SPECTROPHOTOMETRIC DETERMINATION OF STABILITY OF ANIMAL TISSUE LYSOSOMES AND ITS pH DEPENDENCE

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In the pathogenesis of myocardial infarction an important role is ascribed to the lysosomal apparatus of the cardiomyocytes [1]. The basic assumption of the "lysosomal" hypothesis of myocardial infarction [7] is that the pH level is a leading factor in the labilizing action of ischemia on lysosomes. A fall of pH induces more intensive destruction of lysosomal membranes, with release of hydrolytic enzymes into the cell cytosol. This leads to abnormal hydrolysis of cell structures, and this determines and intensifies the process of tissue necrosis. This explains the importance of quantitative assessment of the sensitivity of lysosomes to a change in pH of the medium, but difficulties arise because of the lack of any rapid and direct method of determination of the structural integrity of these particles. Such an assessment is carried out indirectly, by determining the ratio between activities of different forms of marker enzymes [2]. The level of nonsedimented or latent forms of enzyme activity as a fraction of the total, expressed as a percentage, is taken as the measure of integrity of the lysosomes. The main disadvantages of this method are the long duration and laboriousness of the analysis, the use of expensive reagents in short supply, and the low level of specificity of the determination. The last of these features is due to the fact that a change in pH of the medium may lead to solubilization of membrane-bound hydrolases without destruction of the lysosomes [2]. The results of calculation of the balance between forms of the enzyme in this case will be distorted. Moreover, sensitivity of different enzymes to a change in pH of the medium differs. This makes it impossible to compare results obtained with different acid hydrolases.

The aim of this investigation was to develop a method of estimating the sensitivity of lysosomes, isolated from animal tissues, to the action of acidity of the medium, which will be free from the defect mentioned above.

EXPERIMENTAL METHOD

Intact noninbred male albino rats weighing 120-180 g were used in the experiments after starvation for 48 h. The liver was taken from the decapitated animals, perfused on ice with 0.14 M NaCl, wetted with filter papers, carefully freed from connective tissue, and weighed. A homogenate was prepared in the ratio of 1:10 (weight of tissue to volume of homogenization medium). Homogenization was carried out in a Potter–Elvehjem glass homogenizer with Teflon pestle (gap 0.21 mm) for 90 sec at 1200 rpm in 0.33 M sucrose solution, pH 7.4, containing 1 mM EDTA. All manipulations with the tissue were carried out at 0-4°C. An enriched lysosomal fraction was obtained by differential centrifugation [6] and resuspended in 0.7 M sucrose, pH 7.0, in the ratio of 1:0.2 (wet weight of tissue to volume), to a concentration of lysosomal protein of 5 mg/ml, then kept at 0-4°C and used as the initial suspension.

Structural integrity of the lysosomes was judged by the change in absorption of their suspension [3]. The initial suspension was added to a solution of phosphate-citrate buffer, previously heated to 37°C, in the ratio of 1:(100-150) (v/v), so that the initial extinction was about 0.4. This value was taken as 100% absorbance. The degree of lysosomal destruction corresponded to the fall of optical density of the samples expressed as a pecentage of this initial value. Changes in extinction were recorded continuously during incubation for 3 min

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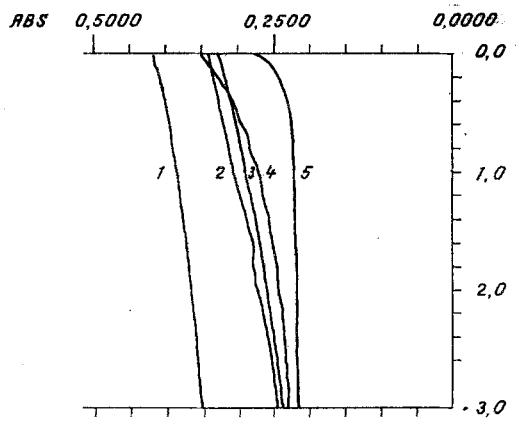

Fig. 1

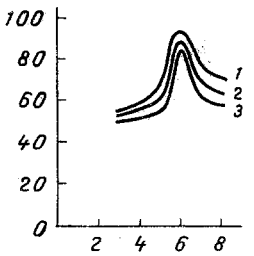

Fig. 2

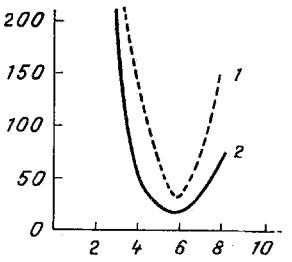

Fig. 3

Fig. 1. Initial, serial recording of change in A_{520} of suspension of intact lysosomes depending on incubation time and acidicity of incubation medium. Abscissa, optical density (A_{520}) of lysosomal suspension measured at 520 nm (in relative units); ordinate, incubation time (in min). pH of incubation medium; 1) 6.0, 2) 7.2, 3) 8.0, 4) 5.0, and 5) 3.0.

Fig. 2. Optical density of suspension of intact lysosomes as a function of pH of incubation medium, at different times of incubation. Abscissa, pH of incubation medium; ordinate, percent of initial value of A_{520}. Incubation time of samples: 1 min (1), 2 min (2), and 3 min (3).

