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THE ACTION OF THERAPEUTIC ULTRASOUND ON THE CATALYTIC ACTIVITY OF THE DISSOCIATING ENZYMES

N. A. ROZANOVA, E. P. CHETVERIKOWA

Institute of Biological Physics, Academy of Sciences of the USSR
(Pushchino, 142292, USSR)

The action of low intensity therapeutic ultrasound (0.88 MHz; 0.05–1.0 W/cm^2) on the activity and dissociation of the enzymes with labile quarternary structure was investigated (creatine kinase – CK, pyruvate kinase – PK, hexokinase – HK and urease). It was shown that ultrasound does not affect the activity of these enzymes in solution. Dissociation of CK was activated by ultrasound when equilibrium forms in solution were not reached. Activation of the rate of the catalytic reaction in the case of immobilized HK was also obtained. This effect may consist in enhancement of the reaction product transport from the bottom of the experimental cell up to where the aliquote volume was replaced for measuring.

1. Introduction

In the investigations of the mechanisms of the biological action of low intensity therapeutic ultrasound it is important to reveal the primary targets. In the publications of BELEVA-STAIKOWA [1] and POSPISHILOVA [7] changes of activity of different enzymes *in vivo* under the action of therapeutic ultrasound were reported. However, in these publications it is impossible to separate the primary and the secondary effects of sonication and consider the possibility of direct action of ultrasound on the enzyme itself.

The work of DUNN and MC LEOD published 15 years ago was the first investigation devoted to the problem whether enzymes in aqueous solutions and enzymic reactions can be affected by noncavitation ultrasound [3]. They showed that a wide range of ultrasonic frequencies and intensities had no effect upon the catalytic rate of five purified enzymes *in vitro* (α-chymotrypsin, trypsin, lactate dehydrogenase, aldolase and ribonuclease). These enzymes have stable quarternary structure. The conditions of the experiments were not va-

ried: concentrations of enzymes, its substrates and pH were constant. The latter is especially important, because the enzymic reaction, in some conditions, can be more sensitive than the enzyme molecule. Besides, there is a special group of dissociating enzymes having very labile quarternary structure which was not investigated. Without investigation of these sensitive and important enzymes in a wide enough range of conditions, it is impossible to make a final decision about the absence or existence of direct action of ultrasound on the enzymic reactions.

Dissociation of these labile enzymes is accompanied by significant changes in catalytic activity. The property of dissociating enzymes to change their catalytic activity according to the oligomeric state is the basis of one of their regulation mechanisms [6]. The change of activity occurs by allosteric mechanism. The allosteric effectors are known: pH, metabolites, changes of the protein concentration and ionic strength [6]; these are very weak factors. Fig. 1

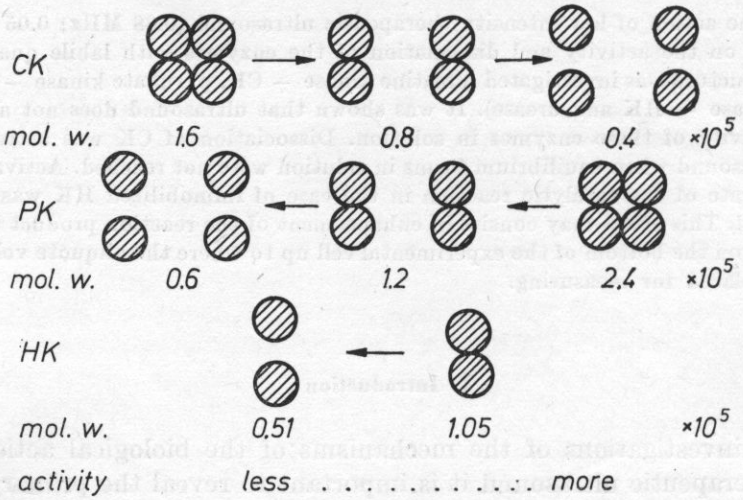


Fig. 1. Two ways of the enzyme dissociation. CK — creatine kinase dissociation accompanied by a high increase in catalytic activity; PK, HK — pyruvate kinase and hexokinase dissociation accompanied by loss of enzyme activity in monomeric form

shows two ways of dissociation of enzymes: creatine phosphokinase (CK), pyruvate kinase (PK) and hexokinase (HK). Dissociation of PK and HK is accompanied by a decrease in their activity; monomeric form has no activity at all [5, 4]. Dissociation of CK is accompanied by a high increase in catalytic activity: monomer is hundred times more active than tetramer [8].

