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Cerebral Metabolic Studies *in Situ* by ^{31}P-Nuclear Magnetic Resonance after Hypothermic Circulatory Arrest[1]

F. STOCKER, N. HERSCHKOWITZ, E. BOSSI, M. STOLLER, T. A. CROSS, W. P. AUE, AND J. SEELIG

Department of Pediatrics, University of Bern [F.S., N.H., E.B., M.S.] and Department of Biophysical Chemistry, Biocenter of the University [T.A.C., W.P.A., J.S.], Basel, Switzerland

ABSTRACT. Cerebral high energy phosphates were studied in the intact rabbit brain using nuclear magnetic resonance spectroscopy. The effect of hypothermia on degradation kinetics in total ischemia due to circulatory arrest was examined, measuring phosphocreatine, adenosine triphosphate, and inorganic phosphate as a function of time at three different temperatures (35, 24, 21° C). Phosphocreatine- and ATP-decays followed single exponential functions at all three temperatures. The half-life times increased by approximately a factor of three upon lowering the temperature from 35 to 21° C with activation energies of 15–20 kcal/mol, which corresponds to values of Q_{10} between 2.4 and 3.2. In the temperature range studied, no critical temperature was found below which metabolism would stop completely. We conclude that nuclear magnetic resonance spectroscopy allows, in the intact animal, quantitative assessment of the influence of hypothermia on energy metabolism in the brain. This influence is a major concern in the field of cardiac surgery in infants and children who are often operated in total circulatory arrest under deep hypothermia. (*Pediatr Res* 20: 867–871, 1986)

Abbreviations

NMR, nuclear magnetic resonance
PCr, phosphocreatine
ATP, adenosine triphosphate
P_i, inorganic phosphate

In infants and small children open heart surgery is often performed with total circulatory arrest under deep hypothermia (1–10). One important open question concerns the effect of this procedure on cerebral energy metabolism (11–13). Norwood *et al.* (14) have investigated the effect of hypothermia on the degradation kinetics of cerebral high energy phosphates after total circulatory arrest using NMR spectroscopy. These studies were performed on isolated and perfused neonatal rat brain. Several other authors have shown that NMR spectroscopy of the cerebral phosphates can also be performed in humans and in intact animals under varied conditions (15–29).

As there are however no reports of kinetic studies in intact animals under deep hypothermia, we have chosen to investigate degradation of cerebral high energy phosphates by NMR spectroscopy in the hypothermic rabbit.

MATERIALS AND METHODS

Male New Zealand rabbits (average body weight 3.4 kg, range 3.0–3.7) were anaesthetized with 0.85 g/kg Urethane intraperitoneal, 30 min later with the same dose intravenous in an ear vein. The neck, thorax, and abdomen were then shaved, tracheostomy performed, and a 3.5-mm tube inserted. An internal jugular vein was catheterized with an umbilical vein catheter (Pharmaseal, CH 5, AHS Herstal, Belgium). The catheter was filled with saline, the syringe remained attached to the catheter without flushing. A femoral artery was prepared and a catheter (Vygon intravenous catheter G 18, Steriflex O.R.X., Ecouen, France) inserted up to the abdominal aorta. This catheter was connected via a transducer to a monitor displaying the arterial blood pressure curve, systolic, mean and diastolic pressure as well as heart rate (Tektronix 414). A temperature probe was inserted through the mouth into the pharynx and the temperature was displayed on the same monitor. Preparation of the animal lasted for an average of 41 min (range 30–55) and was similar for all temperature groups. During these procedures, the animal was breathing spontaneously in room air. Then, muscular relaxation was induced with pancuronium, 2 mg/kg. From here on, the animal was ventilated with a Loosco Amsterdam Infant Ventilator MK2. The following settings were used: F_{IO_2} 1.0, flow 10–13 liter/min, rate 18–25/min, peak inspiratory pressure 10–16 cm H_2O, inspiration: expiration rate = 1:2 or 1:2.5, no exspiratory pressure. ECG was controlled and recorded approximately every 30 min. The animals were divided into three groups. In the first group, four animals were kept at room temperature and introduced into the magnet with a pharyngeal temperature of 36° C at an average of 19 min after initiation of ventilation. Since, after the 2-h experiment, the temperature had dropped to 34° C, an average temperature of 35° C was assumed. In the second group, four animals were put on a plastic bag containing ice and their abdomens were covered with two to three ice packs. When a pharyngeal temperature of 26° C was reached, the animals were introduced into the magnet. During the 4-h experiment, the temperature dropped to 22° C, and an average temperature of 24° C was assumed. The average time from beginning of ventilation until the introduction into the magnet was 63 min (range 55–80 min). The four animals in group three were treated like the ones of group 2, however, the pharyngeal temperature was allowed to drop to an average of 21° C where it remained during the experiment. The average duration of cooling from initiation of ventilation was 97 min (range 85–118 min). Average time from introduction into the magnet

