Biochemical Pharmacology, Vol. 21, pp. 1543–1548. Pergamon Press, 1972. Printed in Great Britain.

INFLUENCE OF SOME HYDROSOLUBLE SUBSTANCES WITH VITAMIN P ACTIVITY ON THE FRAGILITY OF LYSOSOMES *IN VITRO*

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(*Received* 28 *September* 1971; *accepted* 14 *December* 1971)

Abstract—A method is described for measuring *in vitro* the stabilizing capacity of hydrosoluble pharmaceutical products on the lysosomes and this has been applied to substances with vitamin P action. The flavonoids [rutin, tri(hydroxyethyl)rutosid, magnesium flavonic chelates] exert a stabilizing influence on the lysosomes. The anthocyanosides on the other hand show no such effect because they exhibit weak chelating power in our experimental conditions. To this end the chelating capacity for copper, the influence on the —SH groups of cystein and the stabilizing power in the presence of Na_2 EDTA of vitamin P substances have been studied. No parallelism seems to exist between the test of stabilization applied *in vitro* and the methods usually employed *in vivo* to find a vitamin P action.

SINCE substances with vitamin P action influence the fragility and permeability of the capillary blood-vessels, we have compared the action of some of these drugs on the fragility of lysosomes *in vitro*, which can be regarded as vesicles surrounded by a lipoprotein membrane, like many other biological membranes. In our opinion these organelles were particularly suitable for a comparative and especially a quantitative study of the fragility of their membrane. Exposed to the action of various chemical or physical agressions, they can release more or less easily their enzymes, which may then be measured in the surrounding medium. This release evidently depends on the permeability and stability of the lysosomes. Their stability can be modified by different agents such as Na_2 EDTA or cortisone.[1–3] Furthermore, these properties of lysosomes can easily be studied *in vitro*.

MATERIALS AND METHODS

Material

The following figures cover ten tests, taking into account the volumes for high speed centrifugation. The livers (about 20 g) of two male Wistar rats of 300 g were removed immediately after decapitation. All operations were then executed in the cold (0°–4°). The livers are homogenized in a Potter homogenizer with Teflon pestle in 4 vol. of sucrose 0·25 M (w/v). Using the technique described by Weissmann and Thomas,[1] the homogenate was first centrifuged at 800 *g* for 20 min and the supernatant then centrifuged at 15,000 *g* for 20 min.

Labilization of the lysosomes

Our method was inspired by those of Hyttel and Jørgensen.[4] The pellet containing the lysosomes was suspended in 110 ml of Sørensen phosphate buffer pH 7·4, $\Gamma = 0{\cdot}348$. The pH 7·4 was chosen because it is considered as a "physiological" pH. The suspension was shaken at moderate speed by a magnetic stirrer for 5 min and after decantation divided into test tubes, 10 ml per tube. To each of these tubes 10 ml of the same buffer were added, in which 40 mg of one of the vitamin P substances were dissolved. From each tube 9 ml were taken and introduced into two other tubes, one containing 1 ml of phosphate buffer and the other 1 ml of 1% Triton X-100 in the same buffer. After mixing, the tubes were incubated for 40 min at 37° and then centrifuged at 25,000 *g* for 20 min.

In order to study a possible influence of a vitamin P substance on the enzymatic distribution between the supernatant and the granules, the sediment containing the lysosomes was suspended not in a phosphate buffer, but in sucrose 0·25 M adjusted to pH 7·4 with tris buffer 0·02 N. In a sample of the granular suspension thus obtained the enzymes were liberated by three successive freezings (to −20°) and thawings (to 37°). Centrifugation at 25,000 *g* yielded a supernatant rich in cathepsins.