Fig. 3. Dependence of rate of destruction of lysosomes on H^+ concentration in incubation medium and on times of keeping initial suspension at 0-4°C. Abscissa, pH of incubation medium; ordinate, rate of fall of A_{520}, expressed in ΔA_{520}/min. Samples kept at 0-4°C for 16 h (1) and 40 h (2).

in a thermostatically controlled cuvette (diameter 1 cm) at 520 nm on a computerized recording spectrophotometer ("Beckman"), controlled by a "Time Drive" program. The blank (background) sample contained the same components, except lysosomes. The rate of destruction of the lysosomes was determined as the rate of decrease of absorbance at 520 nm (ΔA_{520}/min), controlled by a "Kinetics-II" program during incubation of the samples under the same conditions but for 1 min. The buffer mixture was made up in 0.6 M sucrose containing 1 mM EDTA. The pH varied from 3.0 to 8.0.

EXPERIMENTAL RESULTS

Changes in optical density of the lysosomal suspension during incubation in medium at different pH values for 3 min are shown graphically in Fig. 1. Since the initial values of A_{520} for plotting the graphs did not coincide, for convenience of analysis changes in A_{520} with time were expressed as percentages of the initial value. It will be clear from Fig. 1 that the process of lysosomal destruction under these conditions is linear in character at pH 6.0. A rise or fall of the H^+ concentration in the incubation medium caused sharp change in the character of this process. It became biphasic. Thus during the first 30 sec of incubation, lysis of the lysosomes took place rapidly, but later more slowly. The most significant changes in the structural integrity of the lysosomes occurred at pH 3.0. Under these conditions lysis of the lysosomes reached its maximal possible level (50%) after only 30 sec. Dependence of the degree of destruction of the lysosomes on acidity of the medium is shown in Fig. 2. It is complex in character. The apex of the "bell" defines the range of pH values within which the lysosomal suspension is most stable. Lengthening the incubation time narrows this interval of pH values. The sharpest changes in resistance of the lysosomes to acidity of the medium occurred during a change of pH from 6.0 to 5.0, and rather less, at pH 6.2-7.0. At the boundary values of the pH range chosen for investigation (from 5.0 to 3.0 and from 7.0 to 8.0) differences in the degree of damage to the lysosomes were smoothed out, and the value of this parameter tended toward the limiting value of 50%. This rule was manifested more clearly in the dependence of the rate of lysis of lysosomes on pH of the incubation medium (Fig. 3). The minimal rate of destruction of lysosomes occurred within a narrow interval of pH values and increased with a change in pH from 6.0 to 3.0 or to 8.0. It is also clear from Fig. 3 that after additional long-term incubation (24 h) at 0-4°C (Fig. 3, 2) the

sensitivity of the lysosoms to acidity of the incubation medium fell throughout the range of the artificial pH interval.

The relationships characterizing acid resistance of isolated intact lysosomes thus obtained are relatively simple and sufficiently informative. For instance, it follows from Fig. 1 that after incubation of a suspension of lysosomes for only 30 sec under the conditions specified, easily measurable changes in A_{520} can be obtained. This shortens the total time of analysis compared with the enzymic method from 5-6 h to 30 min. The measurements can be made with the instrument working under the two conditions specified and acid resistance of the lysosomes can be estimated as a percentage of the initial value of A_{520} (Figs. 1 and 2) or relative to the initial rate of destruction (Fig. 3). For comparative estimation of the action of different physicochemical factors on lysosomes in vivo and in vitro, an index such as the biochemical half-period ($t_{1/2}$) also can be used. It can be calculated from data in Fig. 1 as the time during which the number of lysosomes is reduced by half. The fact that a limiting value of the degree of destruction of the lysosomes exists in Fig. 2 indicates that the residual value of A_{520} is determined by fragments of lysosomal membranes. On the addition of Triton X-100 to the samples, the value of A_{520} is zero. Consequently, the mechanism of destruction of lysosomes on a change in acidity of the medium is osmotic and not detergent. In the enzymic method sensitivity of lysosomes to a change in pH of the medium is measured by incubating a suspension of lysosomes at 37°C in acetate buffer, pH 5.0, for 15-60 min [2]. It will be clear from Fig. 2 that under these conditions there are virtually no intact lysosomes. In that case, consequently, it is not the parameter of integrity of the lysosomes that is measured, but individual sensitivity of acid hydrolases to the solubilizing action of H^+.

The results (Figs. 2 and 3) confirm the "lysosomal" hypothesis of the pathogenesis of myocardial infarction. Acidification of the medium leads to lysis of lysosomes. The time course of this process (Fig. 2) suggests that changes in the state of the lysosomal apparatus in myocardial ischemia precede irreversible morphological disturbances [4, 5], i.e., that they are primary relative to them.

The sensitivity of lysosomes to a change in pH of the medium can be used as a dynamic indicator of the state of the lysosomes under the influence of various physicochemical factors, both in vivo and in vitro. By varying the pH of the incubation medium it is possible to estimate the force of the labilizing (at pH 6.0) and stabilizing (at pH 3.0) action of lysosomotropic substances, and also to determine the character of the action (stabilization or labilization) of chemical preparations at pH values of 5.0, 7.2, or 8.0. Expressing the change in A_{520} and the value of ΔA_{520}/min as a percentage can also be used as the measure of such action.

Compared with the enzymic method, the method proposed above significantly shortens the time of analysis and reduces its laboriousness, which saves expensive reagents, and increases the specificity and universality of the determination.

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