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Correspondence N. Herschkowitz, Department of Pediatrics, University of Bern, CH-3010 Bern, Switzerland.
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[1] Dedicated to Prof. E. Rossi on his seventieth birthday.

to registration of the first spectrum was 35 min (range 17–55 min) and did not differ between the groups. After registration of initial control spectra, circulatory arrest was induced by bleeding the animal from the jugular and femoral catheters. Circulatory arrest was defined as occurring at the time of appearance of a flat blood pressure curve. This occurred 7–10 min after initiation of the bleeding.

All NMR experiments were performed in a 1.9 T/24 cm bore superconducting Oxford Instruments magnet, which was interfaced to a Bruker BNT-80 spectrometer. In order to restrict the sensitive volume of the measurement to the brain, a 2-cm diameter surface coil (30) was placed firmly on the scalp, directly overlying the brain. Possible artefacts of the brain spectra by contributions from the skin were minimized by applying a radiofrequency pulse with a 180° flip angle in the center of the coil. This was verified by means of a small sphere in the center of the surface coil filled with a solution of hexachlorocyclotriphosphazene in benzene. The 180° pulse leads to a suppression of resonances in the immediate vicinity of the surface coil. In order to obtain optimum sensitivity and resolution in the spectra, the homogeneity of the main magnetic field was optimized by ^{1}H NMR at 80 MHz on the same surface coil and with the same flip angle as used later for ^{31}P observation at 32.4 MHz. ^{31}P data were acquired in the Fourier mode with 50–200 scans coadded in order to improve sensitivity. The delay between the scans was 4 s, which did not cause differential saturation of the different phosphorus resonances. The raw data were processed with convolution difference processing (14/140 Hz) in order to improve sensitivity and to eliminate broad features in the spectra. Chemical shifts were referred to the PCr resonance defined as 0 ppm. For quantitative evaluation the signal areas, which correspond to metabolite concentrations, were calculated as the product of full line width at half height, times the peak height. For ATP, the β-peak at 16.1 ppm was evaluated, since near the positions of the α- and γ-peaks the signals of other metabolites of possibly significant concentration might also occur. The intracellular pH in the brain was determined from the chemical shift difference δ between the PCr and the P_i peak using the relation (31): $pH = 6.75 + \log [(\delta\text{-}3.27)/(5.69\text{-}\delta)]$.

RESULTS

The ^{31}P-NMR spectrum of rabbit brain before circulatory arrest exhibits resonances from the three phosphates of adenosinetriphosphate (α, β, γ-ATP), PCr, and P_i (*top traces* of Fig. 1). The unlabeled peaks to the left and right of the P_i resonance are attributed to phosphomonoesters and phosphocholine diesters, respectively. This assignment is based on the current literature of ^{31}P-NMR of muscle (31) and brain (19, 32, 33). Figure 1 also provides a comparison of the temporal variation of the metabolites at 35 and 21° C. The top spectrum of each series corresponds to the situation immediately before circulatory arrest. The most obvious changes after induction of ischemia are a decrease of the signal intensities of PCr and ATP and a concomitant increase of P_i.