The purpose of our work was to investigate the action of low intensity therapeutic ultrasound on dissociating enzymes: CK, PK, HK and urease.

2. Materials and methods

a. Enzymes and methods of measuring their activity

1. *Creatine kinase* (E.C.2.7.3.2) was purified from rabbit skeletal muscle by the method described by CHETVERIKOVA [2]. The rate of the enzymic reaction was registered by measuring pH changes in a typical reaction mixture consisting of 5 mM ATP, 6 mH magnesium acetate, 36 mM creatine and 2 mM Tris buffer maintaining the initial pH at 8.35.

2. *Pyruvate kinase* (E.C.2.7.1.40) was purified from rabbit muscle and supplied by "Reanal". The rate of reaction was estimated by pH changes. The reaction mixture consisted of 3 mM ADP, 5 mM magnesium acetate, 0.7 mM PEP, 100 mM KCl, 1.3 μg/ml of protein and 2 mM Tris buffer to maintain the initial pH of 7.8.

3. *Hexokinase* (E.C.2.7.1.1) from yeasts was purchased in purified form from "Fluka" and "Boeringer". The concentration of ATP in the reaction mixture was varied between 0.3 and 3.0 mM. Other components were 15 mM glucose, 5 mM magnesium Acetate and 2 mM Tris buffer. The rate of this reaction was measured by pH changes and spectrophotometrically with glucose-6-phosphate dehydrogenase of NADPH absorbance at 340 nm. This reaction mixture consisted of 66 mM glucose, 3 mM ATP, 6 mM magnesium acetate, 0.3 mM NADP, 2.5 μg/ml HK and 0.5 μg glucose-6-phosphate dehydrogenase. The aliquote of the solution was stopped by addition of 50 mM EDTA in 0.5 N NaOH and then changes in absorbance at 340 nm were measured. Hexokinase was also supplied by "Sigma" in an immobilized form lined to agarose.

4. *Urease* (E.C.3.5.1.5) was obtained from a water-melon as a tissue preparation in lyophilised form. This enzyme was kindly suppied to us by Prof. KURGANOV. The reaction mixture consisted of 0.8 per cent urea in 2 mM Tris buffer, pH 7.5. The rate of the reaction was measured by pH changes.

b. Characteristics of acoustic system

The experiments was performed in two chambers.

Chamber I. The measuring cell was placed in a thermostated box containing a reflector and absorber for ultrasound (Fig. 2*a*). It was made of lucite and had a very thin glass window transparent to ultrasound. Irradiation was performed by using a commercial therapeutic device with a piezoceramic transducer with a 1.5 cm diameter and a frequency of 0.88 MHz within the intensity range 0.05 —0.7 W/cm^2. The distance between transducer and measuring cell was 3 cm. The ratio of the spatial peak to the spatial averaged intensity over the irradiated surface of the cell was about 3. Samples were sonicated during catalytic reaction.

Chamber II. This chamber is shown in Fig. 2*b*. The sample for sonication was placed in a vertical cylindrical lucite tube with a thin glass bottom for ultrasound. This chamber was thermostated as well and samples were sonicated in it before contact with the reaction mixture.

