Rate Equation for Creatine Kinase Predicts the in Vivo Reaction Velocity: ^{31}P NMR Surface Coil Studies in Brain, Heart, and Skeletal Muscle of the Living Rat†

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ABSTRACT: Brain, heart, and skeletal muscle contain four different creatine kinase isozymes and various concentrations of substrates for the creatine kinase reaction. To identify if the velocity of the creatine kinase reaction under cellular conditions is regulated by enzyme activity and substrate concentrations as predicted by the rate equation, we used ^{31}P NMR and spectrophotometric techniques to measure reaction velocity, enzyme content, isozyme distribution, and concentrations of substrates in brain, heart, and skeletal muscle of living rat under basal or resting conditions. The total tissue activity of creatine kinase in the direction of MgATP synthesis provided an estimate for V_{max} (23.4 ± 2.8, 62.4 ± 4.5, and 224 ± 16 mM/s) and exceeded the NMR-determined in vivo reaction velocities by an order of magnitude (4.1 ± 1.2, 5.1 ± 1.6, and 18.4 ± 2.4 mM/s for brain, heart, and skeletal muscle, respectively). The isozyme composition varied among the three tissues: >99% BB for brain; 14% MB, 61% MM, and 25% mitochondrial for heart; and 98% MM and 2% mitochondrial for skeletal muscle. The NMR-determined reaction velocities agreed with predicted values from the creatine kinase rate equation ($r^2 = 0.98$; $p < 0.001$). The concentrations of free creatine and cytosolic MgADP, being less than or equal to the dissociation constants for each isozyme, were dominant terms in the creatine kinase rate equation for predicting the in vivo reaction velocity. Thus, we observed that the velocity of the creatine kinase reaction is regulated by total tissue enzyme activity and by the concentrations of creatine and MgADP in a manner that is independent of isozyme distribution.

Creatine kinase (EC 2.7.3.2), present in high activity in brain, heart, and skeletal muscle, catalyzes the transfer of a high-energy phosphate group from creatine phosphate to MgADP,[1] forming MgATP and free creatine:

$$H^+ + \text{MgADP} + \text{creatine phosphate} \rightleftharpoons \text{MgATP} + \text{creatine} \quad (1)$$

The BB isozyme predominates in brain, the MM form dominates in skeletal muscle, and a combination of MM, MB, and mitochondrial isozyme activity is present in heart (Jacobs et al., 1964; Eppenberger et al., 1967). The intracellular localization of enzyme activity differs among the tissues: About 50% of the creatine kinase activity is bound to mitochondria and myofibrils in heart, but only 10% is bound in skeletal muscle (Scholte, 1973). Brain contains about one-tenth and heart about one-fourth the total tissue creatine kinase activity of skeletal muscle (Jacobs et al., 1964; Eppenberger et al., 1967; Scholte, 1973). The cellular concentrations of the reactants for the creatine kinase reaction also vary among brain, heart, and skeletal muscle: The guanidino substrates vary 5-fold and the nucleotide substrates vary 2-fold among the tissues, with brain having the lowest and skeletal muscle the highest concentrations (Veech et al., 1979; Ingwall, 1982). Thus, the creatine kinase reaction is catalyzed in brain, heart, and skeletal muscle with marked differences in total enzyme activity, isozyme composition, enzyme localization, and substrate concentration. Which of these factors determines the velocity of the reaction in vivo?

The velocity of the creatine kinase reaction in vivo has been estimated with the ^{31}P NMR technique of magnetization transfer for brain (Shoubridge et al., 1982; Balaban et al., 1983), heart (Nunnally & Hollis, 1979; Matthews et al., 1982; Kupriyanov et al., 1984; Degani et al., 1985; Bittl & Ingwall, 1985; Ugurbil et al., 1986), and skeletal muscle (Brown, 1982; Meyer et al., 1982; Balaban et al., 1983). In most cardiac studies, the velocity of the creatine kinase reaction has been related to the level of cardiac performance, but changes in velocity have not been fully explained by substrate regulation, changes in pH, or enzyme activation (Nunnally & Hollis, 1979; Kupriyanov et al., 1984; Bittl & Ingwall, 1985).

