*Biochimica et Biophysica Acta*, 293 (1973) 150–159


BBA 66796

# MULTIPLE EFFECTS OF IONS ON ATP: L-ARGININE AND ATP: CREATINE PHOSPHOTRANSFERASES

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(Received May 31st, 1972)
(Revised manuscript received September 18th, 1972)

## SUMMARY

1. Cations inactivate *Sipunculus* arginine kinase (mol. wt 84 000) (ATP: L-arginine phosphotransferase, EC 2.7.3.3). The order of increasing effectiveness is $K^+$, $NH_4^+$, $Li^+$, guanidinium cation, tetravalent spermine cation. The action of lithium acetate and guanidine·HCl is expressed by a two-step inhibition: an initial immediate inhibition which is of the non-competitive type with respect to L-arginine and $Mg^{2+}$–ATP, and a slower inactivation. While the first stage can be observed at 10 °C, the rate of the secondary one increases with temperature. This inhibition is similar to that observed with anions. The modification of the protein absorption spectrum which characterizes the action of anions is displayed with guanidine·HCl and spermine·4HCl but not with $K^+$, $NH_4^+$, $Li^+$.

2. The effects of KCl on *Sipunculus* arginine kinase in $H_2O$ and in $^2H_2O$ are qualitatively similar but quantitatively different. In $^2H_2O$ the decrease of $V$ by KCl is greater than in $H_2O$. However, the modification of the protein absorption spectrum is less sharp in $^2H_2O$ than in $H_2O$.

3. Rabbit muscle creatine kinase (ATP:creatine phosphotransferase, EC 2.7.3.2) shows a susceptibility towards anions and cations comparable to that of *Sipunculus* arginine kinase. The order of effectiveness of anions and cations on both enzymes is the same. The kinetic behaviour of the action of $Cl^-$ is identical for both enzymes. Nevertheless no modification of the protein absorbance spectrum is observed by the interaction of $Cl^-$.

## INTRODUCTION

In a recent report[1], we have described the effects of anions on three arginine kinases (ATP: L-arginine phosphotransferase, EC 2.7.3.3), the one from *Homarus* (mol. wt 43 000) and the other two (mol. wt 84 000) from *Sipunculus* and *Solen*. The *Sipunculus* enzyme has been shown to be particularly advantageous for this study. The action of anions on these three arginine kinases exhibited an initial phase of fast inhibition, which is of non-competitive type. Furthermore, with the *Sipunculus*

enzyme only, the immediate inhibition was followed by a second step of slow inhibition, concomitant with a modification of the protein absorption spectrum.

In the present work, the effects of cations on *Sipunculus* arginine kinase are studied in comparison with those displayed by anions. Furthermore the effects of KCl on *Sipunculus* enzyme in $H_2O$ are compared with those obtained in $^2H_2O$.

Since the activity of creatine kinase (ATP:creatine phosphotransferase, EC 2.7.3.2) is slowly decreased by the action of anions, as recently shown by Milner-White and Watts[2], it appeared interesting to know if, in experimental conditions different from those used by these authors, the kinetic behaviour of the inhibition of this enzyme was similar to that observed in the case of *Sipunculus* enzyme and could be interpreted as the manifestation of a destabilization of the protein structure by ions.

## MATERIALS

Dilithium ADP, disodium ATP, disodium NADH, phosphoenolpyruvate, crystalline rabbit muscle lactate dehydrogenase (L-lactate:NAD oxidoreductase, EC 1.1.1.27), crystalline rabbit muscle pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) were obtained as previously described[1]. Potassium, ammonium and lithium salts were analytical reagent grade and they were used without further purification. Guanidine·HCl was obtained from Mann and spermine·4HCl from Hoffmann-La Roche. 99.84% $^2H_2O$ was purchased from C. E. A., Saclay, France. *Sipunculus* body muscle arginine kinase and rabbit muscle creatine kinase were prepared as described[3,4].