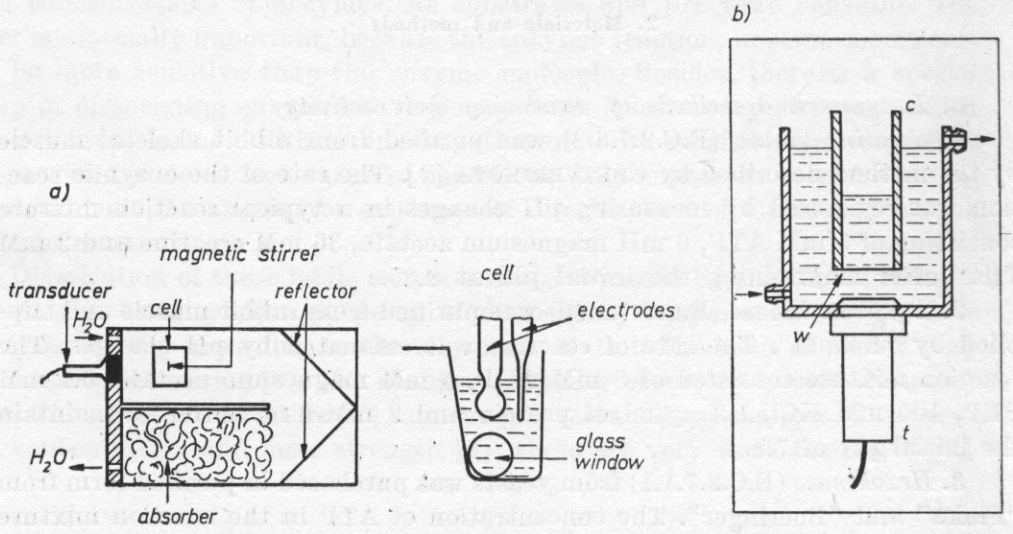


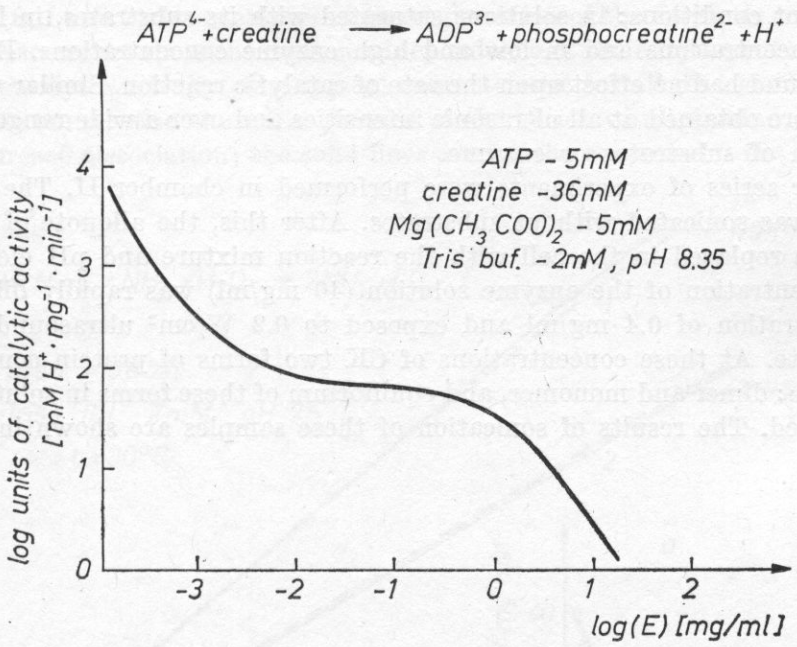
Fig. 2. Schematic diagrams of the chambers for sonication: *a* — chamber I for sonication during catalytic reactions; *b* — chamber II for the preliminary sonication, *c* — cell for the sample, *w* — window for ultrasound, *t* — transducer

3. Results and discussion

The dissociating enzymes are characterised by nonlinear dependence of enzymic activity on the protein concentration. Our results on the dissociation of cytoplasmic CK are shown in Fig. 3. This dissociation is caused by dilution of the enzyme solution. In the range of high protein concentration (about a few mg/ml) where the enzyme is mainly in tetrameric form, according to the data of sedimentation analysis, $S^0_{20,w} = 8.7$ (Fig. 3, the first line under the diagram), the activity was low. The decrease of CK concentration is accompanied by an increase in its activity. The plateau in this diagram (Fig. 3) characterises the stable dimer both by catalytic activity and sedimentation data $S^0_{20,w} = 5.25$. The further substantial increase in activity and the change in the Swedberg coefficient ($S^0_{20,w} = 3.5$) is evidence of dissociation of the dimer and formation of a highly active monomer. The dissociation of the polymeric forms of creatine kinase following dilution takes up to an hour to reach equilibrium.

These samples were sonicated in chamber I during the catalytic reaction. Irradiation of solution with different enzyme concentration and therefore of different quarternary structure of enzyme produced no changes in the catalytic activity (Fig. 4). The left bars in each pair show the activity of the CK in control, and the right bars show the activity in sonicated samples.

Similar negative results were obtained in the case of sonication of the reactions catalysed by PK and HK. The experiments on HK were carried out



sedimentation constant	3.57 ±0.3	5.25 ±0.16	8.7 ±0.6
olygomeric structure	M	D	T
molecular weight ·10^5	0.4	0.8	1.6

Fig. 3. Dissociation of creatine kinase; increase in catalytic activity of creatine kinase

