Low Apparent Creatine Kinase Activity and Prolonged Lag Phases in Serum of Patients with Metastatic Disease: Elimination by Treatment of Sera with Sulfhydryl Agents

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We find lag phases exceeding 20 min in measuring creatine kinase activity, by using the kinetic creatine phosphate → creatine assay, in sera of some patients with carcinoma metastatic to the liver. Such long lag phases are accompanied by a decreased apparent enzyme activity. These problems are eliminated by adding sulfhydryl agents to the serum before assay, but not by adding more of such agents to the assay reagent. β-Mercaptoethanol is superior to Cleland's reagent, glutathione, and cysteine. The long lag phases could not be explained by inadequate activity of the coupling enzymes, interference with the coupling steps, high proportions of cardiac isoenzyme activity, simple oxidation of the enzyme, low concentrations of albumin, or increased concentrations of glutathione reductase, lactate dehydrogenase, or uric acid. We conclude that the prolonged lag phases reflect inadequate reactivation of the enzyme by sulfhydryl agents under the usual assay conditions. Reactivation before assay can prevent potentially serious negative errors in the assay of creatine kinase.

Additional Keyphrases: β-mercaptoethanol • analytical error • cancer • reactivation of creatine kinase by sulfhydryl-containing compounds

The causes of erroneously high results for CK have been well investigated and excellently reviewed (1). Falsely low results have also been described, the most common cause being inactivation of the enzyme during storage of serum. Addition of sulfhydryl compounds to the reaction mixture has been shown by many investigators to re activate the enzyme, although the exact “optimal” concentration of them may vary (2). At about 10 mmol/liter in the incubation mixture, little or no difference in reactivating ability among various thiol compounds was noted whether the “forward” reaction, creatine → creatine phosphate (3, 4), or the more widely used “reverse” reaction, creatine phosphate → creatine (2, 5–11) was used. Other possible causes of low values that have been suggested are increased glutathione reductase (EC 1.6.4.2) activity when reduced glutathione is used as a CK activator in the reverse assay (11, 12), increased lactate dehydrogenase activity when NADH is used in this assay (11), and a sulfhydryl-reversible inhibition by uric acid (13). An association between depressed CK activities and increased alkaline phosphatase (EC 3.1.3.1) activity has also been described (14) but does not appear to be caused by any direct effect of alkaline phosphatase on the assay (11, 15, 16).

We noticed a low CK value in a fresh serum from one patient which showed a lag phase longer than 13 min in the reverse reaction. This report describes an identifiable group of such patients (low CK, prolonged lag phase) and presents data relevant to the etiology of the phenomenon and to its elimination by preincubation of the sera with sulfhydryl reagent.

Materials and Methods

Apparatus

For enzyme assays we used either a Gilford 2000, Gilford 300-N (Gilford Instrument Laboratories, Inc., Oberlin Ohio 44074) or GEMSAEC (Electro-Nucleonics, Inc., Fairfield, N. J. 07006) analyzer, in all of which temperature is controlled. The choice of instruments did not affect the results. The change of absorbance at 340 nm was monitored with a chart recorder (in the case of the Gilford 2000 or 300-N) or a digital computer (GEMSAEC). In addition, when the Gilford 300-N was
used, the change in reaction rate with time was followed with an analog-circuit rate meter.

