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# PHOSPHATE METABOLISM IN THE ELECTRIC ORGAN

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SUMMARY

1. The concentrations of ATP, ADP, AMP, creatine, creatine phosphate and $P_i$ were determined in slices of the main organ of *Electrophorus electricus*. At rest, they were 0.83, 0.080, 0.012, 18.2, 19.3 and 8.28 $\mu$moles/g wet tissue, respectively.

2. The calculated steady-state equilibrium constants for ATP:AMP and ATP:creatine phosphotransferases were $K_A = 1.21$ and $K_C = 0.12$, respectively.

3. Upon electrical stimulation, dephosphorylation of the phosphates of creatine and adenosine occurred with the release of $P_i$.

4. During recovery after stimulation there was some rephosphorylation of creatine and adenosine.

## INTRODUCTION

The biochemical events following electrical stimulation of the electroplates of *Electrophorus electricus* have been examined by KEYNES and his associates[1–3]. Within 1 or 2 sec after the initiation of electrical activity, an evolution of recovery heat is found[1]. After another 10 sec or more, fluorescence changes can be detected which indicate that a reduction of cytoplasmic pyridine nucleotides is taking place[2]. Later still, there is a measurable increase in the lactate content, presumably due to an increased rate of glycolysis[3]. It seems likely that the sequence of events on stimulation of the electric organ is: (1) changes in internal ionic concentrations resulting from the flow of electric current; (2) activation of the sodium pump, driven by phosphate-bond ($\sim$ P) energy, and drawing initially on the reservoir provided by creatine phosphate (CrP); and (3) activation of glycolysis to restore the ATP/ADP ratio. However, it is still far from clear precisely what chemical change is responsible for setting off the final chain of biochemical events, and which compound exerts a controlling influence on the overall reaction rate of this chain of reactions. The experiments described here were designed to examine the time course of changes in the concentrations of ATP, ADP, AMP, creatine phosphate and $P_i$ during and after a period of stimulation in an attempt to decide which of them might predominate in controlling metabolism activated by stimulation.

Abbreviations: CrP, creatine phosphate; Cr, creatine.

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## MATERIAL AND METHODS

Specimens of *Electrophorus electricus* were obtained from British Guiana and Brazil, and were kept in tanks at Babraham for 1–3 years. They were mostly 50–150 cm in length. The fish was decapitated with a cleaver about 15 cm from the head end, thus removing the viscera with the head. The central part of the body was then chopped into 20-cm segments. The main electric organ was dissected out and carefully freed from muscle and skin. The segments of main organ were kept in saline solution[4] (composition in mM: NaCl, 169; KCl, 5.0; $CaCl_2$, 3.0; $MgCl_2$, 1.5; sodium phosphate, 3.0; pH 7.6) at room temperature. Each segment was then mounted in a special holder and divided into ten slices 18 mm thick shortly before the slices were used. The group of ten slices provided material for several different periods of stimulation and recovery, and several resting controls. The electrical excitability of every slice was checked, and when stimulation was called for, 20-V pulses of 0.1 msec duration were applied at 100 times per sec through chlorided silver plates. After an appropriate period of stimulation and sometimes a further period of recovery, the central 5 mm of the slice was cut out with a double-bladed knife and immediately dropped into isopentane cooled in liquid $N_2$. The outer parts of the slice, which might have given misleading results because of relatively rapid biochemical changes in the damaged electroplates at the cut surfaces, were rejected. The frozen central parts were powdered and stored at the temperature of solid $CO_2$.

The frozen powder (2 g) was extracted at −20° in a Duall homogenizer (Kontes Glass Company) with 4 ml of 0.5 M $HClO_4$ in 95 % acetone. After complete homogenization, 8 ml of ice-cold 0.5 M $HClO_4$ in water were added. The homogenate was well mixed and transferred to a stainless-steel Servall centrifuge tube chilled in an ice-bath. The homogenizer was rinsed with another 8 ml of 0.5 M $HClO_4$ in water and the wash combined with the extract. After centrifugation at 0° to pack the precipitate, the supernatant was transferred to a 500-ml round flask with standard tapered joint and kept in an ice-bath. The precipitate was suspended in 12 ml 0.5 M $HClO_4$ and recentrifuged. The supernatant was again transferred to the flask. The combined extract was then neutralized carefully with 5 M KOH in an ice-bath with the aid of indicator papers. The neutralized extract was then lyophilized, and the dry powder taken up in 4 ml of ice-cold water. The insoluble material was centrifuged off and the final solution was used for all determinations.

