



## Non-enzymatic hydrolysis of creatine ethyl ester <sup>☆</sup>

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### ABSTRACT

The rate of the non-enzymatic hydrolysis of creatine ethyl ester (CEE) was studied at 37 °C over the pH range of 1.6–7.0 using <sup>1</sup>H NMR. The ester can be present in solution in three forms: the unprotonated form (CEE), the monoprotionated form (HCEE<sup>+</sup>), and the diprotionated form (H<sub>2</sub>CEE<sup>2+</sup>). The values of pK<sub>a1</sub> and pK<sub>a2</sub> of H<sub>2</sub>CEE<sup>2+</sup> were found to be 2.30 and 5.25, respectively. The rate law is found to be

$$\text{Rate} = -dC_{\text{CEE}}/dt = k_{++}[\text{H}_2\text{CEE}^{2+}][\text{OH}^-] + k_+[\text{HCEE}^+][\text{OH}^-] + k_0[\text{CEE}][\text{OH}^-]$$

where the rate constants  $k_{++}$ ,  $k_+$ , and  $k_0$  are  $(3.9 \pm 0.2) \times 10^6 \text{ L mol}^{-1} \text{ s}^{-1}$ ,  $(3.3 \pm 0.5) \times 10^4 \text{ L mol}^{-1} \text{ s}^{-1}$ , and  $(4.9 \pm 0.3) \times 10^4 \text{ L mol}^{-1} \text{ s}^{-1}$ , respectively. Calculations performed at the density functional theory level support the hypothesis that the similarity in the values of  $k_+$  and  $k_0$  results from intramolecular hydrogen bonding that plays a crucial role. This study indicates that the half-life of CEE in blood is on the order of one minute, suggesting that CEE may hydrolyze too quickly to reach muscle cells in its ester form.

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### Introduction

The efficacy of using creatine as a nutritional supplement for the development of lean muscle mass and strength in athletes has been thoroughly studied and well documented [1–7]. Based on these results, creatine is used widely in the athletic community. However, the bioavailability of creatine taken as a dietary supplement is limited because of its relatively poor absorption properties [8,9]. Furthermore, work by Schedel and coworkers shows that creatine levels in the blood stream reach a maximum of about 2.2 mM approximately 2.5 h after the ingestion of a single 20-g oral dose of creatine [10] indicating very slow absorption. As a result, users of the supplement tend to ingest large doses of creatine in order to attain the desired benefit.

Several patents for the synthesis of creatine ethyl ester (CEE) have been issued recently. Within the patents, it has been suggested that the bioavailability of creatine is improved when creatine ethyl ester is ingested instead of creatine [11,12]. The rationale presented in these patents is that functionalizing the carboxylic acid to the ethyl ester will decrease the “polarity” of the molecule thereby facilitating the transfer of the molecule across cellular membranes. In a recent article, appearing in a popular magazine commonly read by weightlifters and bodybuilders the science editor discussed “new forms of creatine” available to ath-

letes. The author writes, “Because CEE can be absorbed directly into muscle cells, it doesn’t need to rely on insulin and it doesn’t sit outside the muscle cells, which can cause bloating [13]”. Several manufacturers of nutritional supplements now sell CEE even though, as they indicate, their suppositions “have not been evaluated by the Food and Drug Administration”.

An underlying assumption of the claim that CEE can be absorbed into muscle cells more readily than creatine is that the ester can reach muscles without undergoing hydrolysis. However, the rate of hydrolysis of CEE to creatine and ethanol has not been studied. Clearly the rate of hydrolysis of CEE is a fundamental consideration in the evaluation of the claims that it is a better nutritional supplement than creatine.