Reagents

**Assay of creatine kinase.** Unless otherwise indicated, assays were done with Statzyme CPK-15 reagent (Worthington Biochemical Corp., Freehold, N. J. 07728), involving the reverse coupled-enzymatic system hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*), which requires NAD⁺ rather than NADP⁺ (17), and glutathione as the activator (6). Other single-vial reagents for the reverse assay of CK incorporating various sulphydryl compounds at various concentrations and using either NAD⁺- or NADP⁺-requiring glucose-6-phosphate dehydrogenase were obtained from the following manufacturers: "Statzyme CPK n-1" (Worthington), "A-Gent CPK test" (Abbott Diagnostics, S. Pasadena, Calif. 91030), "Eskalab Bulk Reagent" (Smith Kline Instruments, Palo Alto, Calif. 94306), "UV System 10 CPK" (Boehringer-Mannheim Corp., New York, N. Y. 10017), "Fisher Diagnostics CPK No. 18300" (Fisher Scientific Co., Fair Lawn, N. J. 07410), and Sigma Kit 45-UV (Sigma Chemical Co., St. Louis, Mo. 63178). In addition, we obtained a two-part reagent containing the substrate, creatine phosphate, in a separate vial from the other reagents, with and without sulphydryl present.²

**Glucose phosphate isomerase** (EC 5.3.1.9). This was assayed with the "PHI Reagent Set" (Worthington) with fructose-6-phosphate as substrate and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) as the coupling enzyme.

**Sulphydryl compounds** cysteine and reduced glutathione (Fisher Scientific), β-mercaptoethanol, di-thioerythritol, and dithiothreitol (Sigma) were used as fresh aqueous solutions.

In the detailed studies of the lag phase done with the Gilford 2000 or GEMSAEC, the reagent, cuvettes, cuvette holders, etc., were brought to the assay temperature (37 °C unless noted otherwise) before the start of the reaction. These precautions were not taken with the Gilford 300-N because of the rapid temperature equilibration provided by the 3017T thermocuvette. Assays (reconstitution, dilutions, etc.) were performed according to the manufacturers’ directions except that the reaction was monitored continuously and beyond the recommended time. For assays done with the GEMSAEC, dilutions and sample flush volume were chosen to achieve the final concentrations recommended by the reagent supplier.

**Calculation of the Lag Phase**

The lag phase was defined as the time from the start of the enzymatic reaction until the time at which the rate of change of absorbance with time was linear and was determined graphically as shown in Figure 1. The calculations of the lag phase agreed with those obtained by using the analog-circuit rate meter (zero change in rate with time).

**Miscellaneous**

Linear regression was established by means of a Hewlett-Packard Model 9810A calculator, and significance tests were done with use of standard tables (18). pH was measured with a temperature-controlled Radiometer E5021A micro-capillary electrode at 37 °C. Sulphydryl compounds were added to serum before the assay by adding one volume of an aqueous sulphydryl solution to 50 volumes of serum. The side of the serum container was tapped to cause mixing.

**Results**

**Patients with a Prolonged Lag Phase for CK**

Figure 1, curve A, shows the time course of reaction at 37 °C of a serum that showed a 16-min lag phase. Reaction rates measured at 3 and 10 min were only 25% and 75%, respectively, of the final rate. For comparison, curve B, obtained under identical conditions, shows the course for a serum with the same final rate, but with the usual lag phase of 3–4 min. When an equi-volume mixture of sera A and B was assayed, curve C was obtained in which the lag phase was equal to the average of the lag phases of the two individual sera. In other experiments the lag phase was shown to be substantially longer at lower assay temperatures, at 25 °C being twice what it was at 37 °C.

**Correlation of Lag Phase with Glucose Phosphate Isomerase Activity**

The initial serum in which a prolonged lag phase was observed came from a patient with carcinoma of the breast metastatic to the liver, whose serum was known to have an increased activity of glucose phosphate isomerase. Other samples with normal and increased glucose phosphate isomerase activity (>90 U/liter) were tested for their lag phases in the assay of CK. Figure 2 illustrates the correlation found between glucose phosphate isomerase activity and the lag phase in the CK assay of sera from 17 patients. The correlation coefficient was highly significant (P < 0.001). Diagnoses for these patients included breast, lung, colon, and pelvic carcinomas. All had clinical evidence of hepatic metastases if their sera showed abnormally high glucose phosphate isomerase activity. Four additional patients (indicated by triangles in Figure 2) had markedly increased glucose phosphate isomerase activity; they had various diseases but no evidence of carcinoma. Sera from these patients had the usual short (2–4 min) lag phases, which did not correlate with glucose phosphate isomerase activity. This strongly suggested that glucose phosphate isomerase per se did not account for the observed correlation. In these 21 patients we found no correlation between lag phase and CK activity or between the activities of the two enzymes. On review of all patients’ charts we saw no statistically significant correlation of the lag phases with either serum alkaline phosphatase or lactate dehydrogenase activities, al-

