·0	6·4	2·12	2·02	2·40	0·99
III	0	10·0	—	2·22	—	0·45	—
	1	18·0	4·0	2·22	2·08	0·65	0·64
	2	13·0	6·0	1·95	2·10	0·68	1·03

* TBA value of 39,000 rev/min supernate.

† One acid phosphatase unit is equivalent to the enzyme that liberates 1 mg P/hr.
Sp. act. = Units/mg protein.

TABLE 3. EFFECT OF RUTIN ON THE RELEASE OF LIPID PEROXIDES AND ACID PHOSPHATASE BY A LYSOSOME RICH FRACTION (FRACTION I) OF RAT LIVER

Time (min)	TBA value		Protein (mg/ml)		Phosphatase (sp. act.)			
	Control	With Rutin	Control	With Rutin	Supernatant		Sediment	
					Control	With Rutin	Control	With Rutin
0	0	0	465	420	—	5	34·8	32·2
30	9	7	580	610	25	10	35·0	29·5
60	11	7	645	620	22	22	40·5	40·2
120	83	10	630	637	99	80	29·5	26·8
150	87	13	630	650	112	99	29·5	26·8
180	100	14	650	630	145	160	24·1	26·8

Sp. act. in the case of sediment = units/mg wet wt. equivalent of original tissue.

Fraction I was incubated at 37° and at time intervals indicated aliquots spun in the Spinco Model L at 39,000 rev/min. TBA, protein and acid phosphatase were determined in the supernates. Acid phosphatase activity was also assayed in the sediments obtained.

amounts of lipid peroxides. In the absence of a satisfactory method for the identification and quantitation of Rutin in animal tissues, no attempts were made to estimate the Rutin content of tissues receiving the drug by the i.p. or oral route.

Rutin was found to arrest the liberation of lipid peroxides from subcellular particles containing lysosomes and mitochondria but did not prevent the release of soluble

proteins and acid phosphatase from these particles on aerobic incubation. This is in accordance with the earlier finding on the negative effect of Rutin in not preventing the inactivation by ageing of mitochondrial succinoxidase.[2] Peroxidative damage of tissue lipids is expected to cause cell injury and lipid peroxides could, on this assumption, destroy lysosomal structure causing the release of their hydrolytic enzymes. Careful scrutiny of the data presented in this study shows that release of a lysosomal enzyme takes place even under conditions when the output of lipid peroxides is inhibited by Rutin. It was also observed during the present study that Rutin did not significantly affect phosphate-induced swelling of rat liver mitochondria, a phenomenon closely associated with the permeability changes of this subcellular structure (Sharma and Krishna Murti, unpublished). Lipid peroxides extracted from tissue homogenates incubated for three hours were also found not to affect the rate of oxidation of succinate by intact liver mitochondria. Presumably the release of lysosomal enzymes or the loss of succinoxidase of mitochondria under *in vitro* incubation condition is a consequence of membrane injury due to factors other than lipid peroxides.

The mechanism of action of Rutin as an anti-oxidant in tissue peroxidization needs further elucidation. Versene another powerful anti-oxidant protects tissues from lipid peroxidization by complexing with metal ions that mediate such peroxidation.[12] The formation of lipid peroxide in tissue homogenates is largely due to non-enzymic reactions. The mechanism of peroxide formation has been partially elucidated employing systems made up of fatty acids and metal ions.[12] In tissue homogenates or sub-cellular fractions however, very little information is available as to the nature of the catalysts or the primary source of free radicals initiating the chain reactions that lead to the eventual formation of lipid peroxides. A search for these factors may unravel the basic mechanisms of lipid peroxidation in tissues which would permit, in turn, the elucidation of the antioxidant action of Rutin at the molecular level.

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