Modification of Lysozyme Thermostability by Cytochrome c Preparations.* (25547)

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In fractionating rat kidney lysozyme by ion-exchange chromatography, most active fractions contained high concentrations of Cytochrome c(1) subsequently resolved after rechromatography(2). Because of similarities in physico-chemical properties, it was considered that Cytochrome c might affect or imitate the functions of lysozyme in some way. Many basic polypeptides and small peptides exert bacteriolytic activity(3-5). Antibacterial activity has been attributed to hemoglobin and related compounds, including Cytochrome c, which affects oxidizing capacity of *Bacillus subtilis*(6). Kammerer(7) and van Heyningen(8) claimed that mesohematin and hematin, respectively, inhibited bacteria. This report describes 2 effects of Cytochrome c: (a) growth of *Micrococcus lysodeikticus*, frequently used as a substrate for lysozyme, and (b) *in vitro* activity and thermostability of lysozyme.

Materials and methods. Crystalline egg white lysozyme was obtained from Armour Research Labs., Chicago. Two preparations of Cytochrome c were used: beef heart Cytochrome c (Sigma Chem. Co., St. Louis) and horse heart Cytochrome c ("Wyeth Injection Cytochrome c," Philadelphia, 10 mg/ml). The redox state of Cytochrome c was determined by measurement of absorption spectrum in the visible range. Reduction of Cytochrome c was accomplished by excess of $Na_2S_2O_4$. *M. lysodeikticus* (strain 19, Rutgers Inst. of Microbiology) was cultured in liquid media at 28°C and growth followed photometrically at 660 mμ by previously described procedure(9). Cytochrome c or lysozyme was dissolved in sterile water and passed through bacterial filter before addition to culture medium. *M. lysodeikticus* cells used for lysozyme substrate were prepared and standardized by published method(10,11). Lysozyme activity was measured by reduction in turbidity of a bacterial suspension(10) and may be defined as change in percent transmission at 645 mμ during 30 to 60 seconds of reaction in standard assay system (1 unit = change of 1% transmission in this interval) (10). Enzyme activity measurements were replicated to within ± 0.2 units. Following incubation of lysozyme in presence or absence of Cytochrome c at various temperatures for various periods, samples were transferred to ice-water bath, pH was measured, and 0.25 ml aliquot assayed in standard assay system buffered at pH 6.2(10). Heat treatments were performed on aqueous solutions or on solutions buffered by either 0.1 *M* acetate at pH 5.0, or 0.066 *M* phosphate at pH 7.0.

Results. Effect of Cytochrome c upon growth of M. lysodeikticus: A mutational lag phase occurred after lysozyme was added to cultures of *M. lysodeikticus*. Eventually, resistant organisms developed(9). Inclusion of levels of Cytochrome c in cultures of the organism inhibited growth, without a lag, in contrast to results of lysozyme action. Concentrations of Cytochrome c in the culture varied from 0.1 to 0.4 μg/ml. Inhibition of bacterial growth at these levels and extrapolation of Cytochrome c concentration as a function of growth rate (logarithmic phase) revealed that 100% inhibition of growth occurred at Cytochrome c concentration of about 1.1 μg/ml of culture medium. Similar results were obtained if Cytochrome c concentration was expressed as a function of total cellular Kjeldahl nitrogen.

Thermostabilization of lysozyme activity in presence of Cytochrome c: 100 μg of lysozyme were combined with various concentrations of Cytochrome c in an aqueous, unbuffered solution (total volume = 4 ml) and subjected to 100°C water bath for 10 minutes. Results obtained are shown in Fig. 1. The control curve containing oxidized Cytochrome c in-

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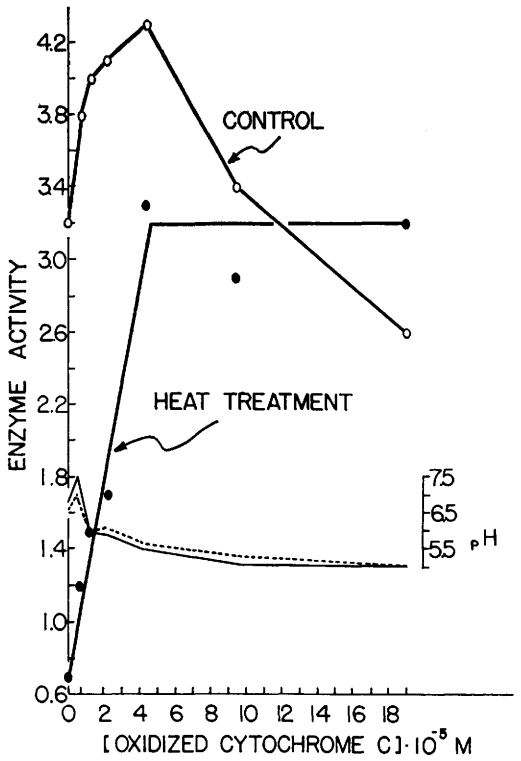

FIG. 1. Thermostabilization of lysozyme activity in aqueous solution by Cytochrome c. Control curve represents enzyme activity resulting from preparations not treated by heat. Heat treatment, 10 min. at 100°C with varying concentrations of oxidized Cytochrome c. pH curves reveal values of systems (after incubation) prior to enzyme assay. Solid curve = control; dashed line = heat treatment. See text for further details of aqueous system.