The purpose of this study was to determine whether the velocity of the creatine kinase reaction in vivo, as measured with ^{31}P NMR methods, is regulated by the same factors that determine reaction velocity in vitro. The rate equation for creatine kinase (Morrison & Cleland, 1966; Kupriyanov et al., 1984) predicts that the reaction velocity is directly related to enzyme activity and to the ratios of the substrates to their K_m and K_i values. The rate equation accurately predicts the reaction velocity as measured with isotope-exchange studies (Morrison & Cleland, 1966) and with NMR methods (Kupriyanov et al., 1984) in experiments performed in vitro, but the rate equation has not been applied to in vivo conditions. In this study, we observed that the NMR-determined reaction

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; CK, creatine kinase; CrP, creatine phosphate; Cr, creatine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; NADPH, reduced nicotinamide adenine dinucleotide phosphate; *k*(for), rate constant for the creatine kinase reaction in the direction of MgATP synthesis; *k*(rev), rate constant for the direction of MgADP synthesis; *v*(for) and *v*(rev), reaction velocity for creatine kinase; mito, mitochondrial; T_1, longitudinal relaxation time.

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velocity for creatine kinase in vivo agrees with the predictions from the rate equation. The results suggest that the velocity of the creatine kinase reaction, measured under in vivo conditions that approach basal metabolism, is controlled directly by total enzyme activity and by substrates present in unsaturating concentrations.

Experimental Procedures

Brain. Male Sprague-Dawley rats, weighing ~250 g, were initially anesthetized with 15 mg of sodium methohexital tracheostomy and 2% halothane vaporized (Fluotec Mark 2, Cyprane Ltd., Keighley, England) and forced through a nasal cone with air. To eliminate contamination of the NMR spectra with skeletal muscle, we incised the skin over the cranium sagitally and excised the temporalis muscle bilaterally (total wet weight of the muscle was 1.0 g). Bleeding was controlled with local compression. The rat was then secured in an aluminum probe with a 1.4-cm, two-turn surface coil placed over the exposed cranium. Halothane anesthesia was continued throughout the course of the study. The magnetic field homogenization was improved by maximizing the water signal at 360 MHz. ^{31}P NMR spectra were obtained at 145.75 MHz by signal-averaging 80 scans with an interpulse delay of 12.5 s. Magnetization-transfer experiments were performed by selectively saturating the spectrum at either the CrP or [γ-P]ATP resonance with low-power, narrow-band radio frequency for 0, 0.3, 0.6, 1.2, 2.4, 3.0, 3.6, or 4.8 s. The duration of the broad-band, high-power pulse was 25 μs. At the end of the experiment, the rat was given a lethal dose of 20 mg of sodium pentobarbital intraperitoneally, and 0.5-g biopsies of cerebral cortex were excised and stored at −70 °C until enzyme and metabolite analyses were performed.

Studies with a five-chambered phantom showed that the pulse sequence investigated a superficial volume with 50% of the signal arising from a volume 2 mm from the coil and 75% of the signal from a volume 4 mm from the coil. Our preliminary NMR studies of the head of the anesthetized rat showed a CrP:ATP ratio of 4:1, suggesting that skeletal muscle was substantially contaminating the brain spectra. Pulse sequences with broad-band pulse durations as high as 150 μs reduced the rato, but magnetization-transfer studies were not possible because of incomplete selective saturation. Resection of the temporalis muscle bilaterally (total wet weight 1.0 g) eliminated skeletal muscle contamination. All magnetization-transfer studies of rat brain under halothane anesthesia were performed after temporalis resection. The spectra showed a substantial base-line "roll" that was eliminated after selective saturation at the inorganic phosphate resonance for greater than 3 s, but only partial elimination was observed for shorter saturation times (not shown).

