# Activation of Creatine Phosphokinase by Sulfhydryl Compounds in Normal and Muscular Dystrophy Sera.* (29933)

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In recent years the determination of serum creatine phosphokinase (CPK) activity has become a very useful diagnostic tool in discriminating between normal subjects and subjects with muscular dystrophy because of its superiority in this respect to all other serum enzymes thus far studied(1-5).

There are conflicting reports regarding the requirement of a sulfhydryl compound in the reaction mixture for a reliable estimation of CPK(6-9). However, it has recently been shown that addition of a sulfhydryl agent to the assay medium ensures a reliable and true determination of serum CPK(10). In the course of studies on CPK we could extend the previous observations(10), thus this report is a further confirmation of the use of sulfhydryl compounds to enhance the activity of this enzyme and give more reproducible results.

*Materials and methods.* Eleven normal sera were analyzed from males ages 40 to 75 years. All persons were without evidence of muscular or neuromuscular disorders or other serious disease. Fifteen pathological sera were studied, 13 from young boys with Duchenne muscular dystrophy, one child with juvenile limb-girdle dystrophy and one adult with a congenital neuromuscular atrophy.

Anterior thigh and cardiac muscles were obtained at autopsy within 10 hours after death from a person with a disease other than that of the neuromuscular system. The preparation of tissue extracts was done as described previously(11).

Fresh solutions of cysteine hydrochloride and reduced glutathione were used after neutralization with sodium bicarbonate.

CPK measurements of sera and muscle extracts were based upon the spectrophotometric method described by Tanzer and Gilvarg (12). A Unit of CPK is defined as that amount of enzyme which will catalyze the transformation of one μmole of substrate/1000 ml of serum or tissue extract in one minute at 25°C and one mU (milli-unit) is equivalent to 1/1000th of a Unit.

*Results. Effect of various sulfhydryl compounds.* It can be seen from Table I that all the sulfhydryl compounds tested, *e.g.*, mercaptoethanol, cysteine and reduced glutathione (GSH) are almost equally efficient in enhancing serum CPK activity. The optimum concentration of the sulfhydryl agent for the activation of serum CPK has been found to be $7 \times 10^{-4}$ *M*.

*Effect of freezing and thawing.* Storage of serum for 4 weeks at —20°C causes about a 30% decline in enzyme activity. Table II shows that normal human serum also loses some of its CPK activity after repeated freezing and thawing. On the other hand, when mercaptoethanol is added to the assay system an enhanced and relatively constant value is obtained.

TABLE I. Activation of Serum CPK by Various Sulfhydryl Compounds.

| Addition | CPK (mU/ml of serum) Normal | Dystrophic |
|---|---|---|
| None | .53 | 54 |
| Mercaptoethanol | 2.62 | 250 |
| Cysteine | 2.58 | 268 |
| GSH | 2.49 | 220 |

Sulfhydryl compounds used at a concentration of $7 \times 10^{-4}$ *M*.

TABLE II. Effect of Repeated Freezing and Thawing on Normal Human Serum CPK Activity.

| No. of samples | Date | CPK (mU/ml of serum) Mercaptoethanol (−) | Mercaptoethanol (+) |
|---|---|---|---|
| 1 | 3. 9.64 | .53 | 3.49 |
| | 3.10.64 | .45 | 3.18 |
| | 3.12.64 | .32 | 3.50 |
| 2 | 3. 9.64 | .74 | 3.33 |
| | 3.10.64 | .64 | 3.50 |
| | 3.12.64 | .53 | 3.50 |

Mercaptoethanol used at a concentration of $7 \times 10^{-4}$ *M*.

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TABLE III. Serum CPK Values of Normal and Dystrophic Patients Before and After Mercaptoethanol Stimulation.

| Disease | No. of persons | CPK (mU/ml of serum) Mean value and range: Mercaptoethanol (−) | Mercaptoethanol (+) |
|---|---|---|---|
| Normal | 11 | .63<br>.21–2.01 | 3.23<br>1.06–9.33 |
| Duchenne MD | 13 | 67<br>19–140 | 323<br>90–624 |
| Juvenile limb-girdle | 1 | 66 | 360 |
| Congenital neuromuscular disease | 1 | 1.0 | 4.3 |

Mercaptoethanol used at a concentration of $7 \times 10^{-4}$ *M*.

TABLE IV. Stimulation of CPK Values in Crude Extracts of Human Muscles with Mercaptoethanol.

| Extracts obtained from | CPK (U/g of wet tissue): Mercaptoethanol (−) | Mercaptoethanol (+) |
|---|---|---|
| Anterior thigh | 5.3 | 16.4 |
| Heart | 1.27 | 6.68 |

Mercaptoethanol used at a concentration of $7 \times 10^{-4}$ *M*.

