Creatine kinase and ATPase in human seminal fluid and prostatic fluid

J. P. Kavanagh and C. Darby

Department of Urology, University Hospital of South Manchester, Nell Lane, Manchester M20 8LR, U.K.

Summary. A creatine kinase assay based on estimation of creatine liberated from creatine phosphate was accurate and reproducible for use with seminal or prostatic fluid, after allowance was made for acid phosphatase interference. Comparison of this method with one which relies on enzymic coupling of ATP formation to NADP⁺ oxidation shows that the latter under-estimates creatine kinase activity by a factor of about 3. This discrepancy could be due to the high ATPase activity found in prostatic and seminal fluid. Uncritical use of the NADP⁺ assay might account for different seminal creatine kinase values reported in the literature.

Interrelationships between ATPase, creatine kinase and zinc suggest that seminal ATPase is a prostatic secretory product while creatine kinase may be multiglandular in origin.

Introduction

Creatine kinase (EC 2.7.3.2) is a dimeric enzyme occurring as the 'muscle type' (MM), 'brain type' (BB) and the hybrid form (MB). The enzyme found in prostatic tissue and seminal plasma is exclusively or predominantly the BB type (Smith, 1972; Jockers-Wretou & Pfeleiderer, 1975; Burgett & Kwan, 1979; Soufir, 1979; Kimler & Sandhu, 1980; Asseo, Paridis, Papadimas & Ikkos, 1981).

The reported values for creatine kinase in semen vary, while evidence for the origin of seminal creatine kinase is also conflicting (Lehmann & Griffiths, 1963; Rosalki, 1966; Bostrom & Andersson, 1971; Soufir, 1979; Asseo *et al.*, 1981). The early report of Lehmann & Griffiths (1963) did not take into account any contributory activity of acid phosphatase in an assay measuring creatine liberated from creatine phosphate. Most other authors have used assays based on the coupling of ATP formation to NADPH production. Consideration of this assay suggests that significant levels of ATPase could seriously interfere with creatine kinase estimation by this method. Observations of raised serum levels of creatine kinase (BB form) in men with prostatic cancer (reviewed by Henderson & Nealon, 1981) suggested that creatine kinase assays of prostatic fluid could be clinically useful in the diagnosis of prostatic carcinoma. We have therefore considered various assays for creatine kinase activity.

Materials and Methods

Enzymes, NADH and NADP⁺ were purchased from the Boehringer Corporation (London). ATP, ADP, glutathione, creatine phosphate, α -naphthol, diacetyl, phosphoenol-pyruvate and L(+)-tartaric acid were obtained from Sigma Chemicals (London). All other reagents (highest grade available) were bought from BDH, Poole, Dorset, U.K.

Semen samples were obtained from specimens collected from healthy donors for artificial insemination. Seminal plasma was prepared by centrifugation. Prostatic fluid was obtained by rectal massage from patients suffering from a variety of urological disorders. All samples were frozen as soon as possible, normally within 2 h. Before analysis the samples were thawed; aliquants were placed into separate containers and refrozen.

The principles of the different creatine kinase assays used are outlined in Text-fig. 1.

Text-fig. 1. Three assay systems for creatine kinase. Bold arrows show intended reaction sequences. Light arrows show sites of interference by contaminating enzymes. Underscored components are added to the assays (— to NADP⁺ assay; ~~~ to NADH assay; --- to α -naphthol assay). Abbreviations: C, creatine; CP, creatine phosphate; PEP, phosphoenolpyruvate; PK, pyruvate kinase; PYR, pyruvate; LDH, lactate dehydrogenase; LAC, lactate; G, glucose; HK, hexokinase; G6P, glucose 6-phosphate; G6P DeH, glucose 6-phosphate dehydrogenase; Gn6P, gluconate-6-phosphate; AP, acid phosphatase.

α -Naphthol assay. This was performed in 0.05 M-Tris, 0.1 M-L(+)-tartaric acid and 10 mM-glutathione at pH 7.5. Substrate concentrations were 5 mM-creatine phosphate, 1 mM-ADP and 10 mM-MgCl₂. Prostatic or seminal fluid was present at a concentration of 1% (v/v). The enzyme reaction (final volume 0.15 ml) was initiated by addition of ADP/MgCl₂, carried out for 20 min at 37°C and stopped by addition of 0.15 ml 20 mM-hydroxymercuribenzoate closely followed by 0.15 ml α -naphthol (20 mg/ml) in 1.5 M-NaOH, 0.5 M-Na₂CO₃, 0.15 ml 0.05% (v/v) diacetyl and 0.9 ml H₂O. The colour was allowed to develop for 20 min at 37°C, the mixture centrifuged (1500 g, 10 min) and the absorbance at 520 nm read within 1 h. Blanks (replacing ADP/MgCl₂ with water) were also performed. Routinely 4 replicates for each sample were analysed and all samples were analysed within 1 month of collection.