² This product with sulphydryl is marketed as “CPK n-1 (2 vial)” by Worthington Biochemical Corp., Freehold, N. J. 07728. They also supplied it to us without sulphydryl.
though these activities were increased in most cases. In addition, the analysis of additional sera with abnormally increased lactate dehydrogenase activity did not show a correlation of this activity with CK lag phase. Patients showing the long lag phases were not receiving any specific drug therapy or class of therapeutic agents, and several of the patients had not been started on therapy at the time the samples were obtained. While some but not all of the patients had a low serum albumin concentration, addition of human serum albumin (Fraction V) up to a concentration of 300 g/liter did not shorten the lag phases. Uric acid, which has been shown to inhibit CK in the absence of sulfhydryl compounds (13), was present in abnormally high concentrations in the sera of some of the patients with prolonged lag phases, but other sera in which uric acid was markedly elevated did not show prolonged lag phases. Moreover, addition of uric acid to pooled sera to concentrations of 240 mg/liter neither affected CK activity nor prolonged the lag phase in this assay, an observation in agreement with that of Warren (13). Lactate dehydrogenase activity could lower apparent CK activity and potentially prolong the lag phase in reverse assays involving NAD$^+$ by catalyzing the oxidation of NADH in the presence of endogenous pyruvate. However, pyruvic acid (up to 1 mmol/liter), when added to sera with normal or high lactate dehydrogenase activities, did not reproduce the long lag phases.

Investigation of the Coupling Reactions

The reactions of the reverse kinetic assay for CK are shown in Figure 3. Interferences in the assay of CK might occur either at the coupling steps or at the primary (CK-catalyzed) reaction. The lag phase might be prolonged by the presence in the sera of inhibitors of the auxiliary enzymes, which would decrease the maximum velocities of the enzymes and (or) increase the Michaelis constants for ATP or glucose-6-phosphate (19). Similarly, the lag phase might be prolonged on removal of reaction products (other than 6-phosphogluconate or creatine) by (e.g.) limited hydrolysis of the ATP formed, removal of NADH, or the isomerization of glucose-6-phosphate via glucose phosphate isomerase (Figure 3). We investigated these possibilities by two independent experiments.

First, CK activity was measured in an automated end-point assay system, which measured creatine directly with the diacetyl–orcinol reaction (20), thus avoiding enzymatic coupling steps and also avoiding the possible interference from removal of ATP. With samples having the usual lag phases, measurement of creatine after either a 5.8- or 12.5-min incubation did not result in significantly different values for CK. In contrast, when we assayed a serum showing a long lag phase with the kinetic method, apparent activity increased proportionately with incubation time: the activity on 12.5-min incubation was twice that with the 5.8-min incubation. This increase in activity with incubation time was similar to that seen when CK activity was calculated from the kinetic assay (see above, Figure 1) and strongly suggests that the long lag phases were not attributable to the coupling enzymes of the reverse kinetic assay.

In the second experiment, we added ATP to the reagent system of the reverse kinetic assay, with and
without serum present, to test the coupling steps. At zero time, 50 nmol of ATP was added to 1.5 ml of assay reagent and the absorbance continuously recorded. As shown in Figure 4, the half-time of the reaction was unaffected by the presence of serum known to have a prolonged lag phase. We saw no effect of these sera when the ATP was added either immediately after serum was added to the reagent (i.e., during the lag phase) or at later times, strongly suggesting that the auxiliary enzymes were not inhibited by the serum. Additionally the analytical recovery of added ATP was quantitative in both cases, which would not be so if glucose-6-phosphate or ATP were being utilized by another reaction or if NADH were being reduced by the serum. Comparable results were obtained when the experiment was performed by adding glucose-6-phosphate. Thus, these experiments present strong evidence that the coupling system was not being affected by serum components in the samples with long lag phases.