*Values in normal and dystrophic sera.* Despite the fact that many dystrophic sera contain very high levels of CPK the activities of this enzyme are stimulated 5 to 6 times by addition of mercaptoethanol in both normal and dystrophic sera (Table III).

*Values in crude muscle extracts.* It can be seen from Table IV that even the enzyme activities of crude extracts from normal human thigh muscle and heart are stimulated by mercaptoethanol almost to the same degree as that observed with the serum enzyme.

*Discussion.* The results reported here show that in normal human serum CPK remains in a partially inactivated form and it could be activated by addition of a sulfhydryl agent like mercaptoethanol. In fact, mercaptoethanol enhances the serum enzyme by about 5 times. The enzyme present in the crude extract of normal human skeletal muscle or heart also behaves in a similar manner. The inactivation of CPK might either be due to the presence of a sulfhydryl blocking agent or to the susceptibility of thiol groups of the enzyme to spontaneous oxidation to -S-S bonds. It has been reported previously(8) that addition of sulfhydryl compounds to the reaction mixture increases accuracy and reproducibility in measuring human serum CPK, but no increase in the activity was reported. Only very recently Okinaka *et al* (10), using a different method of CPK estimation, observed that the serum CPK activity could be activated 2 or 3 times by sulfhydryl agents but in contrast to our findings they did not notice any significant stimulation of enzyme activities of crude extracts from human skeletal muscle. It is interesting in this connection that Nichol(13) has found recently that sulfhydryl compounds enhance the activity of muscle CPK in both dystrophic and control mice but curiously enough, she has not observed any significant activation of mouse serum CPK.

Dialysis of human serum for 20 hours with constant stirring in the cold against 100 volumes of distilled water does not have any effect on CPK activity, which implies that low molecular weight dialyzable compounds do not act as CPK inhibitors. One significant fact that emerges from this report is that in order to have a reliable and reproducible serum CPK activity one should add a sulfhydryl compound to all assay media. The sera obtained from patients with Duchenne muscular dystrophy behave exactly as do those of controls.

*Summary.* CPK activity of sera from normals and subjects with myopathies was enhanced about 5 times by mercaptoethanol or other sulfhydryl compounds. The enzyme present in crude extracts from normal human skeletal muscle and heart behaved similarly in this respect. Addition of a sulfhydryl compound to the assay medium resulted in increased accuracy and reproducibility in measuring the true value of serum CPK.

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## Studies of Vaccinia Hemagglutinin Obtained from Various Vaccinia Infected Tissues. Density Gradient Centrifugation and Electron Microscopy.* (29934)

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Because of its role as a by-product of viral infection and its properties as a lipoprotein, vaccinial hemagglutinin (VHA) has attracted the interest of several investigators. VHA was first described by Nagler(1) and subsequently the dissociation of VHA from infective vaccinia virus was demonstrated by adsorption onto red cells(2), centrifugation(2, 3), or by column chromatography(4). It has been characterized as a lipoprotein(3,5) and its density estimated as 1.1 and its size as 65 m$\mu$(3). Stone(5) noted that lipids extracted from non-infected tissues would also agglutinate chicken red cells sensitive to VHA. Since only red cells from certain chickens were affected, she termed such cells "lipid sensitive" and the general phenomenon "lipid hemagglutination."

VHA can, however, be differentiated serologically from normal tissue lipid hemagglutination using specific immune serum(3,5). VHA is inhibited by specific immune serum; normal tissue lipid hemagglutination is inhibited by certain serum or tissue components which occur in some pathologic conditions(6,7,8). Lecithinase or snake venom phospholipase will destroy the activity of VHA and normal tissue lipid. The hemagglutinating activity of both is relatively heat stable at least in crude preparations.

Gillen *et al*(9) reported the occurrence of 2 viral specific particles, both with hemagglutinating activity and separable by filtration or high speed centrifugation. They did not differ serologically but were unlike in that the filterable particle which was non-sedimentable at 17,000 RPM was destroyed after a short incubation at 56°C. Briody(10) suggested that these differences were attributable to the degree of aggregation in each preparation and not to some inherent difference.

Youngner and Rubinstein(11,12) have described 2 hemagglutinins in infected chorioallantoic membrane separable by an ultracentrifugal flotation technique. The first or "viral" hemagglutinin is sedimented at 30,000 RPM and retains its serologic specificity. The second or "soluble" hemagglutinin floats to the surface at this speed (in a medium of 1.063 density) and serologically resembles the lipids of non-infected tissue. The activity of this soluble hemagglutinin is enhanced by dialysis or treatment with trypsin and is heat labile unlike viral hemagglutinin which is unaffected by any of these treatments. It would appear that these 2 hemagglutinins are the same as those originally reported by Stone and Burnet(2,5).

The only published electron micrographs (13,14) purporting to show vaccinial hemag-

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