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STABILIZATION OF RAT LIVER LYSOSOMES BY (+)-CYANIDANOL-3 *IN VIVO*

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Abstract—(+)-Cyanidanol-3, [(+)-catechin], administered by subcutaneous injection at a dose of 100 to 200 mg/kg/day for 6–20 days, exerts *in vivo* a stabilizing effect on lysosomal membranes in rat liver. This was demonstrated in normally fed rats and in animals whose lysosomes had been rendered fragile by intoxication with galactosamine and ethanol. Under these circumstances, the discharge of acid phosphatase, β-glucuronidase and β-*N*-acetylglucosaminidase by the lysosomes was significantly reduced, by 15–20 per cent, compared to the controls. Moreover, *in vitro*, products of the oxidation of (+)-cyanidanol-3 exert a strong stabilizing effect on the lysosomal membrane. Such stabilization of the lysosomes may have a beneficial effect in various hepatic disorders involving abnormal fragility of the lysosomes.

Lysosomes play an increasingly prominent role in the field of pathology. These cytoplasmic vesicles contain some forty hydrolytic enzymes capable of digesting all the categories of macromolecules in the organism: polysaccharides, proteins, nucleic acids and lipids [1]. These enzymes exert maximal activity at an acid pH and are discharged by the lysosomes only under appropriate cytochemical conditions, with the membrane of the lysosome playing a very important role in controlling their function.

The mechanism for the discharge of these hydrolytic enzymes, if disturbed, may be responsible for a variety of disorders affecting the cell or extracellular connective structures. Thus, abnormal fragility of the lysosomes and increased extra-cellular activity of the lysosomal enzymes have been implicated in a number of pathological phenomena, involving in particular the inflammatory processes [2], rheumatoid arthritis, osteoarthritis, gout and silicosis [3], muscular dystrophy and nervous atrophy [4], allergic reactions [5], conditions of shock [6] and all diseases involving damage to connective tissue, such as atherosclerosis [7], the varicose syndrome [8] and diabetes with vascular complications [9].

Abnormal fragility of the lysosomes, with increased discharge of their enzymes into the serum, is a common characteristic of a number of hepatic conditions [10–12], especially fibrous hepatitis and cirrhosis [13, 14], acute alcohol intoxication [15], acute hepatitis [16], biliary tract disorders [17] and viral hepatitis [18].

It was of interest therefore to induce in rats forms of hepatitis characterized by fragility of the lysosomes in order to test the effect of (+)-cyanidanol-3, [(+)-catechin; (3,3′,4′,5,7,-flavanpentol)], a flavonoid with hepatoprotective qualities. This interest was enhanced by the fact that other flavonoids, notably derivatives of rutin, have demonstrated their capacity for stabilizing the lysosomes of rat liver *in vitro* [19].

There are various models in the literature for animal experiments which make it possible to produce increased fragility of the hepatic lysosomes *in vivo*, notably through prolonged inanition, necrosis or ischemia [20], poisoning by carbon tetrachloride, nitrosamine, thioacetamide or beryllium [21], intoxication by aflatoxin or mitomycin [22] and intoxication by D-galactosamine [23].

In our experiment we used galactosamine and ethanol. D-Galactosamine appeared to be especially suitable as a means of producing lesions in rat liver similar to those provoked by viral hepatitis in man [24] and in the case of prolonged intoxication producing symptoms similar to those of cirrhosis [25].

The effect of ethanol on the lysosomes of liver cells has not been extensively examined. Platt *et al.* [26] were not able to demonstrate, in the rat, a definite activity affecting the permeability of the lysosomal membrane following the daily intraperitoneal injection of 1 g of ethanol for 14 days. On the other hand, some authors [27, 28] found an increase in the fragility of the membrane in the course of chronic alcohol intoxication. Recently, Geokas *et al.* [15] showed that there was a great increase in the activity of arylsulfatase and β-glucuronidase in the plasma of persons suffering from acute alcohol intoxication. Since such an increase could logically result only from abnormal fragility of the hepatic lysosomes, it seemed interesting to verify this hypothesis in rats subjected to acute alcohol intoxication and to observe the effect in this context of (+)-cyanidanol-3.