Dosage of the cathepsins

The cathepsins released by the lysosomes during incubation are dosed by polarography using the method described by Homolka and Soušek,[5] with slight modifications. 0·2 ml of the supernatant were incubated in 6·5 ml of Sørensen citrate buffer pH 3·8, and 0·5 ml of a 2·4% bovine albumine solution Calbiochem in NaCl 9% was used as substrate. After incubation, 2 ml were taken and the proteins precipitated by the same volume of a 20% ice-cold sulfosalicylic acid. After centrifugation, 0·5 ml of the supernatant were polarographed in 11 ml of Brdička solution. A Metrohm polarograph with synchronized drop controller and superimposed alternating current (sensitivity 1×10^{-8} or 2×10^{-8} A/mm; tension range −1·40 to −1·85 V; dropping time 0·2 sec), with a saturated silver chloride reference electrode was used. The cathepsin activity is measured in arbitrary units. The activity measured in the supernatant which has been incubated with Triton X-100 is considered as representing the total cathepsin activity (= 100 per cent), it corresponds to the activity of about 0·5 mg of liver. This activity is compared to that of the sample incubated without Triton and the latter is expressed in percentage of the total activity. In order to estimate the influence of a vitamin P substance on the fragilization of the lysosomes, the percentages obtained with and without the substances are compared.

Substances

The following substances with vitamin P action have been tried: hydrosoluble rutin Merck (sodium salt of rutin sulfate, mol. wt. 815), tri(hydroxyethyl)rutosid (vit. P_4, Zyma, Nyon, Switzerland, mol. wt. 743), magnesium flavonic chelates (Flacitran®, from the Laboratoires Français de Thérapeutique, Bordeaux, France) and anthocyanosides of *Vaccinium myrtillus* (Chibret, Clermont-Ferrand, France). These substances were chosen for their solubility in water. Rutin Merck serves as reference: 40 mg in 20 ml of lysosome suspension correspond to a concentration of $2{\cdot}5 \times 10^{-3}$ M/l. Though it would have seemed more logical to use equimolecular

concentrations for comparison of the different substances, this was not possible because of the imprecision of molecular weights of the last two drugs mentioned above, which are not uniform molecular substances and contain impurities. The first three substances are glycosides with aglycones derived from flavone, while the fourth contains derivatives of anthocyanosides, the structure of which is related to that of flavone. At the given concentration the solutions of the last two products are saturated. In these cases the actual concentration is therefore slightly below that indicated.

Chelating power measurement

The chelating power of vitamin P substances for copper has been measured after incubation during 40 min at 37° of 5×10^{-6} M $CuSO_4$ and 2×10^{-5} M rutin or the equivalent weight of another vitamin P substance in 10 ml KNO_3 0·1 N, the pH of which was adjusted at 7·4 with NaOH N. The remaining free Cu^{2+} is then dosed in the solution with a polarograph (sensitivity 1×10^{-8} A/mm; tension range $-$ 0·5 to $+$ 0·5 V). Furthermore, the total amount of copper in the samples of vitamin P substances has been dosed by atomic absorption spectrophotometry* (Perkin–Elmer No. 303). The results obtained by these methods will only be referred to in the discussion.

Dosage of the thiol groups

The influence of vitamins P on cystein has been studied after inoculation in the phosphate buffer pH 7·4 (15 mg of vitamin P substance and $2{\cdot}5 \times 10^{-6}$ M of cystein in 10 ml of buffer). The dosage of the thiol groups of cystein has been done by the polarographic method (sensitivity 1×10^{-8} A/mm; tension range $+$ 0·25 to $-$ 1·75 V).

Analysis of the results

The results presented in Table 1 are mean values $\pm$ their standard errors. We have applied a variance analysis and compared the results obtained in the presence of vitaminic substances with the controls. An asterisk after an average means that it is significantly different from the control mean ($P < 0{\cdot}05$), and two signify that this difference is highly significant ($P < 0{\cdot}01$).