ATP, ADP and AMP were determined according to the methods described by ADAMS[5]. The DPNH used was prepared with alcohol dehydrogenase[6] (EC 1.1.1.1) from DPN which was preincubated with AMP aminohydrolase (EC 3.5.4.6) to remove contaminating AMP[7]. Creatine and creatine phosphate were determined according to a slight modification of the Ennor and Stocken method[8]. $P_i$ was determined after precipitation with $Ca^{2+}$ and HCl solubilization, and the molybdate color was developed in the presence of $Fe^{2+}$ (ref. 9).

## RESULTS

A preliminary investigation with various extraction methods showed that extraction at −20° with $HClO_4$ in acetone gave the most satisfactory results. ATP concentration was the highest and ADP and AMP concentrations were the lowest. The

TABLE I

THE CONCENTRATION OF ADENOSINE AND CREATINE PHOSPHATES IN THE RESTING ELECTRIC ORGAN AND SOME OF THEIR RATIOS

All values are mean ± standard deviation with $n = 26$.

| *Concn. (μmoles/g wet tissue)* | | | *ATP/ADP ratio* | $K_A$ | *Concn. (μmoles/g wet tissue)* | | *CrP/Cr ratio* | $K_C$ | *Concn. (μmoles/g wet tissue)* |
|---|---|---|---|---|---|---|---|---|---|
| *ATP* | *ADP* | *AMP* | | | *Cr* | *CrP* | | | $P_i$ |
| 0.83 ± 0.18 | 0.080 ± 0.020 | 0.012 ± 0.007 | 10.9 ± 3.5 | 0.84 ± 0.33 | 18.2 ± 5.4 | 19.3 ± 4.4 | 1.19 ± 0.56 | 0.118 ± 0.057 | 8.28 ± 4.46 |

TABLE II

EFFECT OF STIMULATION ON THE CHANGES OF ADENOSINE AND CREATINE PHOSPHATES

| *Treatment* | | *Concn. (μmoles/g wet tissue)* | | | *ATP/ADP ratio* | $K_A$ | *Concn. (μmoles/g wet tissue)* | | *CrP/Cr ratio* | $K_C$ | *Concn. (μmoles/g wet tissue)* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Temp.* | *Rest or stimulation (sec)* | *ATP* | *ADP* | *AMP* | | | *Cr* | *CrP* | | | $P_i$ |
| 16 | Rest | 0.87 | 0.10 | 0.01 | 9 | 1.08 | 31 | 16 | 0.52 | 0.058 | 16.2 |
| | Rest | 0.90 | 0.08 | 0.01 | 11 | 0.76 | 28 | 16 | 0.59 | 0.053 | 16.4 |
| | 10 | 0.88 | 0.08 | 0.01 | 12 | 0.82 | 27 | 17 | 0.63 | 0.059 | 15.8 |
| | 30 | 0.95 | 0.09 | 0.01 | 10 | 1.04 | 30 | 12 | 0.41 | 0.040 | 17.0 |
| | 60 | 0.73 | 0.11 | 0.01 | 7 | 1.37 | 37 | 8 | 0.22 | 0.031 | 19.6 |
| 18.5 | Rest | 0.90 | 0.08 | 0.01 | 11 | 0.71 | 16 | 19 | 1.16 | 0.105 | 11.4 |
| | Rest | 1.02 | 0.11 | 0.01 | 9 | 0.89 | 17 | 17 | 0.98 | 0.105 | 9.3 |
| | 10 | 0.86 | 0.09 | 0.01 | 9 | 1.49 | 21 | 16 | 0.77 | 0.084 | 7.3 |
| | 30 | 0.80 | 0.14 | 0.01 | 6 | 1.17 | 26 | 9 | 0.33 | 0.060 | 16.3 |
| | 60 | 0.59 | 0.08 | 0.01 | 7 | 1.17 | 28 | 11 | 0.40 | 0.054 | 16.3 |

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TABLE III

BIOCHEMICAL RECOVERY AFTER ELECTRIC WORK

| *Treatment* | *Concn. (μmoles/g wet tissue)* | | | *ATP/ADP ratio* | $K_A$ | *Concn. (μmoles/g wet tissue)* | | *CrP/Cr ratio* | $K_C$ | *Concn. (μmoles/g wet tissue)* |
|---|---|---|---|---|---|---|---|---|---|---|
| | *ATP* | *ADP* | *AMP* | | | *Cr* | *CrP* | | | $P_i$ |
| Rest | 0.63 | 0.06 | 0.01 | 11 | 0.74 | 12 | 17 | 1.37 | 0.130 | 4 |
| Rest | 0.73 | 0.09 | 0.01 | 8 | 1.50 | 14 | 15 | 1.09 | 0.135 | 4 |
| 30-sec stimulation | 0.51 | 0.10 | 0.02 | 5 | 0.94 | 24 | 11 | 0.45 | 0.092 | 12 |
| 60-sec stimulation | 0.65 | 0.20 | 0.01 | 3 | 7.34 | 22 | 10 | 0.44 | 0.135 | 14 |
| 60-sec stimulation, recovery 2 min | 0.63 | 0.12 | 0.01 | 6 | 2.00 | 15 | 7 | 0.49 | 0.089 | 8 |
| 60-sec stimulation, recovery 5 min | 0.51 | 0.12 | 0.02 | 5 | 1.31 | 18 | 11 | 0.62 | 0.130 | 4 |
| 60-sec stimulation, recovery 10 min | 0.69 | 0.08 | 0.01 | 8 | 1.12 | 13 | 12 | 0.93 | 0.111 | 3 |

