

NOVEL REAGENTS FOR CREATINE KINASE

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ABSTRACT

The purpose of this investigation was to develop a simple colorimetric method for creatine kinase (CK). The new method is based on the reaction of creatine, formed enzymatically from creatine phosphate and ADP, with different glyoxal compounds. Hydrated glyoxals, such as para-nitrophenyl, 2-thiophene, 4-biphenyl, 4, 4'-biphenyl, α -naphthyl, β -naphthyl, para-chlorophenyl, and styryl were synthesized and allowed to react with creatine. Among the glyoxals, the 2-thiophene derivative was the best in terms of the stability and intensity of the colored complex which was produced under mild alkaline conditions. The complex absorbed maximally at 460 nm with an extinction coefficient of $1.56 \times 10^4 \text{ M}^{-1} \text{ Cm}^{-1}$. This reagent was used to determine CK in the sera of normal human beings and patients with myocardial infarction. The results obtained were in agreement with those obtained by another available method for CK. However, this new method is simple, less time consuming and employs a single reagent for color development. Such a simple method might be of value in clinical laboratories with little access to sophisticated instruments such as autoanalyzers and spectrophotometers.

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INTRODUCTION

Creatine kinase (CK) (ATP: creatine phosphotransferase, EC 2.7.3.2)



Several reports indicate that dicarbonyl reagents, such as glyoxals, react with guanidino group-containing compounds.¹⁻⁵ By varying the properties of the substituted

groups on the glyoxals, it might be possible to obtain a colored product when glyoxals are allowed to react with creatine.
of CK.

The purpose of this investigation was to develop a new method for determination of CK.
on the reaction of creatine with different glyoxal compounds.

glyoxal (2TG) was the most suitable in terms of the sensitivity and specificity of the reaction condition. The procedure requires no critical handling of chemicals and is performed under mild conditions. There is no need to remove excess reagent because the absorption maximum of the product is sufficiently different from the reagent.

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ment in this procedure is accomplished by addition of a single reagent, thus decreasing the number of chemicals ordinarily used in CK assays.

EXPERIMENTAL PROCEDURES

Materials

All chemicals were of reagent grade and were purchased from commercial sources. All glyoxals were prepared in their hydrated form from the corresponding ketones using selenium dioxide in acetic acid or dioxane according to the established procedures described in the literature (Table I and references therein). All glyoxals were soluble in absolute methanol except p-nitrophenyl glyoxal which was soluble in water up to 5 mg/mL. All buffers and glyoxal solutions were prepared fresh daily. Absorbance measurements were done by a Unicam Model SP recording spectrophotometer, melting points were determined by Bushi Model 510 melting point apparatus and pH measurements were done by a NEL pH meter Model 821. Sigma diagnostic kits were purchased from Sigma Chemical Co (USA). Human serum specimens were collected from local hospitals and were stored under refrigeration.

Methods

Reaction of creatine with glyoxals. For each glyoxal, the effects of buffer concentration, pH, temperature and time of reaction, concentration of glyoxal and sodium ascorbate as color stabilizing agent was studied in order to find the optimum condition for the reaction of creatine with glyoxals. The following general condition was used to study the reaction of creatine with 2-thiophene glyoxal (2-TG): 2.0 mL 0.5 mol/L NaOH, 0.025 mol/L sodium ascorbate and 0.4 mL 0.22 mol/L 2-TG in absolute methanol was added to 1 mL of 0.2 mmol/L creatine and the reaction was allowed to proceed at 25°C. After 30 min the absorbance at 460 nm was read against a blank containing all reagents except creatine. The effects of pH, different concentration of NaOH, temperature, concentration of sodium ascorbate, and time of reaction were studied by varying a single factor while keeping the others constant. The specificity of the color reaction for creatine was tested by substituting other compounds at the same concentration used for creatine and reading the absorbance at 460 nm as described above.

The experiments designed for the reaction of other glyoxals with creatine was essentially the same as described for 2-TG with exceptions indicated in Table I.