Half a century ago, the kinetics of the hydrolysis of the esters of some amino acids was studied and the rate constants reported [14]. In that study, the kinetics of the alkaline hydrolysis of nine amino acid esters at 25° and an ionic strength of 0.1 M in water over a pH range from 8.7 to 11.6 was reported. The rate law was found to be of the form

$$\text{Rate} = -\frac{dC_A}{dt} = k_{+/-}[\text{HA}^+][\text{OH}^-] + k_{0/-}[\text{A}][\text{OH}^-]$$

where [A] represents the concentration of the unprotonated form of the amino acid ester, [HA<sup>+</sup>] represents the protonated form of the amino acid ester and C<sub>A</sub> represents the total concentration of the amino acid ester in both forms. The pK<sub>a</sub> of the amino acid esters without ionizable side chains ranged from 7 to 8. The values of  $k_{+/-}$  and  $k_{0/-}$  for histidine methyl ester, leucine ethyl ester and glycine methyl ester were found to be  $4 \times 10^4$  and  $4.0 \times 10^1$ ;  $5.0 \times 10^4$  and  $3.5 \times 10^1$ ,

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and  $3.5 \times 10^4$  and  $7.9 \times 10^1$ , respectively. In each of these cases,  $k_{+/-}$  is about two orders of magnitude greater than that of  $k_{0/-}$ . This is not unexpected as  $k_{+/-}$  represents a process involving the coming together of a cation and the hydroxide anion, while  $k_{0/-}$  represents the coming together of a neutral species with hydroxide ion. (Over the pH range studied, the histidine side chain remains essentially unprotonated.) In addition to the simple base ( $\text{OH}^-$ )-catalyzed hydrolysis of these esters, ester hydrolysis may also be catalyzed by enzymes [15,16].

Based on the non-enzymatic hydrolysis rate constants for the amino acid esters reported by Hay and coworkers [14], the half-lives of the amino acid esters at a physiological pH of 7.4 would be on the order of 10 h. Should CEE show the same or greater level of stability, then further experiments determining its efficacy would be of value. On the other hand, if the rate of hydrolysis is fast, then the issue of bioavailability becomes moot. It is the non-enzymatic hydrolysis of CEE that is the subject of this study. The objective is to determine the likelihood that CEE taken orally could make its way to skeletal muscle cells before being hydrolyzed to creatine and ethanol.

## Materials and methods

**Reagents.** Commercially available creatine ethyl ester hydrochloride was obtained from Higher Power Nutrition. NMR experiments showed no impurities in the creatine ethyl ester hydrochloride, and it was deemed to be sufficiently pure to be used without further purification. Solutions of sodium hydroxide were prepared by appropriate dilution from 50% NaOH obtained from VWR. The dilute solutions were standardized by titration of dry, certified potassium hydrogen phthalate obtained from Fisher Scientific. Solutions of hydrochloric acid were prepared by appropriate dilution from concentrated ACS Certified Plus HCl obtained from Fisher Scientific. All deuterated reactants were obtained from Aldrich. These include: phosphoric acid- $d_3$  85w% solution in  $\text{D}_2\text{O}$  98 atom%D, acetic acid- $d_4$  99.5 atom%D, deuterium oxide 99.9 atom%D, and sodium deuterioxide 40w% solution in  $\text{D}_2\text{O}$  99+ atom%D.

**Acid/base experiments.** The overall rate of hydrolysis should depend on the extent to which CEE is protonated in the solution, and thus be pH dependent. All pH measurements were made using a model 800 Fisher Accumet pH meter. Solutions were prepared by accurately weighing samples of solid creatine ethyl ester hydrochloride ( $195.68 \text{ g mol}^{-1}$ ) and dissolving them into a known volume of high-purity water. These solutions were titrated with standard NaOH solutions and the pH was recorded as a function of volume of NaOH added. Analysis of the data suggested that the initial salt was amphoteric. A subsequent pH titration was performed in which a 1.0 molar equivalent of creatine ethyl ester hydrochloride was added to 1.3 molar equivalents of standardized hydrochloric acid and titrated with standard NaOH. The pH was recorded as a function of volume added and the resulting titration curve is shown in Fig. 1.