TABLE 1. INFLUENCE OF DIFFERENT VITAMIN P SUBSTANCES ON THE RELEASE OF CATHEPSINS (ARBITRARY UNITS) IN A BUFFER pH 7·4 AFTER 40 MIN OF INCUBATION AT 37° WITH (= TOTAL) AND WITHOUT (= FRAGILIZATION) TRITON X-100

Substances	*N*	Total	Fragilization
Control	8	78·25 ± 3·54	57·63 ± 1·14
Rutin	8	82·50 ± 3·11	25·38† ± 1·27
Tri(hydroxyethyl)rutosid	8	83·50 ± 3·89	23·50† ± 2·28
Magnesium flavonic chelates	8	72·00 ± 3·22	20·38† ± 0·27
Anthocyanosides	8	81·75 ± 3·41	65·50* ± 1·78

* The difference from the control is statistically significant.
† The difference from the control is highly significant.

* These measurements have been made in the Laboratory of Toxicology of the University of Liège.

RESULTS

The results given in Table 1 are averages of eight tests. There is no statistically significant difference between the total cathepsin activities of the control samples and the samples containing vitamin P substances. This shows that in our experimental conditions these substances have only a minor direct influence on the measured enzymatic activity. On the other hand the enzymatic activity of samples not exposed to the action of Triton X-100 is plainly weaker in the presence of vitaminic substances derived from flavone than that of the control samples, which suggests that these substances exercise a stabilizing effect on the lysosomes in our chosen experimental conditions. Contrary to the flavonoids, the anthocyanosides show no stabilizing effect on the lysosomes in our experiments. Figure 1 gives the cathepsin activities in the supernatant in percentage of the total lysosomal activity as explained in Methods. Presented in this way the results are not exactly superposable to those given in Table 1, since they are not compared to the control samples, but to their own total activity. Their general trend however, is the same.

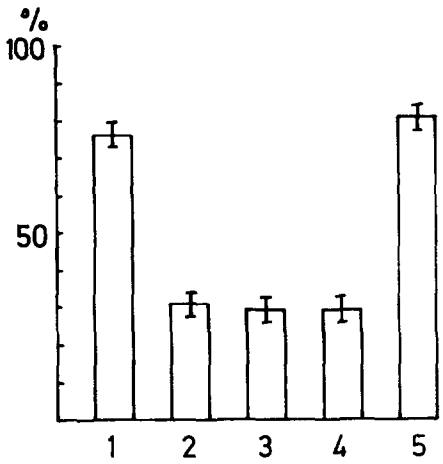

FIG. 1. Cathepsin activity in the supernatant in percentage of the total activity (= 100%). 1: control; 2: rutin; 3: tri(hydroxyethyl)rutosid; 4: flavonic magnesium chelates; 5: anthocyanosides.

Since the membrane of the lysosomes as well as other cellular membranes is sensitive to the action of substances reacting with thiol groups, we have examined the ligant power of vitamin P substances for Cu^{2+}. Cu^{2+} reacts with —SH[6] and fragilizes the lysosomes in our experimental conditions. At the proportions given in the Methods there is hardly any free Cu^{2+} left in the presence of rutin ($2{\cdot}5 \times 10^{-7}$ M) and of tri(hydroxyethyl)rutosid (3×10^{-7} M). The formation of the complex is very rapid. The anthocyanosides on the other hand show little activity (in the same conditions there remain 5×10^{-6} M Cu^{2+}). In the preparation itself we find Cu^{2+}, which is confirmed by dosage with the atomic absorption spectrophotometer (at least 0·1 γ/mg), while the other products do not contain any. We had to give up the study of the ligant power of Flacitran®, this preparation giving a polarographic wave close to

that of copper and interfering with it, but as far as we could see, its chelating power is equal to that of the other two flavonoids.

If Na_2 EDTA (1 mM) is added to the sucrose and to the phosphate buffer, pH 7·4 in the labilization test, the stabilizing effect of rutin and of vitamin P_4 disappears, but the action of Flacitran® and the anthocyanosides on the stability of the lysosomes remain unchanged (Fig. 2).