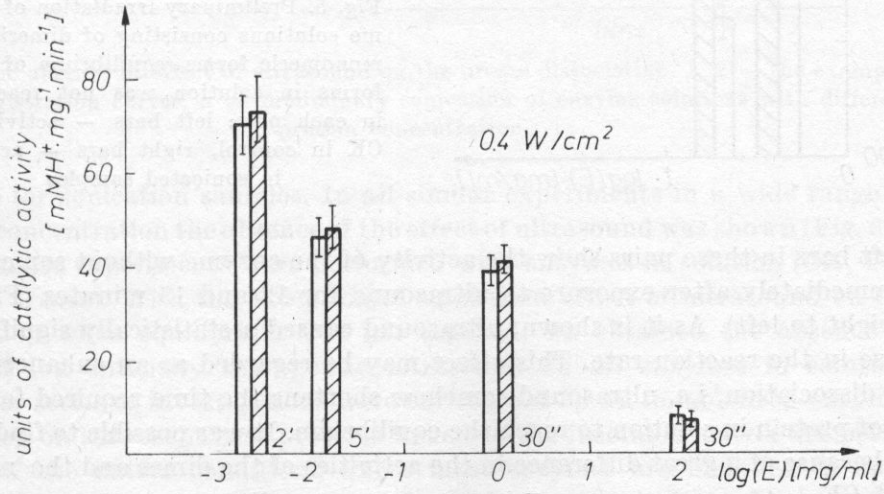


Fig. 4. Irradiation of the reaction mixture with an enzyme in different quarternary structure (equilibrium of forms was reached in every solution); in each pair: left bars — activity of CK in control, right bars — activity in sonicated samples

in different conditions: in solutions saturated with its substrates, in low substrate concentrations and in low and high enzyme concentrations. Exposure to ultrasound had no effect upon the rate of catalytic reaction. Similar negative results were obtained at all ultrasonic intensities and over a wide range of concentration of substrates or enzyme.

Other series of experiments were performed in chamber II. The protein solution was sonicated without substrates. After this, the aliquote of the enzyme was replaced in the cell with the reaction mixture and pH electrodes. The concentration of the enzyme solution (40 mg/ml) was rapidly diluted to a concentration of 0.4 mg/ml and exposed to 0.2 W/cm² ultrasound within one minute. At these concentrations of CK two forms of protein could exist in solution: dimer and monomer, and equilibrium of these forms in solution was not reached. The results of sonication of these samples are shown in Fig. 5.

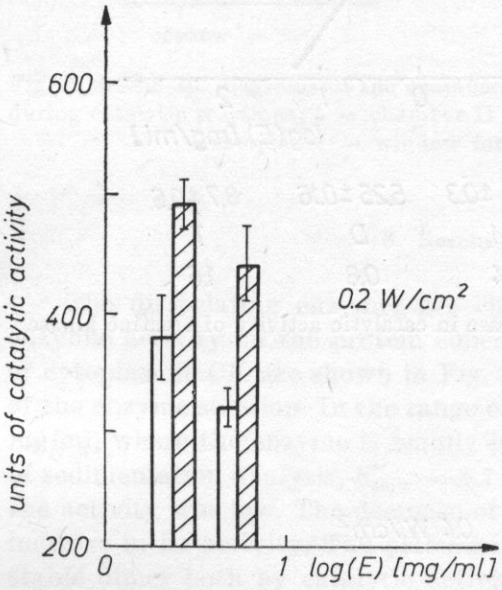


Fig. 5. Preliminary irradiation of enzyme solutions consisting of dimeric and monomeric forms (equilibrium of these forms in solution was not reached); in each pair: left bars — activity of CK in control, right bars — activity in sonicated samples

The left bars in these pairs show the activity of the enzyme without sonication and immediately after exposure to ultrasound for 12 and 15 minutes (Fig. 5, from right to left). As it is shown, ultrasound caused a statistically significant increase in the reaction rate. This effect may be regarded as an enhancement of the dissociation, i.e. ultrasound somehow shortens the time required for the forms of protein in solution to reach the equilibrium. It was possible to find this effect because of a great difference in the activities of the dimer and the monomer of CK.

The action of ultrasound on urease dissociation was investigated by preliminary sonication in chamber II. The results are shown in Fig. 6. These two

curves (Fig. 6, 1 and 2) are examples of the registration of the catalytic reaction kinetics. These curves show the nonlinear dependence of the enzyme concentration on its catalytic activity — a typical characteristic for all dissociating enzymes. These curves also show the absence of the action of ultrasound on the process of urease dissociation; the solid lines are for the control and the dotted