## METHODS

### *Preparation of salt solutions*

Neutral potassium salts, guanidine·HCl and spermine·4HCl solutions were prepared by dissolving the salts in distilled water. Potassium, ammonium and lithium acetate solutions were adjusted with acetic acid to pH 7.6.

### *Protein concentration*

Protein concentration was calculated from the absorbance at 280 nm using $E^{1\%}_{1\,cm} = 10$ and 8.8 for *Sipunculus* arginine kinase[5] and rabbit creatine kinase[6], respectively.

### *Enzyme assay*

Both arginine kinase and creatine kinase activities were measured at pH 7.6 in the forward reaction ($Mg^{2+}$–ATP + arginine or creatine) by estimating the rate of ADP formation with the coupled enzyme method (pyruvate kinase and lactate dehydrogenase) as previously indicated[1]. Creatine kinase solution was diluted in 0.01 M Tris–acetate buffer (pH 7.6), arginine kinase solution was diluted in the same buffer containing 0.1 M 2-mercaptoethanol.

Allowance was not made for the capacity of monovalent cations to form complexes with $ATP^{4-}$ in the calculation of Mg–$ATP^{2-}$ concentrations, but an excess of 1 mM of $Mg^{2+}$ was always present in the incubation mixtures. Thus, the apparent dissociation constant of Mg–$ATP^{2-}$ in the presence of the highest KCl concentration

(0.3 M) becomes 0.0724 mM when the values used for the dissociation constants of K–ATP$^{3-}$ and Mg–ATP$^{2-}$ complexes in the 0.1 M Tris buffer were 100 (ref. 7) and 0.0181 mM (ref. 8), respectively. Consequently, the reduction of Mg–ATP$^{2-}$ concentrations is 6.6%. This makes a negligible contribution to the decrease of the maximum velocity.

The coupled enzyme system of pyruvate kinase–lactate dehydrogenase is affected to some extent by high concentrations of salts and of $^2H_2O$. Accordingly, we have used high concentrations of enzymes and substrates: 30 $\mu$g/ml of both pyruvate kinase and lactate dehydrogenase, 1 mM phosphoenolpyruvate, 0.15–0.20 mM NADH. The linearity of the recording obtained in these conditions ascertained the correctness of the ADP estimation.

*Slow inhibition stage studies and ultraviolet difference spectra measurements*

These measurements were made as mentioned[1].

*Comparative experiments in $^2H_2O$ and $H_2O$*

*Preparation of Sipunculus arginine kinase in $^2H_2O$ and $H_2O$. Sipunculus* arginine kinase solution was divided between two Visking dialysis tubes and dialyzed with magnetic stirring against two changes of 20 volumes of 0.05 M Tris–acetate buffer containing 0.1 M 2-mercaptoethanol (pH or p$^2$H 7.5), 18 h at 10 °C and centrifuged. Enzyme concentration was determined. The enzyme solution was diluted in 0.01 M Tris–acetate, 0.1 M 2-mercaptoethanol buffer at the appropriate pH or p$^2$H.

*Enzyme assay.* In the comparative experiments, the procedure was the same for $^2H_2O$ and $H_2O$. The previously described enzyme assay[1] was slightly modified because the stock solutions of pyruvate kinase and lactate dehydrogenase in $^2H_2O$ were rapidly inactivated. The mixture of pyruvate kinase and lactate dehydrogenase (300 $\mu$g each) was made in aqueous 0.01 M Tris–acetate buffer (pH 7.6).

Assays were carried out in a 1-ml cell of 1-cm light path with the following mixture in $^2H_2O$ or $H_2O$: 100 mM Tris–acetate (at the appropriate pH or p$^2$H), 10 mM KCl, 1 mM magnesium acetate, 0.15–0.20 mM NADH, 1 mM phosphoenolpyruvate, with KCl and substrates ($Mg^{2+}$–ATP, L-arginine·HCl) as indicated in the text. Blank cells contained distilled water.