**Effect of Pretreatment of Serum with β-Mercaptoethanol**

The apparent lag phase in most routine assays of serum CK activity includes the time required for activation of CK by reduced sulfhydryl compounds. To evaluate the possible effect of sulfhydryl reactivation on the prolonged lag phase, we pre-treated sera with β-mercaptoethanol for 30 min before CK assay. Figure 5 illustrates the effect of pretreatment on the time course of the assay of a serum that showed a lag phase greater than 20 min when not pretreated (curve C). Lag-phase was reduced to less than 5 min by pretreatment of the serum with β-mercaptoethanol, either 10 mmol/liter (curve A) or 280 mmol/liter (curve B) final concentrations in the serum. These concentrations were equivalent to 0.3 and 9 mmol/liter, respectively, in the final assay mixture and produced no measurable change in the pH of either the serum or the final assay mixture. Equally important was the observation that the reaction rate of the pretreated samples (curves A and B) exceeded the rate observed with the non-pretreated sample, even after the end of the apparent lag phase (curve C), an observation in agreement with those of Miyada et al. (2) that suboptimal concentrations of sulfhydryl agents resulted in linear reaction rates with a lower apparent enzyme activity than was the case when optimal sulfhydryl concentrations were used.

The observed effect of pretreatment with β-mercaptoethanol suggested several possible explanations. First, the increased rate observed with such pretreatment could not be ascribed to nonspecific reduction of NAD+ or to changes in turbidity, because there was no apparent CK activity when pre-treated serum was added to reagent lacking creatine phosphate. Additionally, samples with the usual or only slightly prolonged lag phases showed little increase in final enzymatic activity on such pretreatment.

Secondly, recent reports that the MB (cardiac) isoenzyme of CK is more resistant to reactivation by sulfhydryl compounds (21, 22) suggested the possibility that samples with increased MB-CK activity may have prolonged lag phases when glutathione is used as the sulfhydryl activator. However, isoenzyme determinations by a sensitive electrophoretic method (23) on the samples with prolonged lag phases revealed no detectable MB-CK, even after pre-treatment of the sera with β-mercaptoethanol.

A third possible explanation of the prolonged lag phases was simple oxidation of the CK molecule. To test this possibility, we bubbled oxygen through normal sera for 30 min to totally inactivate the enzyme (2). When we used the reagent without sulfhydryl, no activity was observed, indicating that the enzyme had been totally inactivated by oxidation. However, the sulfhydryl agent present in the usual reagent fully reactivated the enzyme, and no prolonged lag phases were observed.

Finally, a causative role of glucose phosphate isomerase in the lag phase was further excluded by the observation that pretreatment of sera with β-mercaptoethanol did not affect their glucose phosphate isomerase activity.