Determination of CK activity by 2-TG. The activity of CK in human serum was determined by two different methods. The colorimetric method of Hughes (diacetyl- α -naphthol method, Sigma kit No. 520) was used and compared with the 2-TG method.¹⁴ The incubation mixture for CK contained 5.6 mmol/L creatine phosphate, 0.015 mol/L

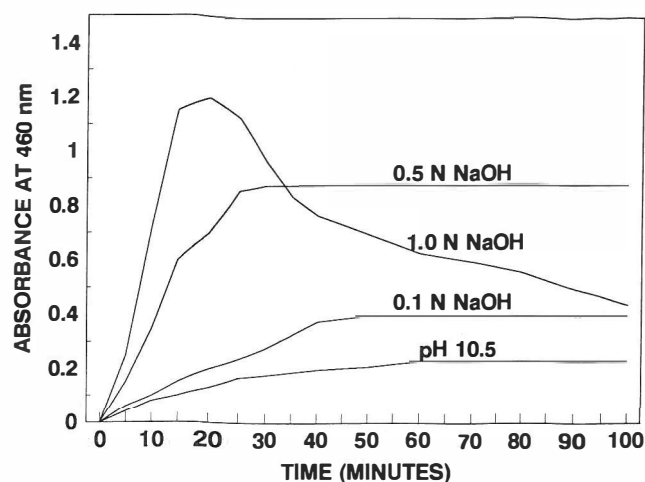


Fig. 1. Absorption spectrum of the reaction mixture of creatine with 2-thiophene glyoxal (2-TG). 2.0 mL 0.5 mol/L NaOH, 0.025 mol/L Na ascorbate and 0.4 mL 0.22 mol/L 2-TG in absolute methanol was added to 1 ml solution of 0.2 mmol/L creatine. The absorbances were scanned after 30 min at 25°C against a blank containing all reagents except creatine in a 1 cm cuvette.

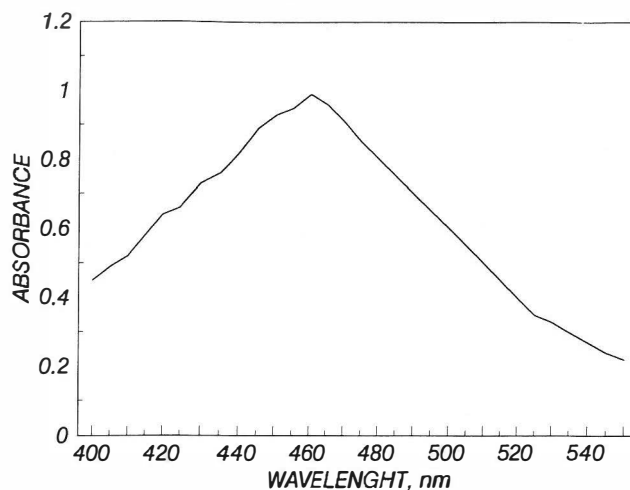


Fig. 2. Effects of pH and concentration of NaOH on the reaction of creatine with 2-thiophene glyoxal (2-TG). 2.0 mL mol/L NaOH, 0.025 mol/L Na ascorbate and 0.4 mL 0.22 mol/L 2-TG in absolute methanol was added to 1 mL solution of 0.2 mmol/L creatine. The reaction was allowed to proceed at 25°C and the absorbances at 460 nm were read against a blank containing all reagents except creatine in a 1 cm cuvette. All alkaline solutions and buffer contained 0.025 M Na ascorbate. The buffer was 0.1 M sodium carbonate, pH 10.5.

MgSO_4 , 4.0 mmol/L ADP, 0.25 mmol/L reduced glutathione and 10 μL serum in 0.8 mL 0.1 mol/L Tris, pH 7.5. The mixture was incubated at 37°C for exactly 30 min. Following incubation, the reaction was stopped by adding 0.2 mL 0.05 mol/L p-hydroxymercury benzoate. The concentration of creatine in the incubation mixture was determined by

Table I. Properties and conditions for reaction of creatine with glyoxals.