The sample cell, the mixture of pyruvate kinase and lactate dehydrogenase, and the enzyme solution were equilibrated for 5 min at the desired temperature. Then 100 $\mu$l of the pyruvate kinase and lactate dehydrogenase mixture were added to 0.8 ml of the medium and mixed. The reaction was started immediately by the addition of 100 $\mu$l of enzyme solution, and the decrease in absorbance recorded after rapidly mixing. Note; we have further observed that it is not necessary to previously dialyze the arginine kinase against $^2H_2O$, since the $^2H_2O$ effect is immediate. Therefore, the enzyme prepared in $H_2O$ was directly introduced into the $^2H_2O$ reaction mixture. Under these conditions, the activity of the extemporaneous enzyme–$^2H_2O$ was slightly higher than that of the enzyme–$^2H_2O$ prepared by dialysis. Probably, the mechanism of the $^2H_2O$ effect on the enzyme would continue, after the rapid inhibition stage, with a very slow inhibition stage at 10 °C. Nevertheless, the KCl inhibition percentage of the extemporaneous enzyme–$^2H_2O$ was of the same order as that is reported in the text for the enzyme–$^2H_2O$ obtained by dialysis.

RESULTS

*Effects of cations on Sipunculus arginine kinase*

*Effectiveness of cations.* The tested cations $K^+$, $NH_4^+$, $Li^+$ were acetate salts in order to minimize the effect of anions. Guanidinium and spermine were hydrochloride and tetrahydrochloride salts, respectively. The increasing order of inhibition potency corresponds to the series: $K^+ < NH_4^+ < Li^+ \approx$ guanidinium$^+$ < spermine$^{4+}$ (Fig. 1).

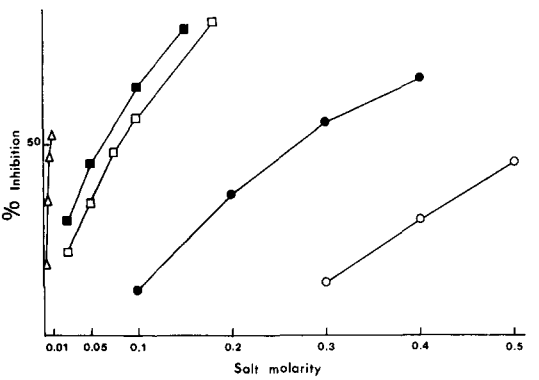



Fig. 1. Inhibition of *Sipunculus* arginine kinase by cations. Temp. 10 °C, 1 mM arginine, 0.3 mM $Mg^{2+}$–ATP (pH 7.6). ○—○, potassium acetate; ●—●, ammonium acetate; □—□, lithium acetate; ■—■, guanidine·HCl; △—△, spermine·4HCl.

*Kinetics of inhibition by cations.* Immediate inhibition stage. The inhibition occurs immediately after the addition of the enzyme to the salt solution. The inhibition by lithium acetate and guanidine·HCl from 0.05 to 0.20 M is of a non-competitive type with respect to arginine and $Mg^{2+}$–ATP, and with $K_m/V$ and $1/V$ being parabolic according to Cleland[9] (Fig. 2). It is necessary to specify that guanidine cannot act as an analogue of arginine, the specificity of these arginine kinases being very strict[3,10]. In fact, with arginine as the variable substrate, the plot of guanidine inhibition is identical to Fig. 2.

Slow inhibition stage. Following the immediate inhibition there is a progressive decrease of activity under conditions of non-saturating substrates, 1 mM arginine and 0.2 mM $Mg^{2+}$–ATP. It becomes more intense with increasing temperature; it is not noticeable between 10 °C and 15 °C, and becomes more marked from 15 °C to 25 °C. Thus, the decrease of activity is not detectable at 15 °C after 25 min with 0.2 M lithium acetate nor at 17 °C after 10 min with 0.12 M guanidine·HCl. However, it is perceptible with 0.2 M lithium acetate at 20 °C and 25 °C after 14 min and 11 min, respectively, with 0.12 M guanidine·HCl at 25 °C after 2 min, and with 0.3 M ammonium acetate at 25 °C after 8 min.