Within the accuracy of the measurement the change of the PCr and ATP concentrations can be approximated by a single exponential function. This is demonstrated in Figure 2 for a set of individual experiments performed at different temperatures. The intensities were normalized with repect to the spectra before circulatory arrest. Figure 2 shows a semilogarithmic plot of the relative intensities *versus* time after circulatory arrest. Ideally, all decay curves should start at 100% relative intensity. However, this is not the case experimentally for two reasons. First, different times have elapsed in the individual experiment between circulatory arrest and the beginning of the NMR measurement. Second, it cannot be excluded that the decay of PCr and/or ATP is biphasic and that an initial fast phase cannot be resolved in the present measurements. From the slopes of the straight lines the time constants τ and the half-life times $\tau_{1/2} = \tau \ln 2$ could be

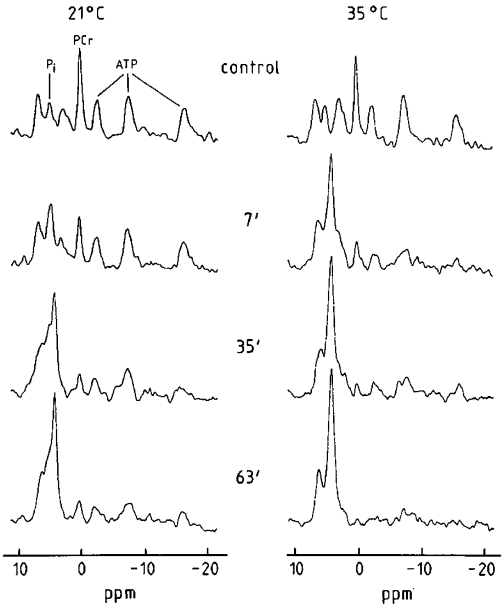

Fig. 1. ^{31}P-NMR spectra obtained from a rabbit's brain before and after circulatory arrest for two temperatures. Data were acquired with a 2-cm diameter surface coil in 200 scans with an interpulse delay of 4 s and processed with convolution-difference 14/140 Hz. The metabolites of interest are labeled P_i, PCr, and ATP.

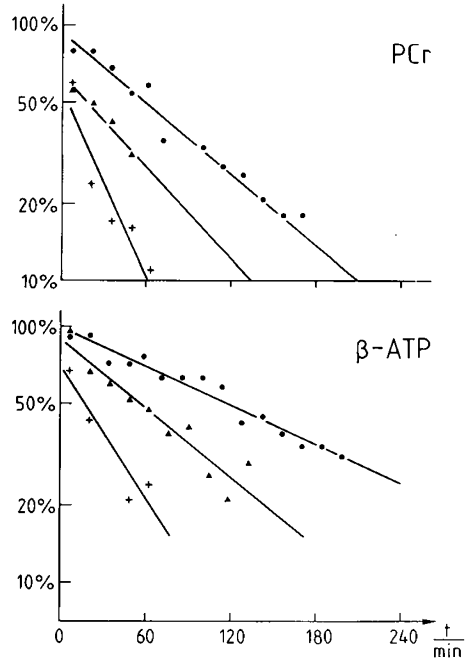

Fig. 2. Semilogarithmic plots of relative PCr and β-ATP intensities as a function of time after circulatory arrest for single experiments. The *solid lines* are the results of linear regression computations. +, 35°C; ▲, 24°C; ●, 21°C.

determined. In our case, the exponential decays were evaluated for the half-life times with the more accurate nonlinear three parameter fitting procedure, whose averaged results for PCr, ATP, and Pi are summarized in Table 1. Inspection of this table reveals: 1) at all temperatures the PCr intensity decays faster than the ATP intensity. The half-life time of PCr is shorter than that of ATP by about a factor of two. 2) The half-life times of ATP and PCr are strongly temperature dependent. They increase approximately threefold upon lowering the temperature from 35 to 21° C.