Glyoxals ^{a,b}	Abb. ^c	MP, °C	Time, min	Optimum Conditions ^d	Wave length, nm ^e	Extinc. Coeff. ^e M ⁻¹ Cm ⁻¹
2-Thiophene (6)	2TG	94	30	0.5 mol./L NaOH, 25°C	460	1.56 X 10 ⁴
p-Nitrophenyl (7)	PNPG	98-99	30	0.1 mol/L pyrophosphate, pH 9.5, 37°C	480	1.18 X 10 ³
α-Naphthyl ^f (8)	ANG	90	-	-	-	-
β-Naphthyl (9)	BNG	108	20	0.5 mol/L NaOH, 37°C	410	0.4 X 10 ³
4-Biphenyl ^g (10)	4BPG	114-116	-	-	-	-
4,4'-Biphenyl bis ^g (11)	BPBG	144	-	-	-	-
1-Styryl (12)	ISG	65-67	20	0.5 mol/L NaOH, 37°C	420	0.8 X 10 ³
p-Chlorophenyl ^f (13)	PCPG	126-128	-	-	-	-

^a Numbers in parentheses correspond to the references used for synthesis of glyoxals

^b The concentration of all glyoxals was 0.22 mol/L.

^c Abbreviation used.

^d All solutions contained 0.025 mol/L Na ascorbate.

^e Wavelengths of maximum absorbance and molar extinction coefficients are for the reaction product when each glyoxal was allowed to react with creatine under the condition given.

^f No reaction occurred.

^g Turbidity of the reaction mixture occurred.

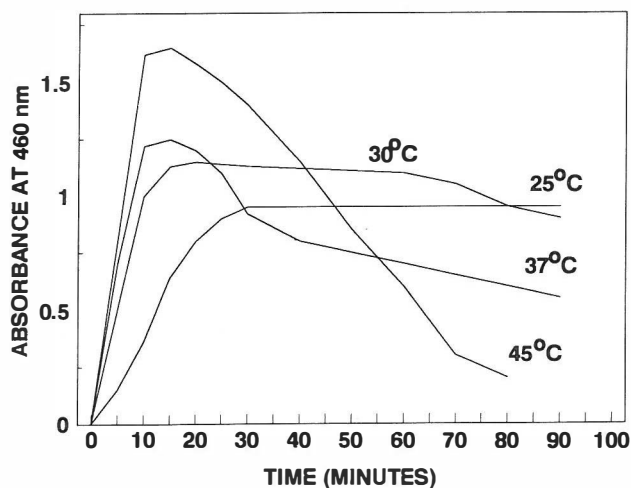


Fig. 3. Effect of temperature on the reaction of creatine with 2-thiophene glyoxal (2-TG). 2.0 mL 0.5 mol/L NaOH, 0.025 mol/L Na ascorbate and 0.4 mL 0.22 mol/L 2-TG in absolute methanol was added to 1 mL solution of 0.2 mmol/L creatine. The reaction was allowed to proceed at indicated temperatures and the absorbances at 460 nm were read against a blank containing all reagents except creatine in a 1 cm cuvette.

2TG method as described in the preceding section, using a molar extinction coefficient of $1.56 \times 10^4 \text{ M}^{-1} \text{ Cm}^{-1}$ (Table I). A blank was prepared which contained all reagents except serum.

In this paper, one unit CK is defined as the amount of enzyme that can produce one μmol creatine per min at 37°C and pH 7.5.

RESULTS

Reaction of creatine with 2-thiophene glyoxal

Creatine reacted with 2-TG in 0.5 mol/L NaOH containing 0.025 mol/L sodium ascorbate to produce a colored complex which absorbed maximally at 460 nm (Fig. 1). The rate of reaction and the intensity of the color was greater at higher alkali concentration (i.e., 1.0 mol/L NaOH), but the absorbance at 460 nm due to the blank was high, the colored product was unstable and the absorbance started to decline soon after reaching its highest value (Fig. 2). The rate of color development was slow at 0.1 mol/L NaOH or 0.1 mol/L sodium carbonate, pH 10.5, even in the presence of 0.025

Reagents for Creatine Kinase

Table II. Absorbance of samples treated with 2-thiophene glyoxal*.