*Spectrophotometric analysis of the interaction of the enzyme with cations.* At 20 °C and 25 °C, 0.20 M and 0.40 M lithium and ammonium acetates promote no modification of the protein absorbance spectrum. In contrast, in the presence of 0.1 M guanidine·HCl and 0.02 M spermine·4HCl at 20 °C, the difference spectrum obtained is identical to that observed with anions[1]. It is characterized by a negative peak at

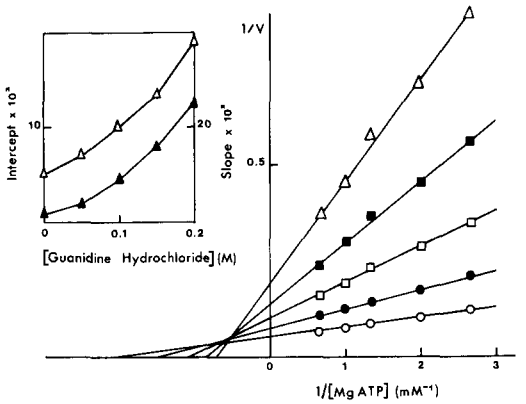



Fig. 2. Kinetics of the inhibition of the forward reaction by guanidine·HCl. Temp. 10 °C, 10 mM arginine as the fixed substrate and 0.375, 0.5, 0.75; 1, 1.5 mM $Mg^{2+}$–ATP as the variable substrate. Primary plots; the concentrations of guanidine·HCl added were: ○—○, none; ●—●, 0.05 M; □—□, 0.1 M; ■—■, 0.15 M; △—△, 0.2 M. Secondary plots: △—△, ordinate intercepts; ▲—▲, slopes.

288 nm with a shoulder at 280 nm. A control made with 0.10 M KCl gives rise only to a scarcely perceptible trace of a difference spectrum.

*Comparative study of the effects of KCl on Sipunculus arginine kinase in $H_2O$ and $^2H_2O$*

*Kinetics of inhibition by KCl.* Fast inhibition stage. The comparative study of the effect of 0.30 M KCl on the kinetic constants in $H_2O$ and $^2H_2O$ performed in the pH and p$^2$H range from 7.0 to 8.0 brings out the following results (Fig. 3).

The decrease of $V$ by 0.30 M KCl, for each of the tested pH values, is 20–22% in $H_2O$ but is greater, 38–43%, in $^2H_2O$. But the increase of $K_m$ for $Mg^{2+}$–ATP is of the same order in $H_2O$ as in $^2H_2O$. It may be noted that the simple transfer of the enzyme from $H_2O$ to $^2H_2O$ leads to a 27% decrease in $V$, allowance being made for the p$^2$H optimum shift from 7.5 to 8.0. (Table I).

Slow inhibition stage. With 0.30 M KCl at 25 °C, in the presence of 1 mM arginine and 0.3 mM $Mg^{2+}$–ATP, we observe the same phenomenon of a progressive de-

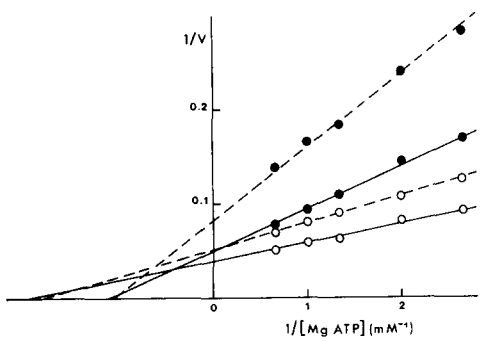



Fig. 3. Solvent dependence of the inhibition of the forward reaction of *Sipunculus* arginine kinase by KCl. Temp. 15 °C, 10 mM arginine as the fixed substrate, and 0.375, 0.5, 0.75, 1, 1.5 mM $Mg^{2+}$–ATP as the variable substrate. ———, $H_2O$, pH 7.5; — — —, $^2H_2O$, p$^2$H 8.0. Concentrations of KCl added: ○, none; ●, 0.3 M.