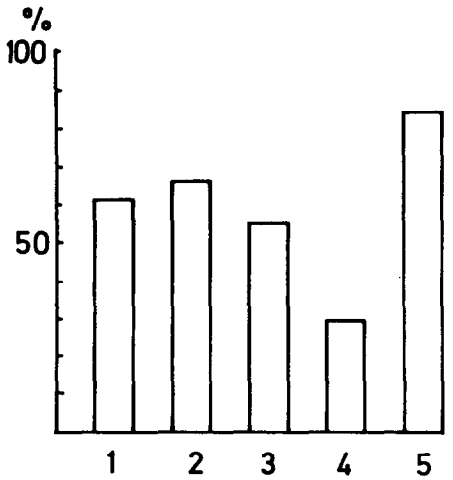

FIG. 2. Labilization test in presence of Na_2 EDTA (1 mM). Same legend as for Fig. 1. The results are averages of four tests.

After incubation with cystein, $2{\cdot}5 \times 10^{-6}$ M, in the presence of a vitamin P substance in the phosphate buffer during 40 min all the cystein has disappeared in the presence of anthocyanosides, while there remain $0{\cdot}78 \times 10^{-6}$ M with rutin, $1{\cdot}1 \times 10^{-6}$ M with vitamin P_4 and $0{\cdot}74 \times 10^{-6}$ M with Flacitran®.

If we compare the cathepsic activity of a supernatant rich in cathepsins dissolved in sucrose-tris (see Methods) and put into contact with the same volume of granular suspension with or without addition of rutin, no difference appears after centrifugation. Therefore rutin does not seem to modify in an appreciable way the distribution of the enzyme between the supernatant and the granular fraction precipitable by centrifugation. Furthermore rutin itself at the given concentration during the enzymatic digestion does not influence directly the cathepsic activity during digestion, a fact which is already revealed by the comparison of the total activities in the presence and absence of vitaminic substances on Table 1.

DISCUSSION

The derivatives of flavone introduced in our study exert a stabilizing effect on the lysosomes, and Fig. 1 shows that the three drugs seem to be about equally active. In this, our results are different from those obtained *in vivo* by other authors,[7] who observed that tri(hydroxyethyl)rutosid was more active than rutin on the capillary resistance *in vivo*. On the other hand we have found no stabilizing action of the anthocyanosides, though the substance seems to be active *in vivo*.[8,9] There seems therefore

to be no correlation or parallelism between the stabilizing power on the lysosomes *in vitro* and the tests usually applied *in vivo* to show a vitamin P action on the capillary blood-vessels.

The fact that the anthocyanosides show no activity in our experiments, though they appear to be active *in vivo*, could be due, at least partly, to a lack of solubility, though this seems hardly probable, since as shown in Table 1, there is rather an increase of fragility than an absence of action. Another hypothesis would be that the inactivity of the anthocyanosides could be connected with the absence of ligant power of these substances *in vitro*. Under these conditions they could not protect the integrity of the membranes against the thiol-inhibition and catalytic action of the traces of metals such as iron and copper.

To this end, the labilization test in the presence of Na_2 EDTA was applied. If the vitamin P substances owe their stabilizing power to their ligant power, it should disappear in the presence of Na_2 EDTA. That is precisely what is found with rutin and vitamin P_4, but not with Flacitran® and the anthocyanosides, which in spite of the addition of Na_2 EDTA preserve their respective stabilizing and labilizing power in comparison with the controls (Fig. 2). But as pointed out before, these two products are not of a uniform composition.

There may perhaps be a connection between the increase of the lysosomal fragility even in the presence of Na_2 EDTA and the particularly strong action of the preparation containing anthocyanosides on the —SH groups.

Acknowledgements—We express our thanks to Mrs. M. de Bruyn for her skilful technical assistance. We also thank the firms Zyma-Galen (Brussels), the Laboratoires Français de Thérapeutique (Bordeaux) and Chibret (Clermont-Ferrand), who kindly provided us with respectively tri(hydroxyethyl)rutosid, magnesium flavonic chelates and anthocyanosides of *Vaccinium Myrtillus*. Grants from the "Fonds de la Recherche Scientifique Médicale" are gratefully acknowledged.

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