Compounds	Absorbance at 460 nm
Creatine	0.92
Arginine	1.66
Lysine	0.006
Histidine	0.01
Creatinine	0.01
Urea	0.002
Creatine phosphate	0.01
Cysteine	0.008
Glycine and other amino acids	<0.002

* 2 mL 0.5 mol/L NaOH/0.025 mol/L Na ascorbate and 0.4 mL 0.22 mol/L 2-thiophene glyoxal was added to 1 mL of the test compound. The reaction was allowed to proceed at 25°C for 30 min and the absorbances were recorded against a blank containing all reagents except the test compound in a 1 cm cuvette.

mol/L sodium ascorbate as color stabilizing agent. The reaction of creatine with 2-TG was also dependent on temperature. At 25°C, the reaction was complete in 30 min, absorbance at 460 nm remained stable for an additional 100 min and started to gradually decrease thereafter (Fig. 3). At higher temperatures, the rate of color development was higher, but the absorbance of the colored product decreased rapidly. The presence of 0.025 mol/L sodium ascorbate in the reaction mixture was necessary in order to increase the rate of color formation, and the intensity of colored product. Higher concentration of sodium ascorbate did not increase the rate of color development nor did it have any effect on the stability of color (Fig. 4). In the absence of sodium ascorbate the color formation was too slow to be of value. It was concluded that 0.5 mol/L NaOH solution containing 0.025 mol/L sodium ascorbate at 25°C was the best condition for reaction of creatine with 2-TG when the color development in the blank was taken into consideration. Under these conditions the reaction product obeyed Beer's law in the range of 0.005-0.06 mol/L creatine with a molar extinction coefficient of $1.56 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (Fig. 5). A 0.4-mL aliquot of 0.22 mol/L 2-TG was considered the best concentration. Higher concentrations caused turbidity of the reaction mixture, probably due to insolubilization of 2-TG. These results as well as the data for other glyoxals are summarized in Table I.

Other compounds that might be present in the samples and that might react with 2-TG, interfering in the determination of creatine, were tested. These included the 20 common amino acids found in the proteins, cholesterol, creatinine, ornithine, urea, and creatine phosphate, which were individually tested at the same concentration. Table II shows the results of the reaction of some of these compounds. Among the compounds tested, arginine reacted

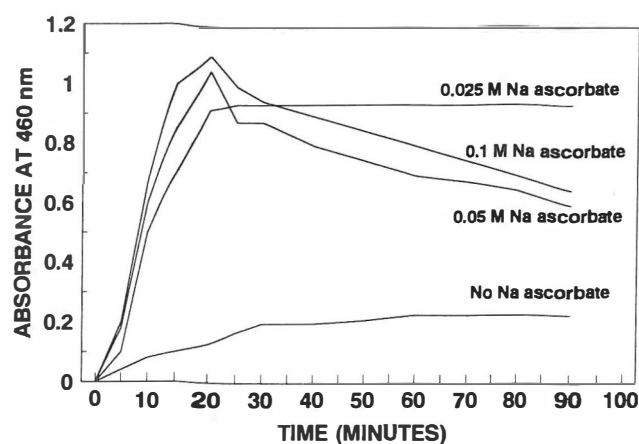


Fig. 4. Effect of Na ascorbate concentration on the reaction of creatine with 2-thiophene glyoxal (2-TG). 2.0 mL 0.5 mol/L NaOH containing different concentrations of Na ascorbate and 0.4 mL 0.22 mol/L 2-TG in absolute methanol was added to 1 mL solution of 0.2 mmol/L creatine. The reaction was allowed to proceed at 25°C and the absorbances at 460 nm were read against a blank containing all reagents except creatine in a 1 cm cuvette.