**Sulfhydryl Requirements for Reactivation**

We investigated the reactivation requirements for CK in a pool of sera with long lag phases by using various sulfhydryl compounds. For these studies sera were
preincubated for 30 min at room temperature in the presence of 0–280 mmol of sulfhydryl per liter. CK activity was then assayed by using the standard reagent containing glutathione. The dependency of both lag phase and final apparent enzymatic activity on sulfhydryl concentration is shown in Figures 6 to 8 for β-mercaptoethanol, dithiothreitol, and dithioerythritol. Regardless of the thiol compound used, activity increased with increasing concentration and was maximal at about 10 mmol/liter of preincubated serum. Concomitantly, the lag phase decreased with increasing sulfhydryl concentration and was minimal at about the same concentration of sulfhydryl compound. The range of concentrations producing full reactivation was different for the three reagents. β-Mercaptoethanol could be used at concentrations up to 280 mmol/liter, giving a broad range of effective concentrations; the other two reagents not unexpectedly (21) gelled the serum at concentrations exceeding 10–15 mmol/liter and thus had a narrow range of usefulness. Glutathione at about 10 mmol/liter was effective in reducing the apparent lag phase to less than 2 min, but the final CK activity was 10–20% less than with the other sulfhydryl agents; cysteine alone neither effectively shortened the lag phase nor increased the CK activity, at concentrations between 0.2 and 190 mmol/liter. With glutathione and cysteine, the reaction rates fluctuated, even after the end of the apparent lag phase, resulting in uncertainty in the estimates of final activity. On pretreatment of the sample with 280 mmol of β-mercaptoethanol per liter, reactivation of CK appeared essentially complete within 30–60 s. This observation was in keeping with the thermodynamically controlled rather than kinetically controlled activation of CK in normal sera (10).

The lag phases of these sera were not overcome by adding corresponding amounts of β-mercaptoethanol, dithioerythritol, or dithiothreitol directly to the reagent itself, regardless of whether or not the reagent already contained glutathione. At final concentrations of these compounds in the reagent similar to the concentrations effective for pretreatment—e.g., 10 mmol/liter—the lag phases were only reduced a little, while at higher concentrations both nonspecific reduction of NAD+ (in the absence of substrate) and loss of apparent enzyme activity were observed. Thus, the ineffectiveness of these compounds when present in the reagent was in striking contrast to their effectiveness when used for pretreatment of the sera.
Use of Other Reagent Formulations

Because all the above work was done with reagents obtained from one manufacturer, other formulations were tested. We used two specimens of pooled serum, one made with sera having normal lag phases, the other from sera with long (12–20 min) lag phases, as measured with the Worthingtor Statzyme reagent. We stored the pools frozen in aliquots and analyzed samples from each pool with and without pretreatment (280 mmol of \( \beta \)-mercaptoethanol per liter, for 30 min), using the kits according to the directions of their respective suppliers. All of the single part kits, which contained glutathione, L-cysteine, dithioerythritol, or some combination of these agents in various concentrations, showed the same phenomena as did the Worthington Statzyme reagent (long lag phases and low CK activities, both of which were corrected by pretreatment with \( \beta \)-mercaptoethanol). With a two-part kit in which the serum was first incubated without creatine phosphate, the lag phase was reduced to less than 3 min. In this single experiment the two-part reagent appeared promising, but further data will be required to determine whether it can substitute for pretreatment of serum. The above experiments and those previously described essentially rule out glutathione reductase (11, 12) or lactate dehydrogenase (11) as causes of the prolonged lag phase and low CK activity, because glutathione and NADH were not present in several of the kits tested.

Discussion

The present study demonstrates that sera from at least one identifiable group of patients (metastatic disease of the liver) may exhibit low apparent enzymatic activity and prolonged lag phases in the “reverse” kinetic assay of CK as it is commonly performed. This phenomenon appears to be the result of slow and incomplete reactivation of CK by sulfhydryl agents and not to other potential causes of low CK activity such as specific drugs, high proportions of the MB isoenzyme of CK (21, 22), deficiency of the coupling enzymes, removal of intermediate reaction products, oxidation of NADH or NADPH, glutathione reductase (11, 12), lactate dehydrogenase (11), uric acid (13), simple oxidation of the enzyme (2), or too-low concentrations of albumin (13, 24). The reports of increased alkaline phosphatase activity in the serum of patients with low CK values (14) may reflect the phenomenon described in this paper, because patients with metastatic disease of the liver often have abnormally high alkaline phosphatase activity in their serum (25), as was seen in the present series of patients. Alkaline phosphatase itself has been excluded as a cause of the low apparent CK activity (11, 15, 16). Similarly, the correlation of lag phase with glucose phosphate isomerase appears to be due to the ability of glucose phosphate isomerase to reflect the severity of metastatic disease of the liver. The severity of disease then correlates with the as-yet-unidentified changes that are etiologically related to the duration of the lag phases.