Unfortunately, the quantitative evaluation of the P_i resonance is rather difficult since the intensity of this peak is small at the beginning of the experiment and, more important, because the final P_i peak height is unknown. Nevertheless, a three-parameter exponential fit of the P_i data was attempted yielding a half-time of approximately 36 min (79 min, 105 min) at 35° C (24 and 21° C). In agreement with the ATP and PCr half-times the P_i half-times increase with decreasing temperatures (*cf.* Table 1).

Immediately prior to circulatory arrest the pH was determined both intracellularly by NMR (from the chemical shift of the P_i resonance) and by conventional pH electrodes in the arterial blood. The NMR measurements consistently yielded a pH value of 7.14 ± 0.07 independent of the temperature. The intraarterial pH values were 7.35 ± 0.50 at 35° C and 7.50 ± 0.10 at 21 and 24° C. At the end of the experiment the intracellular pH had dropped to approximately 6.30 for all temperatures with large variations between the individual animals.

Immediately before circulatory arrest a strong temperature dependence was observed for the arterial CO_2 pressure which measured 40, 25, and 10 mm Hg at 35, 24, and 21° C, respectively. Despite these differences the initial spectra were identical at the three temperatures.

DISCUSSION

A major problem with *in vivo* NMR is the correct localization of the sensitive volume. In the present experiments muscle and skin contributions to the brain spectrum were minimized by application of a 180° pulse in the plane of the surface coil, thus suppressing signals from the tissue layer immediately adjacent to the coil. As an additional control the scalp of the rabbit was removed in two experiments and the temporalis muscle retracted. The surface coil was placed directly on the skull, and measurements were made again with a 180° pulse in the plane of the surface coil. After circulatory arrest the half-life times of PCr and ATP were then found to be 20.8 and 28.4 min (at 35° C), respectively. These data corresponded well to those obtained without scalp removal (Table 1).

Table 2 summarizes typical intensity ratios of various metabolites immediately before circulatory arrest. Since the NMR saturation factors are approximately equal for P_i, ATP, and PCr, the data summarized in Table 2 reflect true metabolic concentration ratios. It may be noted (*cf.* Fig. 1) that the P_i resonance under normal conditions is rather small. Errors in the measurement of P_i are thus amplified in the ATP/P_i and PCr/P_i ratios whereas the PCr/ATP ratio is less sensitive and exhibits smaller fluctuations.

PCr/ATP ratios reported in the literature are 2.1 ± 0.3 for rat brain (34) and 2.3 ± 0.1 for rabbit brain (17); PCr/P_i ratios of rabbit brain are 2.04 and 1.7 (28) and may be compared with the much higher ratio of muscle, which is about 14–10. Finally, the ATP/P_i ratio found in rat brain was 1.68 (34). The intracellular pH in the normal rabbit brain as determined by ^{31}P-NMR was pH = 7.08 (28), which is approximately 0.4 pH units lower than the pH of the jugular venous blood. The data of the present work are in general agreement with these results indicating a proper focussing of the sensitive volume of the surface coil on the rabbit brain. As seen in the initial spectra of Figure 1, the PCr/ATP ratio, and the intracellular pH in Table 2, the metabolic status of the rabbit brain immediately before circulatory arrest is normal and very similar for the three chosen temperatures.