dicates a stimulation in initial lytic activity over a given concentration range of the pigment. Reduced Cytochrome c also caused a slight stimulation in initial lysozyme activity but was inactive in stabilizing lysozyme to heat. The reasons for inability of the reduced pigment to affect thermostabilization of lysozyme are not clear at this time. The reducing agent, $Na_2S_2O_4$, alone had negligible activity in bacteriolysis. Either oxidized or reduced forms of the pigment failed to produce measurable *in vitro* lysis in absence of lysozyme. The curve of results of heat treatment shows that in similar concentration range of oxidized Cytochrome c, stability of lysozyme activity to heat is increased. The optimal level of Cytochrome c for stimulation or thermostability is about $5 \cdot 10^{-5}$ *M*, assuming molecular weight of 13,000(12); at this point the ratio of Cytochrome c molecules to lysozyme molecules is about 10 to 1. The resulting pH values of the systems, with or without heat treatment, rose and then gradually fell to pH about 5.1. The pH after heat treatment was not greatly different from the unheated control.

Systems containing ratios of about one lysozyme molecule to about 13 molecules of Cytochrome c were utilized to study the thermostabilizing effect of the pigment as a function of temperature and pH following exposure periods of 0 to 90 minutes. Significant protection was afforded by Cytochrome c in incubates buffered at pH 5.0 at 28°C, 37°C and 60°C. Compared to zero time control an element of stimulation appeared combined with the thermostabilizing effect. Results at pH 7 were much less dramatic than systems exposed to heat at pH 5.0.

Discussion. Stimulation of initial lysozyme activity by Cytochrome c may result from increased number of $-NH_3^+$ groups provided in the Cytochrome peptide. Ionized amino groups are essential for lysozyme activity(13). It seems possible that some active sites of lysozyme molecules, involving $-NH_3^+$ groups could be adsorbed to inert sites on the bacterial cell wall. If Cytochrome c also could be adsorbed to inert positions on the cell wall, the total concentration of net effective lysozyme active sites might be increased.

Thermostabilization of lysozyme may result because of similar physico-chemical properties which might confer to Cytochrome c a "buffering" protection effect upon the active site of lysozyme. These ideas represent pure speculation.

The inhibitory effect of Cytochrome c upon bacterial growth and the thermostabilizing or stimulating action of this substance may not necessarily involve the same portions of the cytochrome molecule (*viz.* growth inhibitory effect may reside in the heme portion; stimulation or stabilization may involve the peptidic structure).

Summary. Preparations of Cytochrome c from horse heart or beef heart were potent inhibitors of growth of *Micrococcus lysodeikticus*. Preparations of oxidized Cytochrome c from either beef heart or horse heart increased stability of lysozyme activity to heat under

certain conditions. Cytochrome c reduced by $Na_2S_2O_4$ does not appear to stabilize lysozyme activity to heat. Protection of lysozyme activity by oxidized Cytochrome c is afforded from 28°C to 60°C in solutions buffered at pH 5.0 to 90 minutes. Excellent protection is afforded also to aqueous, unbuffered solutions at 100°C for incubation of 10 minutes. The optimal molecular ratio for exertion of beneficial effects upon lysozyme appears to be about 10 molecules of Cytochrome c to 1 of lysozyme. Under certain conditions Cytochrome c preparations appear to exert a slight stimulation of initial velocity of lysis by lysozyme.

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Effect of Beta-Aminopropionitrile and Cholesterol on Lipids and Aortic S^{35}-Sulphated Mucopolysaccharides in Cockerels. (25548)

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When fed to animals and birds, Lathyrus factor beta-aminopropionitrile (BAPN) results in severe osteoporosis, skeletal deformities, dissecting aneurysms of aorta and various internal hemorrhages(1,2). The toxic action of BAPN is related to defective metabolism of connective tissue and lesions in lathyrism are due to abnormal formation or destruction of mucopolysaccharides of ground substance(3,4). There seems to exist an analogy between vascular lesion of connective tissue noted in lathyrism and some changes in vascular ground substance observed in animals with experimental atherosclerosis. Both histological and biochemical data seem to indicate, that alteration in mucopolysaccharides of connective tissue is involved in entrance of lipids into the arterial wall(6,7,8,9). Because of possible analogy between vascular lesions in experimental atherosclerosis and those noted in lathyrism, we studied the effect of BAPN and cholesterol, added separately or in combination to cockerels' diet, on some lipids and mucopolysaccharides of the aorta.

Materials and methods. Cockerels of White-Rock Broiler strain used for this study were obtained from a commercial hatchery when 8 days old and kept, in groups of 8 to 10, in thermostatic brooders. Experimental diets were supplied from 16 days of age. The BAPN-containing diet was prepared by drying a 1% aqueous solution of synthetic beta-aminopropionitrile fumarate (Abbott Laboratories) on a weighed amount of commercial chicken starter. The concentration of 0.025% of BAPN in the diet was used. The cholesterol diet contained 2% of cholesterol and 5% of olive oil. The following 4 diets were given to 4 groups of birds during a 4 week period: 1. Basal diet (normal control); 2. BAPN diet; 3. BAPN + cholesterol diet; 4. Cholesterol diet. Isotope was administered 24 hours before birds were killed. Radiosulphate (S^{35} in H_2SO_4, Atomic Energy, Canada) was injected subcutaneously in dosage of 1 mc/kg of bird's body weight. The