**Kinetic experiments.** The rate of hydrolysis of CEE was determined by measuring the  $^1\text{H}$  NMR spectrum as a function of time using a Varian 400MR. The initial spectrum shows a triplet centered at 1.20 ppm relative to TMS due to the  $\text{CH}_3$  group on the ethyl ester. A second triplet centered at 1.09 ppm relative to TMS appears, increases with time, and is assigned to the  $\text{CH}_3$  group of the ethanol that is produced upon hydrolysis. The appearance and growth of this triplet coincides with the disappearance of the triplet at 1.20 ppm. The area under the triplet at 1.20 ppm was integrated and a plot of  $\ln(A_t - A_{\text{inf}})$  versus  $t$  was constructed. The area under the triplet at time  $t$  is  $A_t$  and  $A_{\text{inf}}$  is the integrated area over the same region following the complete hydrolysis. Rate constants were obtained from the slope of the line.

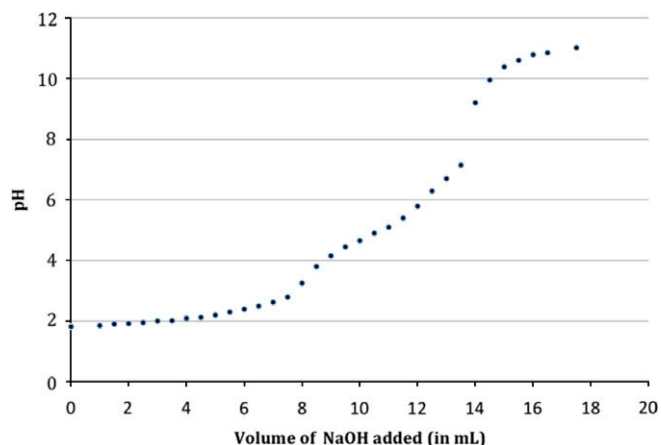


Fig. 1. The calculated values of the acid dissociation constants for the ester from a best fit of the data are  $\text{p}K_{a1} = 2.30$  and  $\text{p}K_{a2} = 5.25$ .

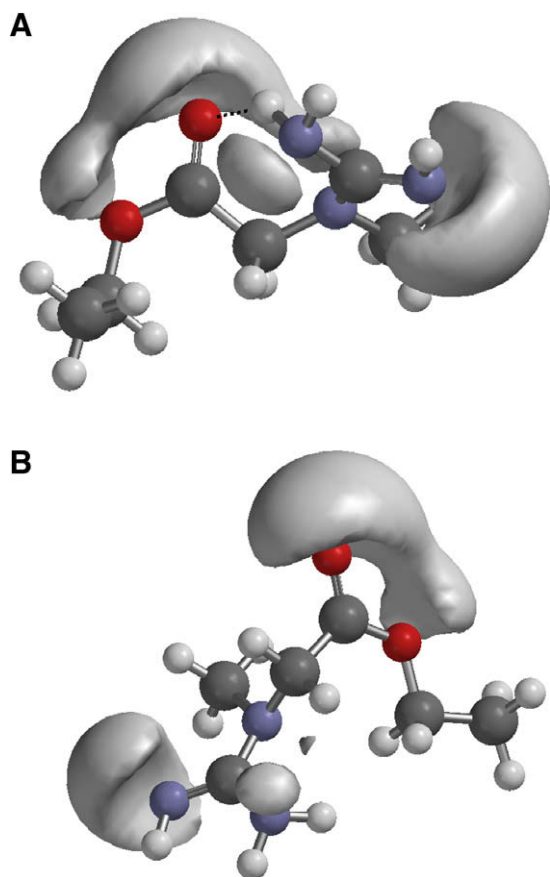
In order to use NMR to measure the extent of hydrolysis, deuterated solvents and buffers are required. Solutions for kinetic experiments were prepared by using varying ratios of phosphoric acid- $d_3$  85w% solution in  $\text{D}_2\text{O}$  (or acetic acid- $d_4$  99.5 atom%D) to sodium deuterioxide 40w% solution in  $\text{D}_2\text{O}$  99+ atom%D in deuterium oxide 99.9 atom%D. The  $\text{p}^2\text{H}$  values were measured using the model 800 Fisher Accumet pH meter. It is important to remember that there will be a kinetic isotope effect on the hydrolysis reaction. The mechanism of hydrolysis involves the nucleophilic attack of the carbonyl carbon atom by either hydroxide ion or water. Since there is no O–H bond breaking involved in the rate determining step, the effect of replacing the hydrogen atom of hydroxide ion with a deuterium atom should be relatively small ( $m_{\text{OD}^-}/m_{\text{OH}^-} = 1.06$ ) and well within the uncertainty of the experimentally-measured rate constants. Additionally, while  $\text{p}^2\text{H}$  values obtained from  $\text{D}_2\text{O}$  solutions will not be equal exactly to pH, the differences are not significant. Other studies using NMR to obtain rate data in deuterated solvents show that the effects of using a deuterated solvent are small [17].