Heart. Male Sprague-Dawley rats of 300-g weight were initially anesthetized with nasal halothane, intubated through a tracheostomy and ventilated with 21% oxygen and 2.0% halothane delivered through a Harvard small animal ventilator adapted to deliver a tidal volume of 3.0 mL 60 times/min. The relatively high dose of halothane was used to produce a low, yet stable, level of cardiac performance. The left common carotid artery was cannulated with a 1.5-cm length of polyethylene tubing (1.14-mm i.d.; 1.57-mm o.d.) that was advanced to the central aorta. The distal end of the cannula was connected to 150 cm of Tygon tubing (3.2-mm i.d.; 6.4-mm o.d.) attached to Statham P23dB pressure transducer for continuous recording of heart rate and central aortic pressure on a Hewlett-Packard 7754B recorder. The transducer system showed excellent frequency and damping characteristics with an undamped frequency response of 68 ± 9 Hz (n = 4; Grossman, 1974), allowing accurate pressure determinations even at heart rates in excess of 300. After a midline sternotomy and ligation of the internal thoracic arteries at the level of the thymus, the anterior chest wall was excised to the anterior axillary lines laterally and to the diaphragm inferiorly. This eliminated any skeletal muscle from contributing ^{31}P NMR signal through a surface coil placed directly over the apex of the left ventricle (data from phantoms and cardiectomized rats not shown). After the magnetic field was shimmed to ^{23}Na at 95.24 MHz, magnetization-transfer experiments were performed by selectively saturating the ^{31}P spectrum at the resonance for either creatine phosphate or [γ-P]ATP for 0, 0.3, 0.6, 1.2, 2.4, 3.0, or 4.8 s. Spectra were obtained by summing 32 scans from a 15-μs broad-band, high-power pulse with a 12.5-s interpulse delay. All magnetization-transfer measurements were made during metabolic and hemodynamic steady state, proved by less than 10% change in creatine phosphate, [β-P]ATP, blood pressure, or heart rate. The four preparations used in this study showed stable cardiac performance and metabolic measurements for 120 min. The animals had a heart rate of 276 ± 20 beats/min and systolic pressure of 71 ± 8 mmHg. Samples of arterial blood obtained directly from the left ventricle through a 26-gauge needle from four preparations showed p_{O_2} = 96 ± 14 mmHg, p_{CO_2} = 37 ± 9, pH 7.38 ± 0.11, and hemoglobin oxygen saturation = 98 ± 1% (Corning pH/blood gas analyzer). At the end of the study, the halothane dose was increased to 4% to produce lethal cardiovascular collapse. Left ventricular tissue was excised, rinsed of blood, and stored at −70 °C until tissue analysis of creatine kinase and creatine was undertaken.

Skeletal Muscle. Male Sprague-Dawley rats, weighing ~250 g, were sedated with 6 mg of sodium pentobarbital intraperitoneally. Each rat was placed in an aluminum probe, machine-tooled to fit the bore of the Nicolet NT360 spectrometer. Within the probe a 1.4-cm, two-turn surface coil was placed against the quadriceps muscle of the sedated rat. The magnetic field was homogenized by maximizing the sodium signal at 95.3 MHz with an Oxford Instruments shim supply. ^{31}P NMR spectra were obtained at 145.75 MHz by recording the signal-averaged sum of 24 scans with an interpulse delay of 12.5 s after narrow-band, low-power, selective saturation at either the CrP or [γ-P]ATP resonance for 0, 0.3, 0.6, 1.2, 2.4, 3.6, or 4.8 s. The duration of the high-power, broad-band pulse was 15 μs. At the end of the study, the rat was given a lethal dose of 20 mg of sodium pentobarbital intraperitoneally, and 1-g biopsies of the quadriceps muscle were taken and stored at −70 °C until analysis of enzyme and metabolite content was performed.

NMR Calculations. Magnetization-transfer measurements of the velocity of the creatine kinase reaction in vivo were based on the two-site chemical exchange model of Forsen and Hoffman (1963). These calculations are described in detail by Bittl and Ingwall (1985). Briefly, variance-weighted computer program for nonlinear regression was used to fit the relationship between magnetization of CrP and time of saturation at [γ-P]ATP, or vice versa, to a single-exponential function whose slope yields the value for τ_1, where $1/\tau_1 = 1/T_1 + k$; T_1 is the longitudinal relaxation time in the absence of exchange for CrP or [γ-P]ATP and k is the unidirectional rate constant for the reaction in the direction of MgATP synthesis (for) or MgADP synthesis (rev) during saturation at [γ-P]-ATP or CrP, respectively.

Biochemical Analysis. From each sample, 5–10 mg of tissue was homogenized in 0.1 M K_2HPO_4 buffer, pH 7.4, with 1

Table I: Isozyme Composition[a]

tissue	total CK	BB	MB	MM	mito
brain	23	99	0	0	1
heart	62	1	14	61	25
muscle	224	0	0	98	2

[a] The percent distribution of the tissue activity of creatine kinase (mM/s) was determined electrophoretically for brain, heart, and skeletal muscle.

mM EGTA, 1 mM β-mercaptoethanol, and 0.1% Triton at 4 °C (final tissue concentration 5 mg/mL). Aliquots were taken for measurement of total creatine kinase activity and isozyme composition prior to the addition of Triton X-100. Tissue creatine kinase activity was measured at 30 °C with the coupled-enzyme scheme of Rosalki (1967) by measuring the rate of appearance of NADPH. The reaction solution contained MgADP and CrP in final, saturating concentrations of 1.2 and 20 mM, respectively, at an optimum pH or 6.5 for the reaction in the direction of MgATP synthesis (Hall et al., 1979; Cook et al., 1981). When the activity was multiplied by 1.75, we obtained estimates for V_{max}(for) at 37 °C.