METHODS

Female Wistar rats, weighing an average of 160 g at the outset, fed with U.A.R. granules and receiving water *ad libitum* were used. The animals were divided into the following groups: three control groups of 16, 15 and 7 rats respectively; one group of 19 rats injected subcutaneously in the tail region with (+)-cyanidanol at a dose of 200 mg/kg per day for 12 days; one group of 16 rats intoxicated with D-galactosamine HCl at a dose of 200 mg/kg per day s.c., for 7 days; one group of 16 rats intoxicated with D-galactosamine, as above, and also receiving 200 mg/kg per day of (+)-cyanidanol-3, s.c., for 6 days; one group of 7 rats intoxicated over a period of 4 weeks with 10% ethanol in their

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drinking water and for the following 6 weeks with 20% ethanol, also in the drinking water; one group of 7 rats intoxicated with ethanol, as described above, and also receiving 100 mg/kg per day of (+)-cyanidanol-3 s.c., during the last 20 days of intoxication. The technique used thereafter was based upon the method originally developed by de Duve *et al.* [29] as discussed by Wattiaux [30].

The rats were decapitated, the liver removed and cooled in a tared beaker containing cold 0·25 M sucrose solution. After weighing, the livers were homogenized in 5 volumes of cold 0·25 sucrose solution with the Potter teflon apparatus (A. H. Thomas Company, Philadelphia) driven by a motor turning at 2000 rev/min, the pestle being slowly moved in and out. The whole procedure required about 15 sec. The homogenate was then centrifuged at 10,000 *g* min, (*g* min = $\int_t^o g.dt$), the equivalent of 10 min at 2000 rev/min, in a Super Minor MSE centrifuge and placed in a cold chamber. After decantation, the sediment was homogenized with 5 vol chilled 0·25 M sucrose and centrifuged in the cold at 6000 *g* min, (10 min at 1700 rev/min in a Super Minor MSE centrifuge). The residue was discarded and the supernatants combined and diluted ten times. These were centrifuged at 250,000 *g* min (2×) i.e., 2 min and 15 sec at 22,000 rev/min, without counting acceleration and deceleration, in a refrigerated MSE 65 centrifuge. The supernatant was decanted as completely as possible and the residue containing the total mitochondrial fraction (ML) of de Duve *et al.* [29] was diluted five times for determination of enzymes activity by the following procedures.

Acid phosphatase. (Orthophosphoric-monoester phosphohydrolase, EC 3.1.3.2). 0·5 ml of diluted ML was preincubated with 0·5 ml of 0·1 M acetate buffer solution containing 0·25 M sucrose at pH 5 for 30 min at 37°.

Free activity was measured by incubating for 10 min at 37° after adding 1 ml of 0·05 M acetate buffer at pH 5 containing 0·25 M sucrose and 0·1 M β-sodium glycerophosphate. Total activity was measured by adding 0·2% Triton X-100 to the preceding mixture. The respective blanks had the same composition except that β-glycerophosphate was omitted. After stopping the reaction with 8 ml of 8% TCA, and adding β-glycerophosphate to the blanks, the solutions were filtered through filter paper. The phosphorus was determined in 1 ml of the filtrate to which was added 1 ml of 0·01 M ammonium heptamolybdate in 2·5 N H_2SO_4, 2 ml of water and 1 ml of a mixture of 0·28 M $NaHSO_3$, 8·5 mM Na_2SO_3 and 2·1 mM aminonaphthalene sulfonic acid in water. The absorption at 650 nm was measured after 15 min.

β-N-Acetylglucosaminidase (2-Acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30). The mitochondrial fraction was preincubated for 15 min at 37° in the same mixture used for the acid phosphatase assay. Free activity was measured by incubating for 10 min at 37° after adding 1 ml of 0·05 M acetate buffer at pH 4·4 containing 2 mg of *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide and 0·25 M sucrose. To measure total activity, 0·2% Triton X-100 was added to the mixture. After stopping the reaction with 6 ml of glycocoll buffer at pH 10 (0·4 M glycocoll, 0·25 M Na_2CO_3, 0·2 M NaCl, dilute 3 times), the solution was centrifuged for 10 min at 6000 *g*. The absorption at 430 mm was then measured.