**Computational methodology.** Theoretical structures and energies reported in this paper were obtained using SPARTAN'06 [18]. Conformational analysis on CEE was performed using MMFF94 molecular mechanics calculations. A Monte-Carlo molecular dynamics technique was used to identify a library of conformations. For each conformation, a single point energy calculation was performed using the Hartree–Fock model at the 3-21G level, and the solvation energy for each species was determined using the Cramer/Truhlar SM5.4 solvation model. Equilibrium structures and energies were obtained using B3LYP density functional theory and the 6-31G\* basis set for only the lowest energy conformers.

## Results

### Acid–base properties

That CEE can undergo two protonation steps in aqueous solution can be seen clearly in Fig. 1. Based on the concentrations and volumes used in the titration the values of  $\text{p}K_{a1}$  and  $\text{p}K_{a2}$  were found to be 2.30 and 5.25, respectively. To identify the most likely sites of protonation, CEE was modeled and the potential surface at a value of  $-160 \text{ kJ mol}^{-1}$  was mapped. The results, shown in Fig. 2, are for two conformations: the lowest energy conformer (in which there is significant intramolecular hydrogen bonding) and a higher energy conformer (in which there is no intramolecular hydrogen bonding). The surfaces suggest strongly that the two most likely sites of protonation will be the guanidinium functionality and



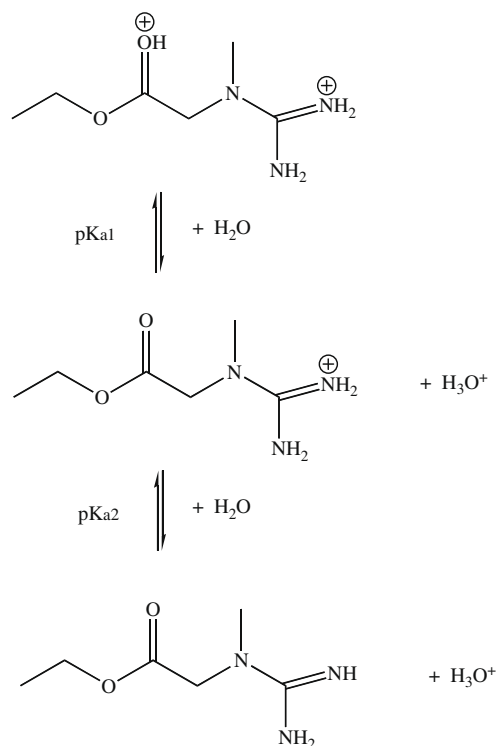
**Fig. 2.** Electrostatic potential surface of two conformers of CEE: (A) a conformer with significant intra-molecular hydrogen bonding (dashed line) between the guanidinium functionality and the carbonyl oxygen atom, and (B) a conformer with no intramolecular hydrogen bonding. The potential surface is for a potential of  $-160 \text{ kJ mol}^{-1}$ .

the carbonyl oxygen of the ester. Modeling the possible doubly-protonated species ( $\text{H}_2\text{CEE}^{2+}$ ) and comparing the energy of each species support that conclusion. That is, the species in which the guanidinium functionality and the carbonyl oxygen atom are protonated is  $27 \text{ kJ mol}^{-1}$  more stable than that in which guanidinium functionality and ether oxygen atom are protonated, and  $57 \text{ kJ mol}^{-1}$  more stable than that in which guanidinium functionality and the asymmetric nitrogen atom are protonated. For the mono-protonated species ( $\text{HCEE}^+$ ), protonation of the guanidinium functionality is about  $240 \text{ kJ mol}^{-1}$  more stable than protonation of the carbonyl oxygen atom. As a result, the predominant species in aqueous solution are expected to be those shown in Fig. 3.