The relative proportions of the creatine kinase isozymes were determined by using cellulose acetate strip electrophoresis coupled with scanning fluorometry (Hall & DeLuca, 1976). To obtain quantitative results, we added 1 mM dithiothreitol to the electrophoresis buffer to prevent oxidation of the B-chain. The addition of diadenosine pentaphosphate (~20 μM) to the staining mixture inhibited the adenylate kinase activity.

The tissue content of CrP was estimated from the CrP:ATP ratio by using index values of 3 μmol of ATP/g wet weight in brain (Chapman et al., 1977; Veech et al., 1979), 5 μmol of ATP/g wet weight in heart (Ingwall, 1982), and 6 μmol of ATP/g wet weight for skeletal muscle (Beis & Newsholme, 1975; Fitch et al., 1974, 1975; Veech et al., 1979). The total tissue content of creatine (creatine plus creatine phosphate) was measured according to the method of Kammermeier (1982).

All values for substrate and enzyme content were converted to units of concentration by using a value of 0.65 g of unbound cell water/g wet weight for blood-perfused heart (Polimeni & Page, 1980), brain (Bradbury et al., 1968), and skeletal muscle (Palmer & Gulati, 1976).

Rate Equation Calculations. The rate equation for creatine kinase is derived from the work of Morrison and Cleland (1966) and Kupriyanov et al. (1984):

$$v(\text{for}) = \frac{V_{max}(\text{for})[\text{ADP}][\text{CrP}]}{DK_m(\text{ADP})K_i(\text{CrP})}$$

$$v(\text{rev}) = \frac{V_{max}(\text{rev})[\text{ATP}][\text{Cr}]}{DK_m(\text{ATP})K_i(\text{Cr})}$$

where

$$D = 1 + \frac{[\text{ADP}]}{k_i(\text{ADP})} + \frac{[\text{CrP}]}{K_i(\text{CrP})} + \frac{[\text{ATP}]}{K_i(\text{ATP})} + \frac{[\text{Cr}]}{K_i(\text{Cr})} + \frac{[\text{ADP}][\text{CrP}]}{K_m(\text{ADP})K_i(\text{CrP})} + \frac{[\text{ATP}][\text{Cr}]}{K_m(\text{ATP})K_i(\text{Cr})} + \frac{[\text{ADP}][\text{Cr}]}{K_m(\text{ADP})K_i(\text{Cr})}$$

Values for V_{max}(for), ATP, CrP, and creatine were determined from the NMR and spectrophotometric methods stated above. Free cytosolic ADP concentration was calculated by using an apparent K_{eq} = 166 (Lawson & Veech, 1979). V_{max}(rev) was estimated by dividing V_{max}(for) by 4 since the ratio V_{max}(for):V_{max}(rev) has been reported to be 4:1 for BB (Jacobs & Kuby, 1970), MM (Hall et al., 1979), and the mitochondrial isozymes (Saks et al., 1975). The K_m and K_i values for each substrate and isozyme were averaged from the literature (Morrison & James, 1965; Jacobs & Kuby, 1970; Jacobus & Lehninger, 1973; Saks et al., 1975, 1984, 1985; Hall et al., 1979; Jacobus & Saks, 1982; Kupriyanov et al., 1984; Basson et al., 1985). All K_m and K_i values were obtained at 30 °C in the presence of Mg^{2+} and, with one exception, at pH 7.4.

Statistical Analysis. Variance-weighted linear and nonlinear regression analysis was aided by the Statistics and Data Management Package (RS/1 Version 2.0) of Bolt, Beranek, and Newman (Cambridge, MA, 1985; VAX 11/780 computer). Unless specified otherwise, all tissue concentrations are given in millimolar or micromolar terms. All data are presented as mean ± SD for four studies of brain, heart, and skeletal muscle.

RESULTS

Enzyme Activity and Metabolite Analysis. The creatine kinase activity varied 10-fold among the three tissues, with brain having the lowest activity and skeletal muscle the highest (Table I). Electrophoretic analysis of the creatine kinase isozymes showed that brain contained 99% BB, heart had 61% MM and 14% MB, and skeletal muscle contained 98% MM creatine kinase. The mitochondrial isozyme comprised 25% of total activity in heart, 0.5% of total activity in brain, and 2% of total activity in skeletal muscle.