β-Glucuronidase (β-D-Glucuronide glucuronosohydrolase, EC 3.2.1.31). The preincubation mixture used in the acid phosphatase assay was maintained at 37° for 25 min. Free activity was measured by incubating for 10 min at 37° after adding 1 ml of 0·05 M acetate buffer at pH 5 containing 0·0025 M phenolphthalein β-D-sodium glucuronate and 0·25 M sucrose. Total activity was measured by adding 0·2% Triton X-100. After stopping the reaction with 6 ml of the glycocoll buffer used in the β-*N*-acetylglucosaminidase assay the solution was centrifuged and the absorption at 545 nm was measured.

The effects of (+)-cyanidanol-3 *in vitro* were measured in a similar manner. The lysosomes were isolated from the liver of normal rats and the flavonoids were added to the preincubation mixture at the rate of 1 mg per ml.

RESULTS

The results are shown in the various tables. The rate of free activity is the percentage of activity of the respective enzymes discharged by the lysosomes, incubated without Triton, in relation to the total activity of the same enzymes when incubated with Triton, which provokes the complete rupture of the lysosomes, resulting in total discharge of the enzymes. The percentage of free activity is a direct reflection of the stability of the lysosomes. The lower the activity, the more stable are the lysosomes.

In normally nourished rats, (+)-cyanidanol-3 is capable of increasing the stability of the lysosomes, and, under our experimental conditions, of reducing by about 15 per cent the discharge of the three enzymes studied (Table 1). D-Galactosamine provokes a fragility in the lysosomes and increases the discharge of the three enzymes by about 30 per cent. (+)-Cyanidanol-3 is capable of reducing the fragility induced by the galactosamine and of diminishing the discharge of the enzymes by 15 per cent (Table 2). Tests carried out

Table 1. Percentage ± standard deviation of free activities of three lysosomal enzymes in rat liver after s.c. injections of (+)-cyanidanol-3 (200 mg/kg per day–12 days)

Enzymes	Control rats	Treated rats
N-acetylglucosaminidase	60·7 ± 17·1 (16)	45·3 ± 14·5 (19)
	←t = 2·9 P < 0·005→	
β-Glucuronidase	75·2 ± 19·4 (18)	61·4 ± 19·6 (20)
	←t = 2·2 P < 0·025→	
Acid phosphatase	89·8 ± 9·4 (18)	77·1 ± 15·6 (20)
	←t = 3·0 P < 0·0025→	

( ): Number of rats studied. P: Probability coefficient (t-test). Results with standard deviations.

Table 2. Percentage ± standard deviation of free activities of three lysosomal enzymes in rat liver after s.c. injections of D-galactosamine-HCl (200 mg/kg per day–7 days) and treatment with s.c. injections of (+)-cyanidanol-3 (200 mg/kg per day–6 days)

Enzymes	Control rats	Galactos. intoxicated rats	Gal. intox. rats treated with(+)-cyanidanol-3
N-acetylglucosaminidase	50·1 ± 15·8 (15)	74·1 ± 20·1 (16)	62·9 ± 22·4 (16)
	←t = 3·7 P < 0·005→	←t = 1·5	0·05 < P < 0·1→
		←t = 1·8 P < 0·05→	
β-Glucuronidase	67·6 ± 19·4 (14)	90·5 ± 15·5 (14)	73·0 ± 25·1 (14)
	←t = 3·4 P < 0·0025→	←t = 2·2 P < 0·025→	
		←t = 0·6 N.S.→	
Acid phosphatase	73·9 ± 18·3 (15)	94·5 ± 4·9 (16)	85·4 ± 16·5 (16)
	←t = 4·3 P < 0·0005→	←t = 2·1 P < 0·025→	
		←t = 1·8 P < 0·05→	

( ): Number of rats studied. N.S.: not significant.