#### Kinetic experiments

The experimental conditions and experimental first order rate constants for each experiment are shown in Table 1. The observed rate law for the hydrolysis of the ethyl ester of amino acids without ionizable side chains consists of two terms. One term is first order in hydroxide and the protonated form of the ester. The other term is other first order with respect to the hydroxide ion and the unprotonated form of the amino acid ester [14]. Since there are three possible species of CEE with varying degrees of protonation, the data were fit to the rate equation

$$\text{Rate} = -d\text{CEE}/dt \\ = k_{++}[\text{H}_2\text{CEE}^{2+}][\text{OH}^-] + k_+[ \text{HCEE}^+ ][\text{OH}^-] + k_0[\text{CEE}][\text{OH}^-]$$



**Fig. 3.** The predominant species in solution depending on pH as determined from density functional B3LYP calculations. Only one of the two equivalent resonance structures is shown for the mono- and di-protonated species.

**Table 1**

The experimental  $\text{p}^2\text{H}$ , observed pseudo-first-order rate constants (at constant  $\text{p}^2\text{H}$ ), and calculated rate constants (based on rate constants and  $\text{pK}_a$  values reported in the text) for the non-enzymatic hydrolysis of creatine ethyl ester. The  $[\text{O}^2\text{H}^-]$  is approximated from the quotient,  $10^{-14}/10^{-\text{p}^2\text{H}}$ .

$\text{p}^2\text{H}$	$[\text{O}^2\text{H}^-]$ (M)	$k(\text{obs}) \text{ expt. (s}^{-1}\text{)}$	$k(\text{obs}) \text{ calc. (s}^{-1}\text{)}$	% Difference
1.6	$4.0 \times 10^{-13}$	$8.1 \times 10^{-7}$	$1.3 \times 10^{-6}$	58
2.1	$1.3 \times 10^{-12}$	$1.5 \times 10^{-6}$	$3.0 \times 10^{-6}$	103
2.4	$2.5 \times 10^{-12}$	$2.2 \times 10^{-6}$	$4.3 \times 10^{-6}$	96
3.2	$1.4 \times 10^{-11}$	$1.1 \times 10^{-5}$	$7.1 \times 10^{-6}$	-50
3.5	$3.2 \times 10^{-11}$	$2.3 \times 10^{-5}$	$8.1 \times 10^{-6}$	-187
4.0	$1.0 \times 10^{-10}$	$1.6 \times 10^{-5}$	$1.1 \times 10^{-5}$	-49
4.3	$2.0 \times 10^{-10}$	$9.3 \times 10^{-6}$	$1.4 \times 10^{-5}$	47
4.7	$4.5 \times 10^{-10}$	$1.1 \times 10^{-5}$	$2.2 \times 10^{-5}$	97
5.2	$1.6 \times 10^{-9}$	$9.8 \times 10^{-5}$	$6.8 \times 10^{-5}$	-43
5.2	$1.6 \times 10^{-9}$	$6.5 \times 10^{-5}$	$6.8 \times 10^{-5}$	6
5.9	$7.1 \times 10^{-9}$	$3.6 \times 10^{-4}$	$3.3 \times 10^{-4}$	-12
6.2	$1.6 \times 10^{-8}$	$6.2 \times 10^{-4}$	$7.5 \times 10^{-4}$	21
6.3	$2.0 \times 10^{-8}$	$2.2 \times 10^{-3}$	$9.5 \times 10^{-4}$	-127
7.0	$1.0 \times 10^{-7}$	$2.2 \times 10^{-3}$	$4.9 \times 10^{-3}$	119