The tissue concentrations of the creatine kinase substrates were measured with ^{31}P NMR and spectrophotometric methods (Table II). The creatine phosphate concentration showed a 3-fold range and ATP showed a 2-fold range among the three tissues. Standard biochemical analysis yielded the values for the tissue concentration of total creatine, which exhibited a 4-fold range from brain at the low end to the skeletal muscle at the high end. To estimate cytosolic MgADP concentration, we used the value of 166 for the apparent K_{eq} at pH 7, 38 °C, 0.25 ionic strength, and 1 mM Mg^{2+}. The estimates for [ADP] in brain, heart, and skeletal muscle were 10, 17, and 31 μM, respectively.

Magnetization Transfer. The creatine kinase system was studied in vivo in three tissues under basal metabolic conditions: in the anesthetized brain, in the open-chest heart at a low level of cardiac performance, and in resting skeletal muscle of living rat. By saturating either the CrP or the [γ-P]ATP resonance for times that ranged from 0 to 4.8 s, we measured the rate of chemical exchange between the two high-energy phosphate reactants of creatine kinase in intact tissue.

The surface coil studies of brain in the living animal yielded spectra with a substantial base-line roll. After saturation at either CrP or [γ-P]ATP for 0–4.8 s, we observed that the [γ-P]ATP or CrP magnetization, respectively, decreased exponentially ($r^2 > 0.99$) with respect to saturation time (Figure 1).

Table II: Enzyme and Metabolite Concentrations[a]

tissue	CK	[CrP] + [Cr]	[CrP]	[Cr]	[ATP]	[ADP]
brain	23.4 ± 2.8	15.0 ± 1.8	10.9 ± 1.3	4.0 ± 0.8	4.6	10 ± 2
heart	62.4 ± 4.5	20.2 ± 1.9	14.7 ± 0.8	5.5 ± 1.5	7.7	17 ± 5
muscle	223.9 ± 16.0	54.8 ± 5.7	35.3 ± 1.5	19.5 ± 4.2	9.2	31 ± 6

[a] The tissue activity of creatine kinase (mM/s) and the concentrations (mM) of substrates were measured by spectrophotometric and NMR techniques. ADP concentration (μM) was estimated from the apparent equilibrium constant of 166 (Lawson & Veech, 1979).

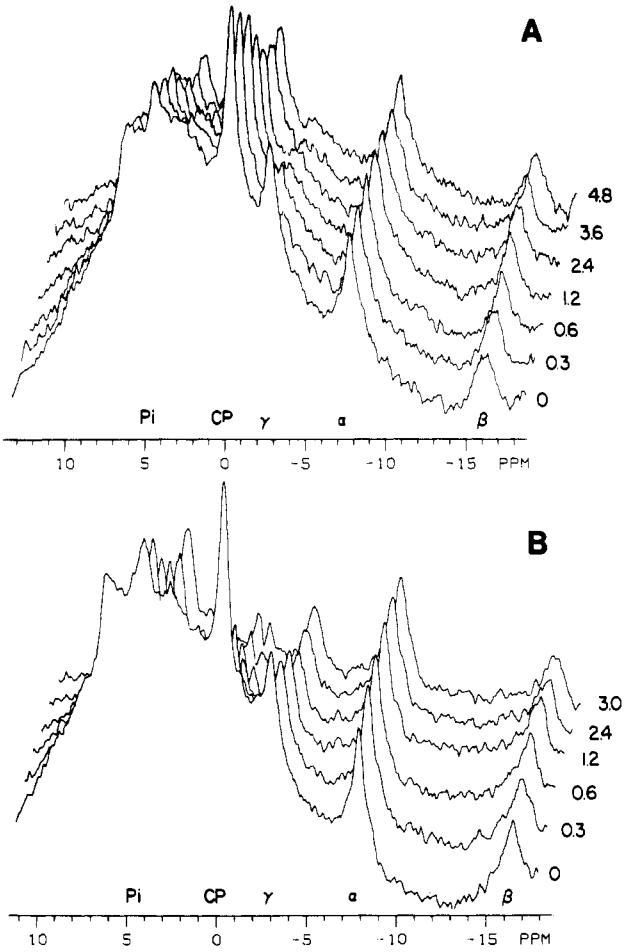

FIGURE 1: Brain. ^{31}P NMR spectra were obtained after selective saturation at either [γ-P]ATP (A) or CrP (B) for 0–4.8 s. The spectra were recorded by using a surface coil that was placed directly on the cranium of the halothane-anesthetized rat after resection of the temporalis muscle bilaterally.