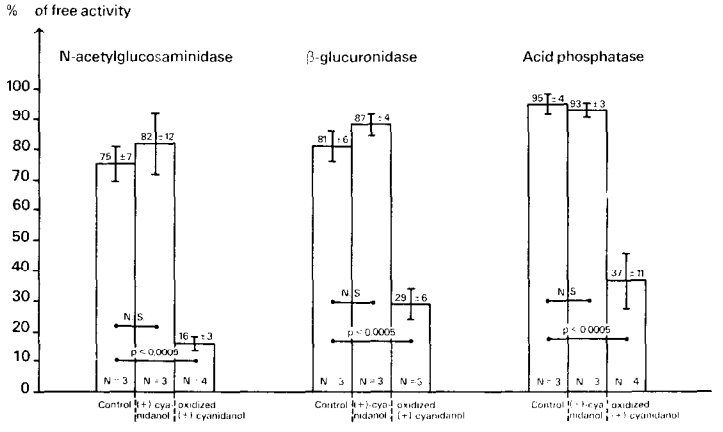

Fig. 1. Percentage ± standard deviation of free activity of three lysosomal enzymes after incubation of rat liver lysosomes in presence of (+)-cyanidanol-3 and the products of oxidation of (+)-cyanidanol-3 *in vitro* (1 mg/ml). N: number of rats; N.S.: not significant.

Table 3. Percentage ± standard deviation of free activities of three lysosomal enzymes of rat liver after ethanol intoxication (10% ethanol in water for 4 weeks and 20% for 6 weeks) and (+)-cyanidanol-3 treatment (s.c. injections of 100 mg/kg per day–20 days)

Enzymes	Control rats	Ethanol intoxicated rats	Ethanol intoxicated rats treated with (+)-cyanidanol
N-acetylglucosaminidase	58·7 ± 15·6 (6)	78·5 ± 12·6 (6)	61·2 ± 17·3 (6)
	←t = 2·6 P < 0·0125→		
		←t = 2·1 P < 0·025→	
		←N.S.→	
β-Glucuronidase	81·9 ± 19·7 (7)	99·4 ± 1·6 (7)	87·4 ± 18·8 (7)
	‹ t = 2·3 P < 0·02→		
		←t = 1·7 P ⩽ 0·05→	
		←N.S.→	
Acid phosphatase	81·5 ± 7·8 (7)	88·6 ± 4·6 (7)	81·1 ± 12·6 (6)
	←t = 2·1 P < 0·025→		
		←t = 1·5 0·05 < P < 0·1→	
		←N.S.→	

( ): Number of rats studied. N.S.: not significant.

Table 4. Total activities ± standard deviation (μM/min per g wet wt) of three lysosomal enzymes in the rat liver after injections of (+)-cyanidanol-3 (200 mg/kg per day–12 days) compared with controls

	N-acetylglucosaminidase	β-Glucuronidase	Acid phosphatase
Control rats	1·35 ± 0·27 (23)	0·51 ± 0·08 (13)	3·56 ± 0·66 (23)
	↑ N.S. ↓	↑ N.S. ↓	↑ N.S. ↓
Treated rats	1·34 ± 0·17 (24)	0·55 ± 0·06 (14)	3·78 ± 0·59 (25)

( ): Number of rats studied. N.S.: not significant.

with ethanol demonstrate that the alcohol provokes an abnormal fragility in the hepatic lysosomes of the rat, with an increased discharge of the three enzymes of 20 per cent compared to the control animals. Here again, (+)-cyanidanol-3 counteracts the effect of the toxic substance and maintains the stability of the lysosomes at values close to those found in the control rats (Table 3).

Figure 1 shows the results obtained when (+)-cyanidanol-3 and its oxidation products are added *in vitro* following isolation of the liver lysosomes and prior to preincubation at pH 5. At a dose of 1 mg/ml, (+)-cyanidanol-3 proved to have no effect on the discharge of β-*N*-acetylglucosaminidase, β-glucuronidase and acid phosphatase from the lysosomes and therefore had no effect *in vitro* on the stability of the lysosomes. On the other hand, products of oxidation and probably of polymerization of (+)-cyanidanol-3, resulting from spontaneous transformation in an aqueous medium and having structures which are not yet known, under the same conditions have a definitely stabilizing effect on the lysosomal membranes and reduce the discharge of the three enzymes by more than 60 per cent compared to the controls.