where  $\text{C}_{\text{CEE}}$  represents total ester concentration in all three forms. The optimal values for the rate constants  $k_{++}$ ,  $k_+$ , and  $k_0$  were obtained from a least squares fit of the experimental data to the three-term rate law using the  $\text{pK}_a$  values determined from the titration data. The resulting values of  $k_{++}$ ,  $k_+$ , and  $k_0$  at  $37^\circ\text{C}$  were found to be  $(3.9 \pm 0.2) \times 10^6 \text{ L mol}^{-1} \text{ s}^{-1}$ ,  $(3.3 \pm 0.5) \times 10^4 \text{ L mol}^{-1} \text{ s}^{-1}$ , and  $(4.9 \pm 0.3) \times 10^4 \text{ L mol}^{-1} \text{ s}^{-1}$ , respectively. The fit to the experimental data using these rate constants and the  $\text{pK}_a$  values reported above is shown in Fig. 4.

#### Discussion

As with free creatine, the first site of protonation (the most basic site) of CEE is the guanidinium functionality. However, the  $\text{pK}_a$

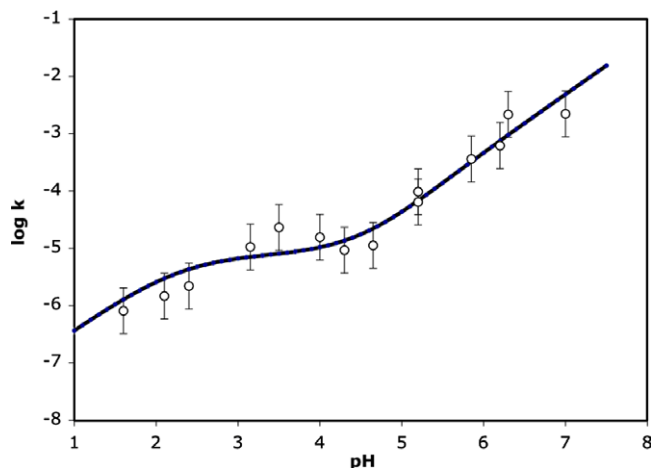


Fig. 4. The fit to the experimental data (open circles) by the calculated rate constant obtained from the least squares fit of the three-term rate law described in the text.

for the deprotonation of this site (5.25 at 25 °C) is seven  $pK_a$  units smaller than that reported by Wang et al. for free creatine (12.90 at 30 °C) [19]. This difference suggests that the free energy change for the transfer of a proton from the protonated guanidinium functionality of creatine is about  $40 \text{ kJ mol}^{-1}$  more positive than that for the transfer of a proton from the protonated guanidinium functionality of HCEE<sup>+</sup>. In comparison, there is a decrease of about two  $pK_a$  units for most amino acid esters compared to the underivatized amino acid [14]. Thus for amino acids and their esters, the difference in free energy for ionization is about  $10 \text{ kJ mol}^{-1}$ . Margaret Robson Wright suggested that the decrease in  $pK_a$  results primarily from the entropy change associated with the ionization of a zwitterionic amino acid to produce the anion of the amino acid and hydronium cation [20]. Her argument is that this entropy change would be much larger than that for the ionization of the protonated ester to form a neutral species and the hydronium cation.

Analyzing the conformers of CEE obtained from modeling experiments, a significant number of conformers show intramolecular hydrogen bonding. One such conformer for CEE is shown in Fig. 2A. For the amino acid ethyl esters, it is assumed that  $\Delta H^\circ$  for the transfer of the proton to water is very small. Since the entropic arguments for creatine and its ester would be analogous to that for an amino acid, a similar change in  $pK_a$  might have been expected. However, the change in  $pK_a$  between that of the protonated creatine ethyl ester (HCEE<sup>+</sup>) and protonated creatine is significantly larger than that observed for the amino acid esters. Models of creatine and its ethyl ester derivative show the presence of significant hydrogen bonding between a hydrogen atom on the guanidinium functionality to the carbonyl oxygen atom. Arguably, the hydrogen bond to the oxygen atom of a deprotonated carboxylic acid, as in creatine, should be stronger than that to the carbonyl oxygen atom of the ester. The presence of this strong hydrogen bond stabilizes the zwitterionic creatine molecule much more than it does the ester. As a result, the  $\Delta H^\circ$  for the deprotonation of the creatine zwitterion should be much more positive than it is for the deprotonation of the creatine ethyl ester. Thus there is both an enthalpic and entropic effect that serves to make the guanidinium functionality much more acidic in the ester than it is in the creatine zwitterion. Furthermore, the presence of this intramolecular hydrogen bonding should affect the electrophilicity of the carbonyl carbon atom in creatine ethyl ester and thereby affect the rate of hydrolysis.