citrate method used for muscle extraction[10] yielded results comparable to the $HClO_4$–acetone method for creatine and creatine phosphate, but less ATP and more ADP and AMP. Table I shows the average contents of ATP, ADP, AMP, creatine, creatine phosphate and $P_i$. The ATP/ADP and CrP/Cr ratios were calculated from the original data and then averaged; they were 10.9 ± 3.5 (5 to 15) and 1.19 ± 0.56 (0.45 to 2.08), respectively. The ratios

$$K_A = \frac{[ADP]^2}{[ATP]\cdot[AMP]} = 0.84 \pm 0.33$$

$$K_C = \frac{[ADP]\cdot[CrP]}{[ATP]\cdot[Cr]} = 0.12 \pm 0.06$$

could also be calculated. *In vitro*, these ratios would correspond to the equilibrium constants of ATP:AMP phosphotransferase (EC 2.7.4.3) and ATP:creatine phosphotransferase (EC 2.7.3.2), respectively. These figures are important since they indicate whether the *in vitro* equilibrium constants can be applied in the *in vivo* calculations.

The effect of stimulation is shown in Table II. In the experiment at 16°, a decline in CrP/Cr was found after 30 sec of stimulation, but the ATP/ADP ratio fell only after 60 sec. In the experiment at 18.5°, both ratios fell after 30 sec of stimulation. Since the AMP concentration in these slices changed very little, the change in ATP/ADP ratio was reflected in the apparent $K_A$ which increased upon stimulation. On the other hand $K_C$ showed a definite decrease. These observations and calculations suggest a transient metabolic process where the steady-state equilibrium has been upset and a new steady level has not yet been achieved. The loss in high-energy phosphates was found mainly in the increase in $P_i$, but there was some unexplained variation in creatine phosphate *plus* $P_i$.

When resting was allowed after stimulation, a biochemical recovery was observed (Table III). The ATP/ADP and CrP/Cr ratios underwent a decrease due to stimulation and then an increase, following recovery. $K_C$ went through a similar but not so marked change. $K_A$ and $P_i$ both went up during stimulation and decreased upon recovery. Apparently, $P_i$ was rephosphorylated into high-energy phosphates upon recovery. Also, both the creatine and adenosine systems were working to regain their respective equilibria.

## DISCUSSION

Before the data are discussed, mention must be made of a serious and unexplained difficulty encountered in the course of this work. All of the electric organ from four of the nine eels that were used, and some of the tissue from the other five, appeared to have a negligibly small creatine phosphate content, and therefore gave unacceptable results. It did not seem possible to attribute this behavior to inadequacies in the extraction procedure, since after the first few trials the standard procedure with $HClO_4$ in acetone was always used. It also seemed unlikely that some fish were more fatigued than others in the brief struggle that preceded their decapitation, because in every case the electric response of each slice of tissue was checked and found to be normal. The only explanation that remains, which is not claimed to be at all satisfactory, is that the biochemistry of some but not all of the fishes that were used

had been changed by a long period spent in captivity. Some support for this suggestion was provided by the observation that the specimens of electric organ that yielded no creatine phosphate also seemed to contain abnormally small quantities of the ($Na^+$-$K^+$)-activated ATPase in which they should have been rich (S.L. BONTING, personal communication). Whatever the cause of this trouble, it reduced sadly the amount of data finally suitable for presentation here.