Following circulatory arrest the coordinate decrease of PCr and ATP metabolite concentrations obeys first-order kinetics, and the half-life times range between 20 and 120 min depending on temperature and metabolite measured. The only known reaction involving PCr in brain is PCr + ADP + $H^+ \rightleftarrows$ Cr + ATP catalyzed by creatine kinase. Hence the decline of PCr concentration should be accompanied by a production of ATP. On the other hand, ATP is involved in many reactions and there is no *a priori* reason that the half-life time of ATP decline could not be equal or even shorter than that of PCr. In fact, the concomitant decrease of ATP and PCr demonstrates that more ATP is consumed in other pathways than is produced by the creatine kinase reaction.

Figure 3 shows Arrhenius plots of the half-life times, *i.e.* a semilogarithmic plot of $(\tau_{1/2})^{-1}$ *versus* the reciprocal absolute temperatures. By means of the Arrhenius equation

$$d(\ln(\tau_{1/2})^{-1})/d(1/T) = -Ea/R \qquad (1)$$

in which Ea and R represent the Arrhenius activation energy and the gas constant, respectively, the slopes of the approximately straight lines can be evaluated for activation energies, which are 16.7 ± 2.8 kcal/mol for PCr, 14.6 ± 2.1 kcal/mol for ATP, and 14.9 ± 4.4 kcal/mol for P_i. Another way of expressing the temperature dependence, which is more familiar to biological readers, is the factor Q_{10}, by which the reaction rate is increased, if the temperature of the system is raised by 10° C. It is defined

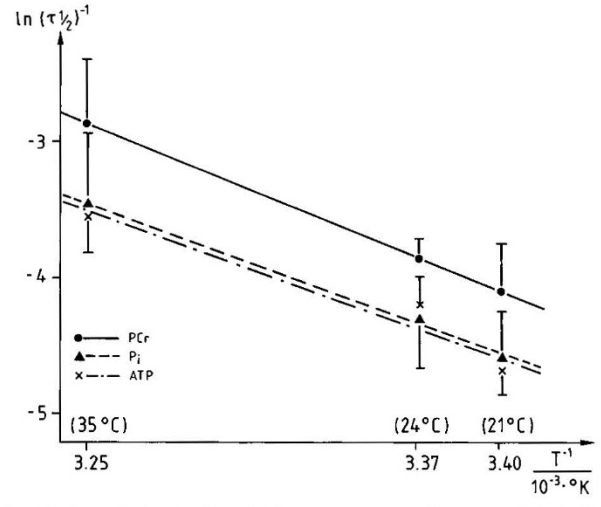

Fig. 3. Logarithmic plot of the average reaction rates $[1n(\tau_{1/2})^{-1}]$ of PCr, P_i, and ATP *versus* the inverse absolute temperature. See text for explanations.

Table 1. *Decay half-times $\tau_{1/2}$ of phosphorus-containing metabolites in rabbit brain after circulatory arrest**

Temperature (° C)	$\tau_{1/2}$ (min) PCr	ATP	P_i
35	19 ± 8	35 ± 10	36 ± 19
24	48 ± 6	69 ± 14	79 ± 26
21	63 ± 19	112 ± 19	105 ± 32

* Experimental errors are SDs.

Table 2. *Concentration ratios of ^{31}P-containing metabolites in rabbit brain immediately before circulatory arrest*

	35° C	24° C	21° C	Literature
PCr/ATP	1.5 ± 0.2	1.3 ± 0.1	1.3 ± 0.2	2.1 [Shoubridge *et al.* (34)] (rat) 2.3 [Delpy *et al.* (17)]
PCr/P_i	1.8 ± 0.1	3.6 ± 0.3	2.1 ± 0.4	2-1.7 [Petroff *et al.* (28)]
ATP/P_i	1.2 ± 0.1	2.7 ± 0.4	1.6 ± 0.6	1.7 [Shoubridge *et al.* (34)] (rat)
pH	7.11 ± 0.03	7.20 ± 0.10	7.07 ± 0.06	7.08 [Petroff *et al.* (28)]

by the equation

$$k/k_o = Q_{10}\,(T - To)/10 \quad (2)$$

With the rate constant $k = 0.69/\tau_{1/2}$, Q_{10} can be related to Ea as follows. We first rewrite the integrated form of the Arrhenius equation 1 for two temperatures T > To:

$$\ln k = \ln A - Ea/RT \quad (3)$$

$$\ln K_o = \ln A - Ea/RT_o \quad (4)$$

which yields

$$\ln (k/k_o) = -(Ea/R)(1/T - 1/T_o)$$

$$= -(Ea/RTT_o)(T_o - T) \quad (5)$$

and for a small temperature range, as it is investigated in our experiments, simplifies to

$$\ln (k/k_o) \approx (Ea/RT_o^2)(T - T_o) \quad (6)$$

This equation can be compared to the logarithmic form of equation 2

$$\ln (k/k_o) = (\ln Q_{10}/10)(T - T_o) \quad (7)$$

and yields

$$\ln Q_{10} \approx 10\ Ea/RT_o^2 \quad (8)$$

The values of Q_{10}, calculated from the Arrhenius activation energies according to equation 8 are 2.6 ± 0.4, 2.3 ± 0.3, and 2.4 ± 0.6 for PCr, ATP, and P_i, respectively. These *in situ* activation energies and Q_{10} values are very similar to those measured for enzyme reactions *in vitro* (35, 36).

The total creatine kinase activity of rat brain homogenates was found as 207 μmol PCr/min per g wet weight (37). With an *in vivo* PCr concentration of approximately 5.5 μmol/g wet weight a unidirectional, pseudo first order rate constant of $k = 0.63\ s^{-1}$ can be estimated for the creatine kinase forward direction (PCr hydrolysis). ^{31}P NMR saturation transfer measurements yield $k = 0.26\ s^{-1}$ for rat brain *in vivo* (34). The latter study also indicates that the reaction catalyzed by creatine kinase is close to equilibrium under *in vivo* conditions. Since the rate constant k is related to the reaction half-time according to $\tau_{1/2} = 0.69/k$, half-times of the order of a few seconds can be calculated for the irreversible PCr hydrolysis *in vivo*. In contrast, the present studies yield half-times between 20 and 60 min for rabbit brain. This difference can be explained by the fact that the present study measures the net change of PCr and not the unidirectional flows. Since the reaction catalyzed by creatine kinase is close to equilibrium even before circulatory arrest, no large change in the PCr concentration is to be expected. Only when ATP is used irreversibly in energy consuming reactions will the PCr ⇄ ATP equilibrium follow.

An isolated perfused brain model of neonatal rats has been used by Norwood *et al.* (14) to study hypothermic circulatory arrest with ^{31}P-NMR. The decay of the metabolites PCr and ATP followed a single exponential with half-times of 6 and 9.6 min, respectively, at 37° C. At 20° C the half-life time of PCr was 9.5 min while that of ATP had increased to 70 min. These half-times are clearly shorter than those measured in the present study, which may be due to the different animals used but also, perhaps, to the difference between perfused and nonperfused brain. Qualitatively, the temperature dependence of ATP is similar in both experiments with approximately the same activation energy. In contrast, the PCr decay is almost equally fast at 37 and 20° C in pH buffer perfused neonatal rat brain, while there is a distinct temperature dependence in *in situ* adult rabbit brain. Thus the suggested isolation of creatine kinase from the ATP pool in rat brain cannot be proven for the rabbit. Another crucial difference between the results of Norwood *et al.* (14) and our measurements is that in normothermic and hypothermic rabbit brain, the pH after circulatory arrest dropped to a value of 6.3 ± 0.2 units, whereas it was not affected in hypothermic buffer perfused neonatal rat brain.

Our results show that by using NMR spectroscopy the influence of hypothermia on energy metabolism in the brain can be measured in the intact animal. This is the basis of further experimental work. With the help of extracorporal circulation in addition to the present preparation, the situation of an operated infant with congenital heart disease can be simulated in the rabbit.

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