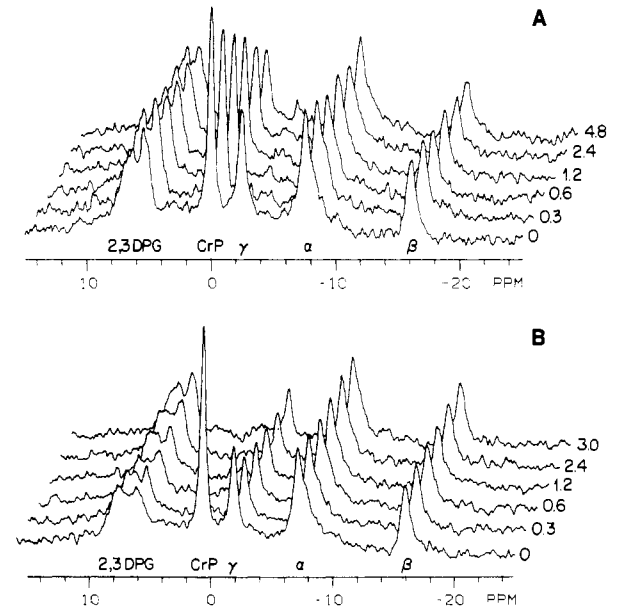

FIGURE 2: Heart. ^{31}P NMR spectra were recorded through a surface coil placed directly over the left ventricular apex in intubated, halothane-anesthetized rats. A wide excisional thoracotomy was performed to eliminate skeletal muscle signal. Magnetization transfer was observed after selective saturation at the resonance for either [γ-P]ATP (A) or CrP (B) for 0–4.8 s.

In the heart of the open-chest rat, the magnetization-transfer technique provided evidence for exchange via creatine kinase

Table III: T_1 Values for CrP and [γ-P]ATP[a]

tissue	T_1(CrP)	T_1([γ-P]ATP)
heart	2.4 ± 0.2	1.0 ± 0.2
brain	1.7 ± 0.2	1.0 ± 0.2
muscle	2.7 ± 0.3	1.3 ± 0.1

[a] ^{31}P NMR magnetization-transfer studies were performed in the heart, brain, or skeletal muscle of living, anesthetized rat to yield T_1 values (s) for the high-energy reactants of the creatine kinase reaction.

Table IV: Kinetics of the Creatine Kinase Reaction Measured by ^{31}P NMR[a]

	k (s^{-1})		velocity (mM/s)	
tissue	k(for)	k(rev)	v(for)	v(rev)
brain	0.37 ± 0.07	0.39 ± 0.06	4.1 ± 1.1	1.8 ± 0.3
heart	0.34 ± 0.11	0.59 ± 0.07	5.1 ± 1.6	4.6 ± 0.5
muscle	0.52 ± 0.03	1.90 ± 0.30	18.4 ± 1.7	17.5 ± 2.4

[a] The unidirectional rate constants and velocities for the creatine kinase reaction were derived from ^{31}P NMR magnetization studies of brain, heart, or skeletal muscle of living rat in the direction of MgATP synthesis [k(for), v(for)] and CrP synthesis [k(rev), v(rev)].

(Figure 2). As the saturation time at CrP or [γ-P]ATP was prolonged, more magnetization was transferred between the high-energy phosphate species. The relationship between magnetization and saturation time defined single-exponential functions ($r^2 > 0.99$) for both the forward and reverse directions of the creatine kinase reaction. To test for the effect of direct saturation of a neighboring peak by the narrow-band low-power pulse, we saturated the base line 350 Hz downfield from CrP in other experiments (not shown). Such experiments showed that there was less than 5% attenuation in the CrP magnetization at saturations times that ranged from 0.3 to 4.8 s, suggesting that the low-power pulse was selective.

Similarly, in skeletal muscle the CrP or [γ-P]ATP magnetization decreased exponentially as the saturation time at the reciprocal resonance increased (Figure 3). The relationship between magnetization and saturation time for both the forward and reverse reactions defined a single-exponential function ($r^2 > 0.99$). In order to determine the selectivity of saturation under the experimental conditions, we placed the position of the saturating pulse 350 Hz (2.7 ppm) downfield from the CrP resonance for 0–4.8 s. The direct attenuation of the saturating pulse on CrP magnetization was <5%, suggesting that saturation of the CrP or [γ-P]ATP resonances during the magnetization-transfer experiments was selective.