TABLE I

EFFECT OF 0.3 M KCl ON APPARENT KINETIC PARAMETERS OF *Sipunculus* ARGININE KINASE IN $H_2O$ AND IN $^2H_2O$

Temp. 15 °C; 10 mM arginine as the fixed substrate and 0.375, 0.5, 0.75, 1, 1.5 mM $Mg^{2+}$–ATP as the variable substrate. $K_m$: $Mg^{2+}$–ATP apparent $K_m$ (mM); $V$: maximum velocity ($\mu$moles/mg per min).

| *pH* | $H_2O$ | | | | $^2H_2O$ | | | |
|---|---|---|---|---|---|---|---|---|
| | *Native enzyme* | | *0.3 M KCl–enzyme* | | *Native enzyme* | | *0.3 M KCl–enzyme* | |
| *p²H* | $K_m$ | $V$ | $K_m$ | $V$ | $K_m$ | $V$ | $K_m$ | $V$ |
| 7.0 | 0.57 | 23 | 1.0 | 18 | 0.55 | 11 | 0.90 | 6.6 |
| 7.5 | 0.51 | 25 | 0.91 | 20 | 0.50 | 16 | 0.85 | 9.1 |
| 8.0 | 0.57 | 23 | 1.0 | 18 | 0.57 | 19 | 0.95 | 12 |

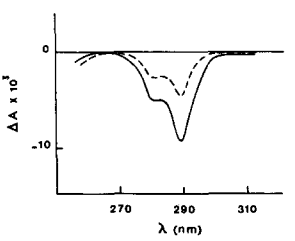
crease of activity in both $^2H_2O$ and $H_2O$. We have not been able to determine precisely the relative importance of these inhibitions. Furthermore, in $^2H_2O$ the activity of the native enzyme decreases with time while that of the native enzyme does not vary in $H_2O$.

Spectrophotometric analysis. The difference spectra of the effect of 0.30 M KCl on the enzyme at 20 °C display the same characteristics in both $H_2O$ and $^2H_2O$. The sole difference lies in the amplitude of the spectra, which is lower in $^2H_2O$ than in $H_2O$ (Fig. 4).

*Effect of ions on creatine kinase*

The order of effectiveness of inhibition of creatine kinase corresponds to the series: acetate anion $<$ $Cl^-$ $<$ $SCN^-$ $\approx$ $SO_4^{2-}$ $<$ $NO_3^-$ for anions, and $K^+$ $<$ $NH_4^+$ $<$ $Li^+$ for cations (Fig. 5).

The immediate inhibition by KCl, between 0.05 M to 0.3 M at 10 °C, pH 7.6,



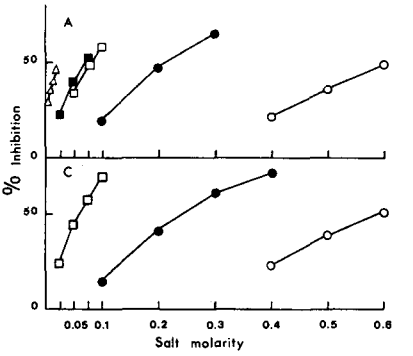




Fig. 4. Difference spectra between native *Sipunculus* arginine kinase and $Cl^-$-treated enzyme. Enzyme 1.36 mg/ml, 0.05 M Tris–acetate, 0.3 M KCl (pH 7.6), temp. 20 °C. ———, $H_2O$; – – –, $^2H_2O$.

Fig. 5. Inhibition of rabbit muscle creatine kinase by anions and cations. Temp. 10 °C, 10 mM creatine, 0.3 mM $Mg^{2+}$–ATP (pH 7.6). A. Inhibition by anions: ○—○, potassium acetate; ●—●, KCl; □—□, $K_2SO_4$; ■—■, KSCN; △—△, $KNO_3$. B. Inhibition by cations: ○—○, potassium acetate; ●—●, ammonium acetate; □—□, lithium acetate.

with 40 mM creatine and $Mg^{2+}$–ATP varying from 0.375 mM to 1.5 mM, is of a non-competitive parabolic type. Thus it is of the same profile as that observed with arginine kinases.

