ON THE EXISTENCE OF FREE RADICALS IN ENZYMATIC HYDROLYSIS OF ADENOSINE TRIPHOSPHATE

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It was shown in the work /1/ that free radicals emerge during the enzymatic splitting of adenosine triphosphate (ATP) by the contractile proteins of the skeletal muscles. At the same time a whole number of substances are known which act as inhibitors of processes, taking place with the formation of free radicals. In connection with this it was interesting to examine the effect of such inhibitors on the ATP-ase reaction of myosin and actomyosin. Propyl gallate (PG) was selected as the inhibitor. It had been used earlier as an antioxidant during the investigation of biochemical processes /2--5/. The proteins myosin and actomyosin were obtained from the muscles of a rabbit by the method of Szent-Gyorgyi. The reaction of splitting ATP was carried out in a borate buffer with a pH of 9.1 for myosin and 7.0 for actomyosin for a period of 10 minutes at 37°. The concentrations of protein were -- 150 mkg, Ca^{++}-- 7 mkM, KCl -- 0.5 M, ATP -- 2250 mkG. Pg was used in concentrations of 0.1 -- 0.5%. The reaction was arrested by the addition of an equal volume of 5% trichloroacetic acid (TKhK). The intensity of the reaction was judged according to the liberation of non-organic phosphate (P_1) from the ATP, determined from the TKhK filter by the Fiske and Subarrow method. For clearing up the specificity of the effect of PG as an inhibitor of free radical reactions we carried out tests, in which we added to the incubation mixture, in place of PG, equimolecular amounts (10.1--0.4%) of benzoic acid, which as is known does not possess within the structure of its own molecule (4) the properties of an inhibitor of free radical reactions. We also investigated the effect of a synergist of PG -- citric acid (0.3%). Each experiment consisted of three series of tests: The test, where the reaction was carried out in the presence of the substances being studied (PG, citric acid, benzoic acid); a control, where these substances were added following the discontinuance of incubation, and the "blank" test, where the enzymatic processes were discontinued prior to the onset of incubation. The results obtained are presented in figure 1. These facts show that with an increase in the concentration of PG in the test, the formation of P from ATP is reduced, while the depressing effect of PG is increased with the addition of acetic acid. Benzoic acid in equimolecular concentrations does not cause the inhibition of ATP-ase activity. Similar results were obtained in the system of actomyosin + ATP.

The resulting reduction of the ATP-ase activity of proteins could possibly be explained by the fact that PG blocks the intermediate free radical products of the reaction. In this case it can be expected that when all the PG which is

found in the test has been used up in this way, P again begins to accumulate. However, a determination of P after every 2.5 minutes showed that the accumulation of P proceeds only in the course of the first minutes of the reaction, while with an increase in the concentration of PG this time is shortened. The resulting data bring up the thought, is not PG a nonspecific inactivator of the enzyme itself, and the time, during the course of which the reaction is nevertheless going on, is necessary for the inhibition of the active centers. The test, in which the ATP was added following a 5-minute incubation of the protein with PG, showed that in this case P is not formed, and in this way supported our assumption. Following dialysis of the enzyme, formed by PG, in the course of 24 hours at + 4° against 0.5 M KCl the activity of ATP-ase equaled zero.

Thus, PG cannot serve as an index of whether or not the reaction of hydrolysis of ATP by myosin and actomyosin is a free radical one, due to its nonspecific effect on fermentation protein. These data correspond with the results of Emanuel and Neufakh /3/ concerning the oxidizing effect of PG on the SH-groups of proteins.

Diphenyl picryl hydrazyl (DFPG), a stable free radical, was used in certain cases for the fixation reactions, going on with the formation of free radicals /6/. Based on a lowering of the concentration (DFPG) it is possible to judge concerning the quantity of radicals at the moment of fixation. The stated method was used for studying the reaction of hydrolysis of ATP by actomyosin. We added 0.15 ml of a solution of ATP (80 mg/ml) to 1 ml of a saline (0.5 M KCl) solution of protein (concentration 20 mg/ml). In 2 minutes following the onset of the reaction it was fixed by the addition of 1 ml of a solution of DFPG in 96% alcohol (10^{18} particles/ml). In the control we added 0.15 ml of a solution of KCl to the protein, or following fixation the same amount of ATP as in the test. Since in the first minutes following fixation, decolorization and, consequently, a lessening of the concentration of DFPG proceeds rapidly both in the control and in the test (figure 2), apparently due to the reduction of DFPG in the protein (SH-groups), then this mixture was fixed in liquid nitrogen following 10 minutes after the addition of the solution of DFPG, when this process had mainly concluded. In the course of these 10 minutes the tests were vibrated intensively for an uniform mixing. The congealed mixture was ground in a porcelain mortar up to a consistency of fine sand. Three samples were taken of each test and the concentration of DFPG in the liquid nitrogen was measured on an EPR spectrometer.

The results of the measuring are presented in the table. A freshly prepared solution of DFPG was used in each test; each value which is cited in the table for the content of DFPG in the test and in the control represents the average figure for 3--5 tests. Since in the course of the reaction additional SH-groups may be liberated and the concentration of SH-groups substantially influences the result of the test, a check was made of the amperometric titration of SH-groups of /?/$AgCl_2$ in an ammonium buffer. No difference was detected in the contents of the SH-group in the control and the test experiments. Thus, a lowering of the concentration of DFPG may apparently be connected with the formation of free radicals in the reaction of the hydrolysis of ATP by actomyosin.

Literature

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Figure 1. Change of the ATP-ase of myosin depending on the concentration of: 1) propyl gallate; 2) a mixture of propyl gallate + citric acid; 3) benzoic acid.

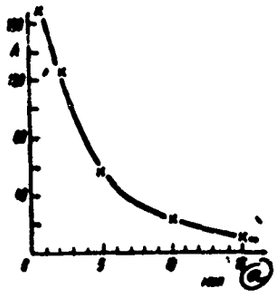

Figure 2. Decrease in the concentration of DFPG (relative units) in the presence of actomyosin over a period of time. a - minutes.

Relative content of DFPG

No.	Test, solution of actomyosin with ATP	Control, solution of actomyosin
1	146±13	333±56
2	107±7	150±14
3	169±17	130±13
4	98±23	225±46
5	42±4	77±6