DISCUSSION

(+)-Cyanidanol-3 has been found to have a stabilizing effect *in vivo* on the lysosome membranes. This action has been demonstrated by measuring the discharge of three lysosomal enzymes, β-*N*-acetylglucosaminidase, β-glucuronidase and acid phosphatase, in control rats, in rats treated with cyanidanol-3, in rats intoxicated with galactosamine or with ethanol, and in rats which have been intoxicated and treated with (+)-cyanidanol-3.

Taken in conjunction with tests carried out *in vitro* with (+)-cyanidanol-3 and its oxidation products, it is possible to suppose that the action of (+)-cyanidanol-3 may be due to transformation of this molecule in the organism, attended by the appearance of such oxidation products as can be obtained through the spontaneous transformation of (+)-cyanidanol-3 in an aqueous solution.

The activity of (+)-cyanidanol-3 observed *in vivo* is not due to enzyme inhibition but is clearly a phenomenon of augmentation of the stability of the lysosomal membrane. The total activity of the three enzymes did not vary according to whether the rats had or had not been treated with (+)-cyanidanol-3 and there was therefore no inhibition of the lysosomal enzymes in the liver by (+)-cyanidanol-3 *in vivo* (Tables 4 and 5). Likewise, the activity of the oxidation products of (+)-cyanidanol-3 *in vitro* is definitely due to an increase in the stability of the lysosomal membrane. In the latter case, the action of these products brings about an inhibition in the total activity of β-*N*-acetylglucosaminidase and of β-glucuronidase. The degree of inhibition however is almost the same for total activity and free activity, so that the percentage of free activity in relation to total activity undergoes no change, whether or not there is any inhibition of the particular enzyme in question (Tables 4 and 5).

Although there are references in the literature to a great many toxic substances capable of labilizing the lysosomes, such as glucagon, vitamin A and the endotoxins, there are very few substances which have the capacity for stabilizing these organelles in the face of the various pathological conditions which render them fragile [21]. Most of the tests with such substances, furthermore, were carried out *in vitro* and the results obtained cannot be extrapolated directly to living organisms. It has been demonstrated *in vitro* that glucocorticoids such as cortisol, cortisone and methylprednisolone enhance the stability of lysosomes [31, 32]. Chloroquine [33] as well as certain flavonoids with structures similar to that of (+)-cyanidanol-3 [19] also possess this capacity *in vitro*.

Protective activity on lysosomes *in vivo* has been demonstrated for cortisone [34], hydrocortisone, phenylbutazone and various other anti-inflammatory drugs [35, 36].

This stabilizing action is of interest especially in liver disorders in which lysosomal fragility has often been demonstrated (see introduction). Some authors regard this fragility as a very precocious phenomenon [37], while others believe that the transformation is a retarded one, taking place along with necrosis of the hepatic cells [38]. All researchers, however, recognize this fragility of the lysosomes, attended by the subsequent discharge of lysosomal enzymes into the blood stream as characteristic of liver disorders. The discharge of enzymes takes place much earlier than one

Table 5. Percentage inhibition of the total and the free activity of three lysosomal enzymes after incubation of rat liver lysosomes in the presence of products of oxidation of (+)-cyanidanol-3 *in vitro* (1 mg/ml)

	N-acetylglucosaminidase	β-Glucuronidase	Acid phosphatase
Total activity (%)	38·1	28·7	0
Free activity (%)	49·4	40·2	0

would be led to believe on the basis of morphological studies of subcellular structures [39]. This fact may be important, notably with regard to acute alcoholism [15], ischemia of the liver [40, 41], fibrous hepatitis [13], disorders of the bile tract [17] and hepatic disorders in general [10, 11–16]. There is also a probable parallel between liberation of the lysosomal enzymes and diminution of the mitochondrial function since the enzymes may contribute to degradation of the mitochondria [42].

Due to its stabilizing action on the membrane of the lysosomes and the consequent diminution in liberation of hydrolytic enzymes on the occasion of intoxication by galactosamine and ethanol in the rat, (+)-cyanidanol-3 exercises a protective action on hepatic cells. This action is all the more interesting in the light of the fact that (+)-cyanidanol-3, unlike other products, is non-toxic.

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