The progressive inhibition by 0.20 M KCl was observed between 15 °C and 30 °C in the following conditions: 0.3 mM $Mg^{2+}$–ATP, 2 mM creatine, pH 7.6 and 7 $\mu$g creatine kinase. Its intensity increases with temperature but less markedly than in the case of *Sipunculus* enzyme.

The absorbance spectrum of the enzyme (1.35 mg/ml) is not modified by the action of 0.30 M KCl at 23 °C. pH 7.6, after 15 min.

The difference spectra of the binding of $Mg^{2+}$–ATP (0.114 mM) on the native enzyme (1.5 mg/ml) and on the enzyme–KCl 0.30 M in the presence of 40 mM creatine exhibit the same characteristics. Only a decrease of the amplitude of the peaks by the effect of salt is noted.

## DISCUSSION

### *Action of cations on Sipunculus arginine kinase*

The great susceptibility of *Sipunculus* arginine kinase to inhibition by $K^+$, $NH_4^+$, $Li^+$ could be presumed from the action of anions on the catalytic properties of this enzyme[1]. Guanidine·HCl shows a higher effectiveness than lithium acetate. However, if we admit the additivity of the actions of $Cl^-$ and guanidinium cations, the potency of guanidinium cation should be of the same order or slightly lower than that of $Li^+$. Emphasis must be laid on the excessive susceptibility of the enzyme towards spermine, which could not be explained by the mere consideration that spermine is a tetravalent cation. The great effectiveness is most likely related to the length of the hydrocarbon chain of the molecule. Among other examples allowing this interpretation we quote that of tetraalkylammonium ions, the effectiveness of which on ribonuclease increases with the length of the hydrocarbon chain[11].

The kinetic behaviour of inhibition by cations mimics that of anions[1]. It is characterized by an immediate inhibition of a complex non-competitive type followed by a progressive stage of inactivation. While the first stage is slightly influenced by temperature between 5 and 25 °C, the second one is much more temperature dependent. As in the case of anions, the characteristics of the slow inhibition by cations suggests that it proceeds by a mechanism different from that of the initial effect.

We have reported previously in respect of the effects of anions on *Sipunculus* arginine kinase that the optical rotatory dispersion and the sedimentation coefficient of this enzyme in the presence of 0.3 M and 0.4 M KCl, respectively, did not show any change in respect to the native enzyme[1]. Accordingly, we think that cations used at relatively low concentrations should promote no dissociation but only limited conformation changes.

However, it appears that the conformational change induced by cations might be different from that promoted by anions. The progressive inactivation stage resulting from the action of $K^+$, $NH_4^+$, $Li^+$ on *Sipunculus* arginine kinase did not reveal the perturbation of the protein absorption spectrum observed with anions. In this respect guanidine and spermine hydrochlorides differ from the other cations and produce the same spectral manifestation concomitant to the secondary inactivation stage. It may be questioned that guanidinium and spermine, acting as cations, could

either cause the spectral perturbation themselves or they would reinforce the effect of $Cl^-$. Our analytical tests did not disclose differences between the effects of these compounds and those of anions. However, it is probable that the induced structures could be distinct. Thus Von Hippel and Wong[11] have indicated that guanidinium salts affected the optical rotatory dispersion of ribonuclease in a manner different from neutral salts. Hammes and Swann[12] have obtained ultrasonic relaxation spectra of polyethylene glycol that are not identical in the presence of neutral salts or guanidinium·HCl.

*Change of solvent from $H_2O$ to $^2H_2O$*

The configuration of macromolecules is probably influenced by the hydrophobic interactions resulting from the tendency of non polar regions of the molecule to minimize their interaction with polar solvents[13,14]. The alteration by ions of the structure of water would lead to a remodelling of intramolecular hydrophobic interactions, which could account for the effects observed at the level of the protein submitted to the action of ions. Since the $O-^2H\cdots O$ bond is stronger that the $O-H\cdots O$ bond[15] and since the hydrophobic bonds of proteins would be slightly stronger in $^2H_2O$ than in $H_2O$ (ref. 16), the interactions involving the solvent may be different in $H_2O$ and in $^2H_2O$. In this connection if the hydrophobic interactions were implicated in the effect of ions, they should be reinforced in $^2H_2O$.