NADP⁺ assay. The conditions used were essentially those described by Forster, Bernt & Bergmeyer (1974). The reaction was performed in 0.1 M-triethanolamine buffer, pH 6.9 containing 10 mM-glutathione, 0.8 mM-NADP⁺, 20 mM-glucose, 1 mM-ADP, 10 mM-MgCl₂ and 20 mM-creatine phosphate. Hexokinase and glucose 6-phosphate dehydrogenase were each present at 1 Boehringer unit/ml. Prostatic or seminal fluid was incubated (final concentration 1% v/v) with the buffer and all components except creatine phosphate for 15 min. The absorbance at 340 nm (25°C)

was then monitored for a sufficient time to measure any detectable rate and the reaction was started by addition of creatine phosphate. Any pre-creatine phosphate rate was subtracted from the final rate.

NADH assay. The method of Forster *et al.* (1974) was used, the reaction being followed at 340 nm against a blank from which ATP was omitted.

Comparison of α -naphthol and NADP⁺ assays. The assays used different conditions of pH, temperature and substrate concentrations, so they could not be compared in terms of i.u./ml. Instead the activities of samples were converted from the change in absorbance/min/ml to ng commercial creatine kinase per ml, based on the corresponding calibration curves for each assay. To minimize differences due to any time-dependent decay of enzyme activity, comparative assays were performed within 1–2 days of each other.

ATPase assay. This was carried out in 0.1 M-Tris/HCl buffer, pH 7.4, containing 0.25 mM-NADH, 0.4 mM-phosphoenolpyruvate, 4 mM-ATP, 4 mM-MgCl₂, 10 Boehringer units lactate dehydrogenase/ml and 5.3 Boehringer units pyruvate kinase/ml. The rate of decrease in absorbance at 340 nm (25°C) was measured against a blank containing water instead of ATP. The final concentration of prostatic fluid used was 0.25% (v/v) and of seminal fluid 1% (v/v).

All enzyme activities are reported as i.u./ml (μ mol substrate transformed/sec/ml). Zinc determinations were performed as described by Kavanagh & Darby (1982). Mann–Whitney U tests were used to test the significance of differences between samples.

Results

α -Naphthol assay. Standard curves of creatine concentration against absorbance at 520 nm were reproducible and linear up to at least 0.5 mM (in the initial reaction volume). The reaction with commercial creatine kinase was linear with time for at least 30 min (at 200 ng/ml, initial volume). The reaction rate (with a 20-min incubation period) was proportional to the creatine kinase concentration up to at least 200 μ g/ml (initial volume). The presence of prostatic fluid had a slight effect on the absorbance at 520 nm due to added creatine (in the absence of creatine phosphate). However, recovery of creatine approached 100% at prostatic fluid concentrations (in the initial volume) of $\leq 1\%$ (v/v).

The effect of acid phosphatase was overcome by use of a comparatively low creatine phosphate concentration, inclusion of L(+)-tartaric acid (an inhibitor of prostatic acid phosphatase) and the use of a suitable blank (omitting ADP). Under these conditions the blanks usually gave an absorbance (at 520 nm) reading of 0.13–0.20/20 min/1 cm path length while similar blanks in the absence of prostatic fluid gave values of about 0.11.

The accuracy of the assay was evaluated by considering the recovery of commercial creatine kinase added to prostatic fluid; this averaged 98% for samples ($n = 4$) with a ratio of added to endogenous creatine kinase of 0.1–2.0. Reproducibility was also acceptable. Mean coefficients of variation for prostatic or seminal fluid samples with low, medium or high creatine kinase activities were 8% ($n = 6$), 6% ($n = 8$) and 5% ($n = 6$) respectively (each coefficient based on 8 replicates/sample). The activity in the frozen samples fell by an average of 3% per month ($n = 10$). A lower concentration of glutathione (1 mM) gave much poorer recovery of activity after storage.

NADP⁺ assay. With the commercial preparation of the enzyme this assay gave linear initial rates proportional to creatine kinase concentration up to at least 200 ng/ml. In the absence of prostatic fluid the change in absorbance at 340 nm due to added ATP was as expected based on the extinction coefficient of NADPH. The coefficient of variation for the assay of prostatic fluid was about 5%. Recovery of excess commercial creatine kinase added to prostatic fluid was low and variable (69–84%, $n = 12$).

NADH assay. The rate determined by this method represents the activity due to both creatine kinase and ATPase. In practice the rate due to ATPase was so great that this assay could not be used to give meaningful results.

Comparison of α -naphthol and NADP⁺ assays. The creatine kinase activities based on the naphthol assay were generally 3 or 4 times those obtained by the NADP⁺ method for prostatic and seminal fluid (Table 1). There was only one sample in which the creatine kinase activity estimated by the NADP⁺ assay exceeded that obtained by the α -naphthol assay.