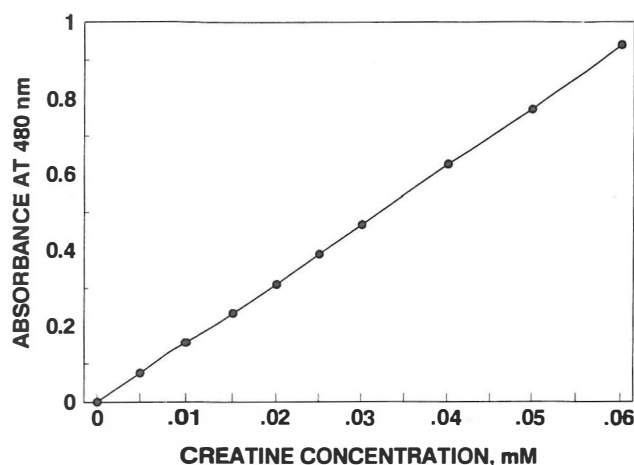


Fig. 5. Standard curve for determination of creatine with 2-thiophene glyoxal (2-TG). 2.0 mL 0.5 mol/L NaOH, 0.025 mol/L Na ascorbate and 0.4 mL 0.22 mol/L 2-TG in absolute methanol was added to 1 mL solution of 0.005 to 0.075 mmol/L creatine. The reaction was allowed to proceed at 25°C and the absorbances at 460 nm were recorded against a blank containing all reagents except creatine in a 1 cm cuvette.

with 2-TG to produce colored product which absorbed at 460 nm. All other compounds tested but not listed in this table showed absorbance of less than 0.001 at 460 nm. Other amino acids, citrulline, as well as many other commonly used biochemical reagents including Tris, borate, Hepes, dithiodierythritol, EDTA, pyridine, and ethanolamine did not react.

Reaction of creatine with other glyoxals

The reaction of creatine with other glyoxals was studied

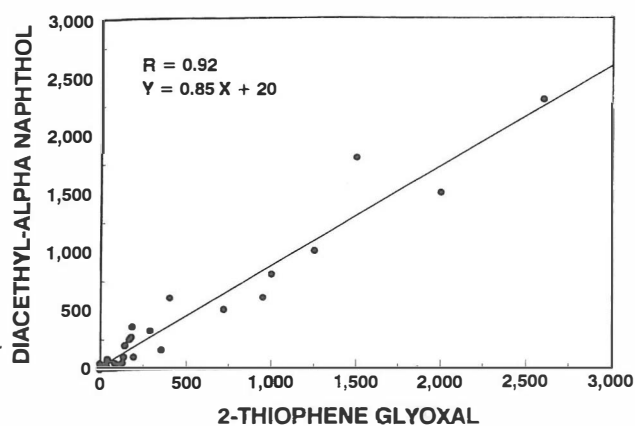


Fig. 6. Correlation between results of 2-TG and diacetyl- α -naphthol method for determination of serum CK.

and the optimum conditions for each was established. The results are summarized in Table I. Among the glyoxals tested, α -naphthol glyoxal (ANG), 4-biphenyl glyoxal (4BPG), 4, 4'-biphenyl bisglyoxal (BPBG), and p-chlorophenyl glyoxal (PCPG) did not react, even in the presence of 1.0 mol/L or higher concentration of NaOH. Also changing other variables such as increasing the temperature to 45°C or above, or changing sodium ascorbate concentration did not improve the reactivity of these glyoxals with creatine. In the case of 4BPG and BPBG the reaction mixture became turbid and lowering the concentration of creatine and/or glyoxal did not decrease turbidity.

Determination of CK by 2-TG method

CK activity was determined in normal sera and sera of patients with acute myocardial infarction. Units per liter (U/L) of CK in each serum sample was determined by 2-TG method as well as diacetyl α -naphthol method (Fig. 6). In both methods CK activity was stopped by p-hydroxymercury benzoate. Activities lower than 100 U/L were considered normal and those above 100 U/L were from patients with myocardial infarction. The slope of the curve in Figure 6 (0.85) indicates the degree of correlation between these two methods. A correlation coefficient of 0.92 shows a linear relationship between the results of two methods.

DISCUSSION

In this paper the results of studies on the reaction of creatine with different glyoxals is reported. Creatine reacted with some glyoxals and produced colored products which could be used to measure CK activity. Among the glyoxals tested, 2-thiophene glyoxal (2-TG), p-nitrophenyl glyoxal, (PNPG), β -naphthyl glyoxal (BNG), and 1-styryl glyoxal (ISG) reacted with creatine under mild alkaline conditions. 4-biphenyl glyoxal (4BPG), 4, 4'-biphenyl bisglyoxal

(BPBG) did not react due to the insolubilization of these glyoxals in the aqueous reaction mixture. Solubility of these glyoxals was a problem and application of solvents such as dimethylsulfoxide (DMSO) did not improve the solubility. Therefore, no further work was performed on these glyoxals. While β -naphthyl glyoxal reacted with creatine in 0.5 mol/L NaOH at 37°C and the colored product absorbed maximally at 410 nm, no reaction occurred with the α -naphthyl derivative. This lack of reactivity might be attributed to the steric hindrance between the hydrogen atom on carbon 8 of the naphthalene moiety and the carbonyl group on carbon 1 of glyoxal.