In fact, arginine kinase is less active in $^2H_2O$ than in $H_2O$, and the effect of KCl is greater on $^2H_2O$–arginine kinase than on $H_2O$–arginine kinase. KCl reduces the maximum velocity and increases the apparent $K_m$ for $Mg^{2+}$–ATP in $H_2O$ and in $^2H_2O$, but the decrease of $V$ in $^2H_2O$ is stronger. These results, *plus* the fact that the immediate inhibition does not vary with the increase of temperature, would not be inconsistent with the hypothesis that hydrophobic interactions might be predominant among various types of bonds accessible to the primary effects of ions.

The secondary effect of anions exhibits the same characteristics in both $^2H_2O$ and $H_2O$, namely the progressive inhibition and the modification of the protein absorption spectrum. We have not determined the relative extents of this second phase of inhibition in $^2H_2O$ and $H_2O$. However, it is clear that the difference spectrum in the presence of KCl is smaller in $^2H_2O$ than in $H_2O$. This difference could arise from the lesser action of KCl on a protein already modified by the change of solvent. It is also possible that the native enzyme in $^2H_2O$ could not exhibit the same absorption spectrum as in $H_2O$, although we have not been able to check this because of technical difficulties.

*Creatine kinase*

The order of effectiveness of inhibition of creatine kinase by anions and cations is the same as that for the *Sipunculus* enzyme[1]. For anions, this order corresponds to that previously indicated[2,17]. However, for cations, our results do not agree with those reported by Milner-White and Watts[2]. For these authors, the inhibition is independent of the cation and it is the same with lithium, potassium, and sodium chlorides. These discrepancies may come from the experimental conditions, the authors having performed enzyme assay with saturating substrates and with $Cl^-$, the potency of which could attenuate the differences between the respective susceptibilities of the enzyme towards cations.

The kinetic behaviour of the action of KCl on creatine kinase, characterized by two stages of inhibition, is similar to that observed with *Sipunculus* arginine kinase[1] and different from the inhibition of arginine kinases from *Homarus* and *Solen* muscle. Nevertheless, with creatine kinase no perturbation of the protein absorption spectrum, characteristic of the *Sipunculus* enzyme, was observed.

The first step of inhibition studied in the forward reaction is of a non-competitive type with parabolic curves and vertical intercepts. Heyde and Morrison indicated an inhibition by NaCl of the non-competitive type also, but with linear slopes and intercepts[18]. Their results are not in disagreement with ours. In fact, we have observed that slopes and intercepts are a parabolic function of the salt concentration, the parabola being very splayed so that the function is sensibly linear at the low salt concentrations used by these authors (NaCl $\leqslant$ 0.09 M) and rapidly becomes curved at salt concentrations higher than 0.20 M.

Milner-White and Watts, studying the inhibition of creatine kinase by anions at 30 °C, have also observed the decrease of activity with time. This led them to the conclusion that there is a formation of an abortive enzyme–creatine–ADP–chloride quaternary complex. This interpretation is not consistent with the rapid removal of ADP in the enzyme assay method used in this work. The slow inhibition of creatine kinase and arginine kinase is not due to the approach of an equilibrium, because the curvature of the progress activity recording is perceptible with a reduction of arginine or creatine to as low as 3%. Also, it is not due to the inhibition by the reaction products, since the potent inhibitor ADP is removed by the coupled enzyme system, and the weak inhibitors (arginine phosphate and creatine phosphate) appear in low concentrations. We ascertained that the activity of native arginine kinase and creatine kinase without salt was constant during the same time and for the same transfer.

ACKNOWLEDGMENTS

This work was supported by a grant from the Centre National de la Recherche Scientifique (G.R. No. 6). The authors wish to thank Mrs V. Le Comte for helpful technical assistance.

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