Using the two-site chemical exchange model of Forsen and Hoffman (1963) and the parameters for the exponential functions for brain, heart, and skeletal muscle above, we calculated the T_1 values for CrP and [γ-P]ATP and the rate constants and the velocity of the creatine kinase reaction in vivo. The T_1 values for CrP were about 2.0 s and the T_1 values for [γ-P]ATP were about 1.0 s in heart, brain, and skeletal muscle (Table III).

In brain, the rate constants for both directions of the reaction were nearly equal (Table IV). In heart, k(rev) was 1.6 times greater than k(for). In skeletal muscle k(rev) was 3.7 times greater than k(for). Multiplying the rate constant by the substrate content yielded the values for reaction velocity. The values for the creatine kinase reaction velocities varied approximately 10-fold among the three tissues. In brain, the velocity of the reaction in the direction of MgATP synthesis was higher than the velocity in the opposite direction; however, in heart at a low level of cardiac performance and in resting skeletal muscle the velocities in both directions were indistinguishable.

We compared values for creatine kinase reaction velocity

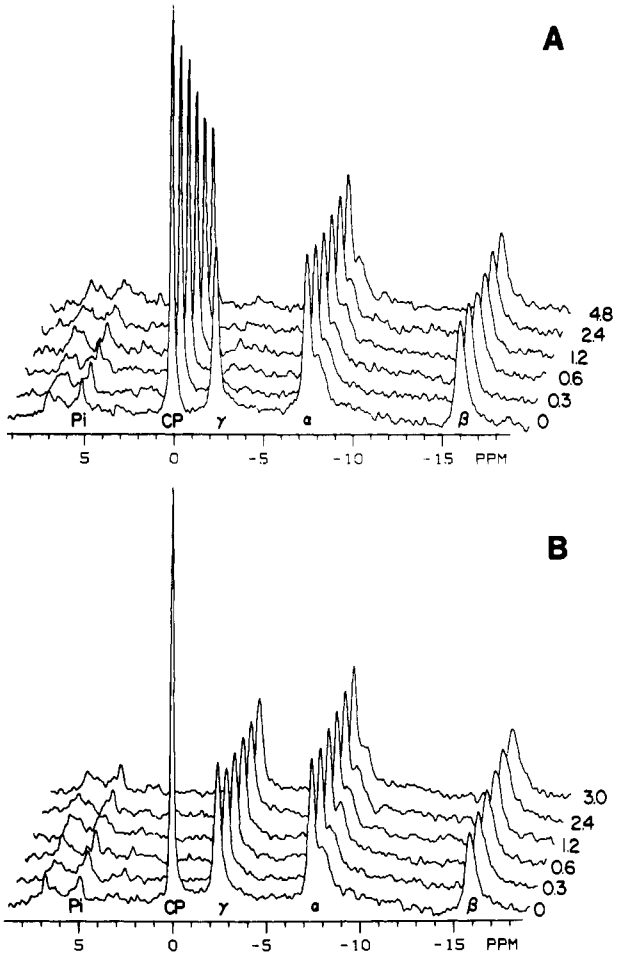

FIGURE 3: Skeletal muscle. A surface coil was placed over the quadriceps muscle of anesthetized rats, and ^{31}P NMR spectra were obtained after 0–4.8 s of selective saturation at [γ-P]ATP (A) or CrP (B).

to the activity of the enzyme and the to the concentrations of the reactants in the three tissues. The relationship between the reaction velocity for creatine kinase in vivo, as measured by ^{31}P NMR magnetization transfer, and the activity of creatine kinase in brain, heart, and skeletal muscle described a linear function that intercepts the origin (Figure 4). This suggests that, under conditions of basal metabolism, the NMR measurement of creatine kinase reaction velocity is closely related to total creatine kinase activity despite the variations in isozyme composition and intracellular localization. The slope of the function that relates reaction velocity to the tissue activity of creatine kinase is 0.075. This suggests that less than 10% of the tissue capacity for chemical transfer by the enzyme is used under conditions that approach basal metabolism.