Table 1. Comparison of α -naphthol and NADP⁺ assays for creatine kinase estimation (ng/ml) in seminal and prostatic fluid

	α -naphthol assay : NADP ⁺ assay			
	No. of samples	Mean	Median	Range
Prostatic fluid	49	4.2	3.2	0.7–21.0
Seminal fluid	7	3.2	3.4	2.1–4.0

ATPase assay. In the absence of ATP, but with added ADP (in the presence of prostatic fluid), this assay gave the expected change in absorbance at 340 nm based on the extinction coefficient of NADH. Reproducibility was acceptable (coefficient of variation < 10%) and activities were stable for up to 1 month.

Origin of seminal creatine kinase and ATPase. Scattergrams of prostatic fluid creatine kinase and ATPase activities against zinc concentration are presented in Text-fig. 2. The correlation coefficient between zinc and ATPase was 0.65 ($n = 72$, $P < 0.001$) and between zinc and creatine kinase it was 0.26 ($n = 32$, $P > 0.1$). Creatine kinase values were similar in normal prostatic and seminal fluids while ATPase activities were significantly lower in seminal plasma (Table 2). Comparison of the ratio of ATPase activity/Zn concentration in seminal and prostatic fluid showed no difference, but the creatine kinase/Zn ratio was significantly higher in seminal fluid (Table 2).

Text-fig. 2. Scattergrams of (a) zinc and ATPase and (b) zinc and creatine kinase in human prostatic fluid samples.

Table 2. Normal values and activities related to zinc concentration of creatine kinase and ATPase in seminal and prostatic fluid

	Prostatic fluid				Seminal fluid				P
	No. of samples	Mean	Median	Range	No. of samples	Mean	Median	Range	
Creatine kinase* (i.u./ml)	9†	24.6	17.8	4.8–70.7	11	17.3	13.2	7.2–37.9	>0.1
ATPase (i.u./ml)	27†	59.1	52.9	14.0–126.9	10	11.6	10.3	5.1–19.2	<0.001
CK:Zn (i.u./mg)	32	62.5	41.4	5.7–369.6	11	160.8	107.3	64.3–380.4	<0.001
ATPase:Zn (i.u./mg)	72	115	103	11–478	10	103	113	59–137	>0.1

* By α -naphthol assay.

† Only samples from men with normal prostates are included (defined by Kavanagh & Darby, 1982).

Discussion

The sites at which ATPase or acid phosphatase could interfere in some of the common creatine kinase assays are shown in Text-fig. 1. Both of these enzymes are abundant in seminal and prostatic fluid. In practice, because of the high ATPase activity the NADH assay could not be used. The α -naphthol assay gives results that are the sum of creatine kinase and acid phosphatase activities, but the effect of acid phosphatase was overcome by using a concentration of creatine phosphate lower than that usually employed, incorporating L(+)-tartaric acid and including blanks which did not contain ADP. Under these conditions the assay was both accurate and reproducible.

In the NADP⁺ assay, both acid phosphatase and ATPase could, in principle, reduce the rate of production of NADPH. We found that addition of L(+)-tartaric acid to this system had little effect. The high ATPase activity could therefore account for the 3–4-fold difference in values obtained by the α -naphthol assay and the NADP⁺ method. Use of the NADP⁺ assay has given rise to reports of creatine kinase values in seminal fluid varying from 'little or no activity' (Rosalki, 1966) to about 10 times that of normal serum (Bostrom & Andersson, 1971; Soufir, 1979; Asseo *et al.*, 1981). These discrepancies could be due to slightly different assay conditions which might favour ATPase to a greater or lesser extent. The validity of the NADP⁺ assay is important in interpreting the value of studies aimed at investigating the relationship between seminal creatine kinase activity and determinants of semen quality (Bostrom & Andersson, 1971; Buitrago *et al.*, 1980; Asseo *et al.*, 1981).

Previous studies have provided conflicting information about the origin of seminal creatine kinase (using the NADP⁺ assay) (Bostrom & Andersson, 1971; Soufir, 1979; Buitrago *et al.*, 1980; Asseo *et al.*, 1981). In the present study the increased seminal creatine kinase/Zn ratio compared to prostatic creatine kinase/Zn ratio indicates that proportionately more seminal creatine kinase than zinc is non-prostatic in origin. Our results therefore favour multiglandular production of seminal creatine kinase.

ATPase activity has been demonstrated in prostatic and seminal fluids, associated with secretory granules and vesicles (Ronquist, Brody, Gottfries & Stegmayr, 1978a, b). Our results indicating a positive correlation of ATPase activity and zinc in prostatic fluid suggests a mechanistic link between them and hence with other prostatic secretory products (Kavanagh & Darby, 1982). This adds further support to the suggestion by Ronquist *et al.* (1978a, b) that this ATPase is closely concerned with the formation and release of prostatic secretory vesicles. These authors also suggested a prostatic origin for most of the seminal ATPase activity and our findings (Table 2) support this conclusion.

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