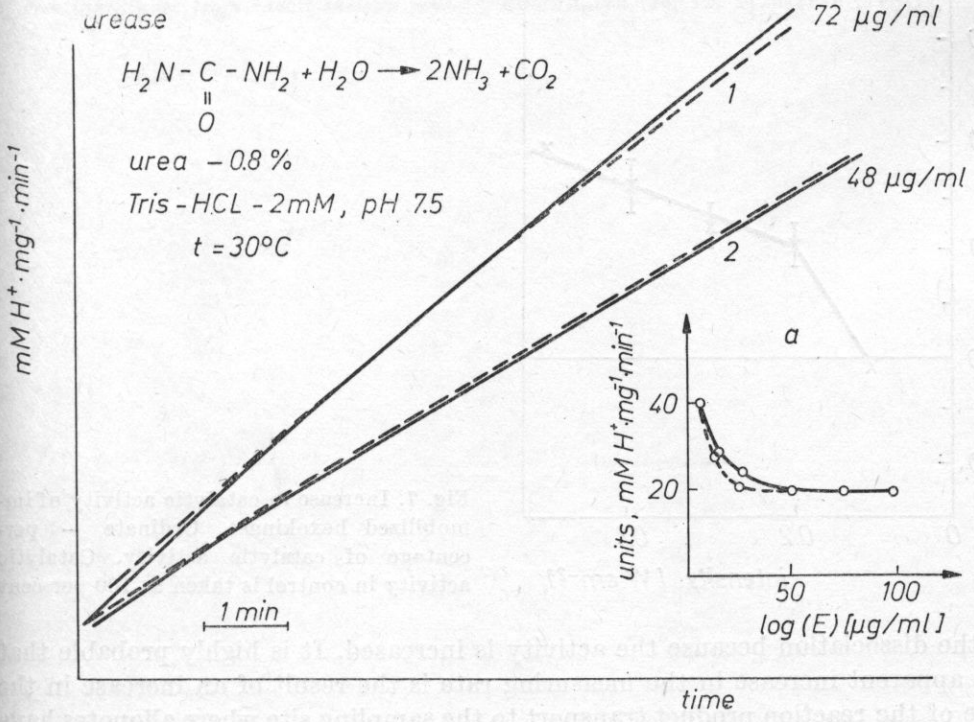


Fig. 6. The absence of effect of ultrasound on the urease dissociation. *1, 2* — the examples of the registration curves, *a* — preliminary sonication of enzyme solutions with different protein concentration

lines are for sonication samples. In all similar experiments in a wide range of protein concentration the absence of the effect of ultrasound was shown (Fig. 6*a*).

All these experiments were performed with enzymes in solution (CK, PK, HK and urease). With the exception of significant effect of ultrasound on CK dissociation when equilibrium was not reached, we obtained the absence of action of low intensity therapeutic ultrasound on all enzymes in solution.

Other series of investigations were carried out on an immobilized enzyme: KH linked on agarose. These proteins linked to the insoluble matrix are better models of the native conditions where most enzymes are included in large complexes in cytoplasm. HK has activity only in dimeric form. If sonication leads to dissociation of this enzyme it must lose the activity. The experiments which were performed in the stable stirring conditions show no effect of ultra-

sound. Ultrasound with the intensities greater than about 0.2 W/cm² caused a small but statistically significant increase in the measured activity of the immobilized hexokinase, but the magnitude of this effect apparently increased with increasing intensity of ultrasound (Fig. 7). These data cannot be a result

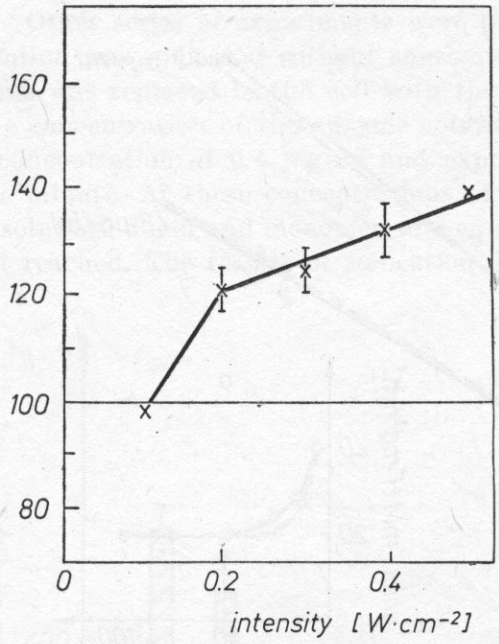


Fig. 7. Increase in catalytic activity of immobilized hexokinase. Ordinate — percentage of catalytic activity. Catalytic activity in control is taken as 100 per cent

of the dissociation because the activity is increased. It is highly probable that the apparent increase in the measuring rate is the result of an increase in the rate of the reaction product transport to the sampling site where aliquotes have been removed for the measuring.

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