These findings indicate that one can make serious errors in most assays for CK in the serum of an unknown proportion of patients. The assay for CK isoenzymes by differential sulfhydryl reactivation (22) would also be expected to be in serious error in these patients. Until the exact etiology of this phenomenon is elucidated it cannot be assumed that other disorders in addition to metastatic liver disease could not cause similar errors. We stress that the error-detection algorithms of most, if not all, commercial instruments would not alert the laboratory to this problem because the rate changes observed are so gradual (Figures 1 and 5) that they would not be detected by such instruments.

Pre-incubation of serum with at least 10 mmol of \( \beta \)-mercaptoethanol, dithiothreitol, or dithioerythritol per liter completely abolishes the problem of prolonged lag phases and low apparent CK activity in the patients studied, \( \beta \)-mercaptoethanol being the best of the four sulfhydryl groups tested, although other sulfhydryl compounds might be as effective or more so (11). The addition of similar concentrations of sulfhydryl to the assay reagent rather than to serum did not correct this problem. Pretreatment of serum with sulfhydryl agents improves recoveries of human CK isoenzyme as compared with sulfhydryl in the reagent (21, 26), and the results with the two methods are different (27). Alternatives to serum pretreatment, such as incubation in reagent without substrate, are now being investigated, as is the requirement for additional sulfhydryl in the reagent. The work of others indicates that sulfhydryl may still be necessary in the reagent (6).

The mechanisms responsible for the prolonged lag phases are not known. A role for a different molecular form of the enzyme in the long lag phases cannot be excluded. An inactive enzyme, which contained only two sulfhydryl groups per molecule rather than the usual four in the native enzyme, was formed after rabbit skeletal muscle CK was kept for a few days at \(-20^\circ\text{C}\) in tris(hydroxymethyl)aminomethane chloride buffer (28). The earlier findings of Watts et al. (28) may also be important in this regard. They found that different preparations of rabbit muscle CK may contain one or two catalytic sites per molecule, each associated with no more than a single sulfhydryl group.

The prolonged lag phases may be related to the inactivation of CK by sera, which has been studied by others. Apparently, some factor in serum is responsible for inactivation, because purified enzyme or tissue extracts are generally not so quickly inactivated and the added sulfhydryl agents do not affect the activity of such preparations (5, 10, 27). Both protein-bound and diffusible species appear involved in this inactivation of CK by sera (13, 24). Our finding of proportional changes in the lag phase after dilution with normal sera (Figure 2) would support a role for a serum factor in the production of the long lag phases. In the usual situation, uric acid appears to be responsible for a large part of the CK inactivation by sera. Because both Warren (13) and ourselves have shown this effect to be completely reversible by the addition of even mild sulfhydryl agents.
to the assay, it cannot be responsible for the prolonged lag phases found in the patients studied.

Oxidized glutathione has been noted to inhibit CK (29); possibly the patients studied had high concentrations of this or of other oxidized sulphydryl compounds. This could account for the need for pre-incubation with relatively high concentrations of strong sulphydryl agents to overcome the effect of such compounds. Such a hypothesis is also supported by the alterations in sulphydryl metabolism that are known to occur in some malignancies (36). Obviously it is important to learn if the low CK activity is only a manifestation produced by serum-based factors, or if the activity of the enzyme in the tissues in vivo is altered, or both.

It is also not known what effect pretreatment of sera has on such phenomena as the "dilution effect" (4, 31, 32), light inactivation of CK (33), or the apparent instability of BB isoenzyme during electrophoresis (34). The dilution effect (disproportionate rise in CK activity upon dilution) has been attributed to insufficient methodology by some (11) but others believe it to be related to the presence of CK inhibitors in specified pathological conditions (32). Studies of these phenomena with sulphydryl pre-treated sera are presently under way.

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References