This intramolecular hydrogen bond manifests itself in another way: the values of  $k_+$  and  $k_0$  in the observed rate law are of the same order of magnitude. This is in sharp contrast to those ob-

served for the corresponding rate constants for histidine methyl ester, leucine ethyl ester and glycine methyl ester in which the value of  $k_+$  is two orders of magnitude greater than that of  $k_0$  [14]. The base catalyzed mechanism most likely involves nucleophilic attack of the carbonyl carbon by the hydroxide ion. With the presence of intramolecular hydrogen bonding, the carbonyl oxygen atom can be considered to be partially protonated in both CEE and HCEE<sup>+</sup>. As a result, the carbonyl carbon atom has a similar electronic structure in both species resulting in a similar rate constant. For H<sub>2</sub>CEE<sup>2+</sup>, the carbonyl oxygen atom is fully protonated, making the carbonyl carbon much more susceptible to attack. This is reflected in a rate constant that is two orders of magnitude larger than that for CEE or HCEE<sup>+</sup>.

Manufacturers have made the claim that for the same dose, more creatine can be absorbed into muscle cells from CEE than from creatine monohydrate. The results of this work cast serious doubts on this claim, and raise the possibility that little or no CEE will survive transport to reach muscle cells. The pH of saliva usually falls between 6.0 and 7.4, while the pH of the stomach may go as low as 1.0 [21]. Because of the buffering capacity of proteins when the stomach is filled with food, the pH of the stomach may rise to 3.0 or 4.0. When compounds reach the small intestine, they are exposed to mucosal tissue with a pH of nearly 8.5. The pH of blood is maintained in a very narrow range from 7.35 to 7.40 [21]. Since athletes using creatine ethyl ester take it orally, and given the  $pK_a$  values found in this work, the molecule predominately will be neutral (unprotonated) throughout the digestive system with the exception of the stomach. Table 2 shows the half-life of the ester as a function of pH calculated from the experimental rate law. Based on these values, it is reasonable to assume that, in the absence of any enzymatic hydrolysis, the molecule should remain intact until it reaches the small intestine. Depending on the rate of absorption into the blood stream, the molecule will have a half-life of somewhere between 14 s and 1 min. To put this into perspective, assuming that the molecule is absorbed from the digestive system to the blood stream before reaching the small intestine (which seems unlikely given that it will be cationic in the stomach) over 99% of the ingested CEE will be hydrolyzed to creatine and ethanol within 7 min. While the neutral ester probably would transport through the muscle cell membranes more efficiently than the zwitterionic creatine should it ever reach them, CEE is very unstable in the blood, and may be degraded long before it is effectively distributed to muscles. As a result, it seems highly unlikely that supplementation with CEE would be in any way superior to supplementation with creatine monohydrate. Furthermore, in a study to be published soon [22], it was found that while the total muscle creatine content was significantly higher in athletes taking CEE and creatine monohydrate compared to a control group without any creatine supplementation, there were no differences between the CEE group and the group supplementing with creatine monohydrate. The results of this work may in fact explain the conclusions reached in that study.

Table 2

The calculated half-life of creatine ethyl ester as a function of pH based on the calculated parameters obtained for the non-enzymatic hydrolysis of the ester.

pH	Half-life
8	14 s
7.4	56 s
7	2 min
6	25 min
5	4 h
4	18 h
3	1 day
2	3 days
1	22 days

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