Electroplates are modified muscles and we shall confine ourselves in the discussion to muscles and nerves. The extracellular space in this tissue was large, about 50 % (KEYNES, unpublished results), so the actual intracellular concentrations were approximately twice those reported here. Complete analyses of the three adenosine nucleotides were lacking for the electroplates. Only the ATP/ADP ratio and their individual amounts had been reported[11]: 1.39 and 1.28 $\mu$moles/g wet tissue for ATP and 0.32 and 0.23 $\mu$mole/g wet tissue for ADP. Although, in this study, the ATP content (0.83 $\pm$ 0.18 $\mu$mole/g wet tissue) is a little lower, the ADP content (0.080 $\pm$ 0.020 $\mu$mole/g wet tissue) is much less (Table I). The net result is a higher ATP/ADP ratio (10.9 $\pm$ 3.5) in these experiments than the 4.3 and 5.6 of the earlier report[11]. Examples of similar values for other tissues are 2.0 and 1.8 for frog muscles[12,13], 9.4 for rabbit muscle[10], 7.2 for rabbit cervical vagus nerve[14], 7.5 for rabbit sciatic[15], 2.6 for frog sciatic[16] and 2.3 and 6.0 for Loligo giant axons[17]. The average ATP/ADP ratio in this study was higher than all those in previous reports, suggesting that our results are closer to the concentrations of these compounds *in vivo*. It is also possible that an ADP-like contaminant[18] was accidentally eliminated at $-20°$ using acetone, so that the apparent concentration of ADP was reduced and a higher ATP/ADP ratio obtained.

Using the data for AMP content (0.012 $\pm$ 0.007 $\mu$mole/g wet tissue), the steady-state $K_A$ could also be calculated. The average is 0.84 $\pm$ 0.33. This value is not far from the equilibrium constant of unity *in vitro* for muscle ATP:AMP phosphotransferase[19,20], suggesting that this enzyme was in equilibrium *in vivo*, and providing some support for the reliability of our ATP and ADP determinations. Previous reports for $K_A$ are, for comparison, 3.69 for frog muscle[12], 0.67 for rabbit sciatic[15], and 0.47 for rabbit C fibers[14].

The average concentration of creatine phosphate (19.3 $\pm$ 4.4 $\mu$moles/g wet tissue) was found to be somewhat higher than that reported by NACHMANSOHN *et al.*[21] and that by CALDWELL AND KEYNES[3], suggesting that there was less breakdown in this extraction procedure. The CrP/Cr ratio (1.19 $\pm$ 0.56), which is probably a better measure for the creatine phosphate breakdown than creatine phosphate concentration *per se*, was larger than 0.65 (CALDWELL AND KEYNES, unpublished results), but less than the value of 3.43 for frog muscle[13], and comparable to that of 1.16 for frog sciatic[16].

When the ATP/ADP and CrP/Cr ratios are taken into account together, the apparent ATP:creatine phosphotransferase steady-state equilibrium constant can be calculated: $K_C = 0.12 \pm 0.06$. This value is in the middle of the range of the *in vitro* equilibrium constant of 0.05–0.25 reported for rabbit muscle[22] and is closer to it than any of the previous reports of 3.69 (ref. 13) and 1.89 (ref. 12) for muscle and 0.44 for frog nerve[16]. The best estimate, not derived from a direct calculation, was 0.05 for frog muscle[23]. The value from this study seems to represent the first piece of concrete evidence that, at rest, the steady-state equilibrium of ATP:creatine phosphotransferase is similar to the equilibrium state of the isolated enzyme. Unfortunately we

were unable to obtain enough of this enzyme *in vitro* to make a direct comparison.

During stimulation, *i.e.*, electrical discharge, an increase in $K_A$ and decrease in $K_C$ were observed. In the two examples cited in Table II, a marked temperature effect was observed. At 16°, the change in $K_A$ became noticeable after 30 sec of stimulation and no definite change was observed in $K_A$ until 60 sec. At 18.5°, both $K_C$ and $K_A$ showed changes after 10 sec of stimulation. Such a change in $K_C$ with respect to the duration of stimulation was in agreement with the decrease in creatine phosphate observed at 21–23° (ref. 3).

In the experiments with stimulation and subsequent recovery (Table III) the adenosine system again gave some indication of a slower response than the creatine system during stimulation and recovery. The single high value for $K_A$ after 60 sec stimulation was due to its high ADP concentration. Since these experiments were difficult to perform, we were unable to get a set of data with perfect figures. However, it became clear from these results that both enzymes involved here tried to keep their respective equilibria at a value similar to those reported *in vitro*. Since creatine phosphate was not replenished unless creatine was rephosphorylated, but ATP could readily be replaced, the first indication was a drop in CrP/Cr ratio upon tetanization. The ATP/ADP ratio then followed with a drop.

When all the data were considered together, the conclusion could be drawn that both ATP:AMP and ATP:creatine phosphotransferase were in equilibria at rest and departed from their equilibria during stress. Deviation from the equilibrium state occurred earlier and the deviations were corrected later in the creatine system. We did not examine the source of energy during recovery, but it seems to have derived mainly from glycolysis[11]. Our data also fail to identify the control mechanism for these biochemical changes.

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