The nature of the complex produced by the reaction of creatine with glyoxals is not known at present. It has been reported that phenyl glyoxal reacts with arginine to produce a derivative containing two phenyl glyoxal moieties per arginine.³ A similar mechanism might be involved in the reaction of the methyl guanidino group of creatine with glyoxals used in this study. In order to absorb in the visible region, some degree of conjugation in the glyoxal derivative is apparently required. While the product of reaction of guanidino group in arginine with phenyl glyoxal³ and p-hydroxyphenyl glyoxal⁵ absorbs at the ultraviolet region, p-nitrophenyl glyoxal reacts with arginine and creatine to produce colored compounds which absorb at 475-480 nm.¹⁻⁴ These considerations might explain why p-chlorophenyl glyoxal did not produce colored compound with creatine (Table I). No attempt was made to follow the reaction of creatine with p-chlorophenyl glyoxal in UV region because of the high extinction of p-chlorophenyl glyoxal itself at wavelengths below 300 nm.

The highest extinction coefficient was obtained for the reaction of creatine with 2-TG. Another suitable candidate was PNPG. However, at pH 9.0 and 37°C, the optimum conditions for reaction of creatine with PNPG (Table I), the extinction coefficient of the product was at least one order of magnitude lower than that produced by 2-TG. At higher pH's the rate of color development and the intensity of the color with PNPG was greater but the color product was too unstable and the reaction lost its specificity.¹⁵ Therefore, it was concluded that 2-TG was the best glyoxal in terms of the simplicity of the reaction conditions and the sensitivity and specificity of the reaction for creatine determination. This reaction is fairly specific for guanidino group-containing compounds. Among the compounds that are present in serum and that might be expected to interfere with CK determination is free arginine. A simple calculation shows that arginine, at the levels present in the human serum (0.6-1.2 mg/100 mL)¹⁶ does not normally interfere with determination of enzymatically formed creatine. If 10 μ L serum is used, the concentration of free arginine in 3.4 mL reaction mixture of 2-TG method would be $1.1-2.2 \times 10^{-4}$ mmol/L. Table II shows that 0.2 mmol/L of arginine will give an absorbance of 1.6 at 460 nm.

2-TG method can be used reliably to determine CK activity in normal sera as well as sera from patients with myocardial infarction. Good correlation was obtained when 2-TG method was compared with diacetyl- α -naphthol method of Hughes.¹⁴ Both methods are based on direct colorimetric determination of creatine. However, 2-TG method offers several advantages over currently used colorimetric methods. In this method, color development is achieved by addition of a single reagent, i.e. 2-TG, to the incubation mixture, thereby obviating the inconvenience of working with such odorous and dangerous compounds as diacetyl and α -naphthol. 2-TG method is more rapid because the color develops in 30 min at 25°C and no turbidity of the reaction mixture occurs, therefore all centrifugation steps that are usually required in other methods are omitted. Furthermore, we have found (unpublished data) that 2-TG inhibits CK. The reason for this action of 2-TG on CK is not known. It is possible that 2-TG reacts with a critical arginine residue of CK. The beneficial effect of 2-TG is that the addition of p-hydroxy mercury benzoate, which normally is used in other assays for stopping CK after incubation, is eliminated.

At present, the coupled enzymatic assay of Rosalki¹⁷ is the method of choice in most laboratories¹⁸ and has been adapted to automation. However, besides the requirement for recording spectrophotometers, this method utilizes two enzymes and many reagents and is more expensive than colorimetric methods. Decreasing the cost of CK tests, which is probably achievable by the 2-TG method, might render this method valuable in clinical laboratories with little access to such facilities as recording spectrophotometers, autoanalyzers, or expensive chemicals.

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