Creatine kinase reaction velocity was also closely related to the total Cr, CrP, and free Cr concentrations ($r^2 = 0.91$, $r^2 = 0.94$, and $r^2 = 0.82$, respectively) and to the concentrations of ATP and ADP ($r^2 = 0.64$ and $r^2 = 0.68$, respectively). At first analysis, it would appear that the reaction velocity is regulated directly by substrate concentration, but the levels of all substrates merely match the total activity of creatine kinase. Brain has the lowest and skeletal muscle the highest enzyme activity and substrate concentrations for the creatine kinase reaction. The relationship between the guanidino substrate concentrations and enzyme activity is shown in Figure 5.

Rate Equation Analysis. In order to identify the biochemical determinants of the reaction velocity for creatine kinase in vivo, we first compared the substrate concentrations

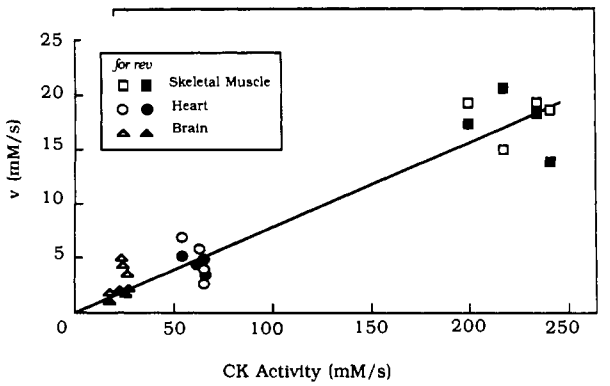

FIGURE 4: Creatine kinase reaction velocity vs. enzyme activity. The velocity (v, mM/s) of the creatine kinase reaction, as measured by NMR magnetization transfer for the direction of both MgATP synthesis (open symbols) and MgADP synthesis (closed symbols), was compared to the tissue activity of the enzyme (mM/s) to define the linear function $v = 0.075(\text{activity}) - 0.8$ ($r^2 = 0.92$; $p = 0.001$).

Table V: Dissociation Constants (mM) for Creatine Kinase Isozymes[a]

		apparent K_m				
tissue	isozyme	MgADP	CrP	MgATP	Cr	ref
brain	BB	0.03	0.52	0.05	3.0	*b*
brain	BB	0.11	0.90	0.16	2.3	*c*
mean	BB	0.07	0.71	0.11	2.7	
heart	MM + MB	0.12	3.6	2.5	33.3	*d*
heart	MM + MB	0.09	2.4	0.6	22.0	*c*
heart	MM	0.03	0.4	0.4	9.0	*e*
mean	MM + MB	0.08	2.1	1.2	21.3	
heart	mito	0.04	0.7	0.1	6.0	*d*
heart	mito	0.07	4.1	0.4	2.3	*c*
heart	mito	0.05	0.5	0.7	5.0	*f*
heart	mito	0.02	0.3	0.06	4.5	*g*
heart	mito			0.02	3.5	*h*
heart	mito			0.01	5.2	*i*
mean	mito	0.05	1.4	0.22	5.0	
weighted mean		0.07	2.0	0.93	17.3	
muscle	MM	0.05	2.9	0.5	6.1	*j*
muscle	MM	0.25	4.7	0.9	8.0	*k*
mean	MM	0.15	3.8	0.7	7.1	

		K_i				
tissue	isozyme	MgADP	CrP	MgATP	Cr	ref
brain	BB	0.04	0.67	0.18	13	*b*
heart	MM	0.09	1.04	0.90	20	*e*
heart	mito		1.40	0.29	29.4	*i*
weighted mean		0.09	1.13	0.75	22.4	
muscle	MM	0.25	4.65	2.70	26.7	*k*

[a] Actual and mean values for the dissociation constants measured at 30 °C in the presence of magnesium at pH 7.4. Weighted mean (heart) was calculated from the formula 0.75(mean MM + MB) + 0.25(mean mito) to reflect the isozyme distribution in heart (Table I). [b] Jacobs & Kuby, 1970. [c] Basson et al., 1985. [d] Jacobus & Lehninger, 1973. [e] Saks et al., 1984. [f] Saks et al., 1975. [g] Hall et al., 1979. [h] Saks et al., 1985. [i] Jacobus & Saks, 1982. [j] Morrison & James, 1965. [k] Kupriyanov et al., 1984.

to their respective apparent K_m and K_i values for each isozyme. The values for the dissociation constants were averaged from 10 studies in the literature in which measurements were made under conditions of temperature, pH and Mg^{2+} concentration that were applicable to this study (Table V). Comparing substrate concentrations (Table II) to dissociation constants for each isozyme, we observed that ATP and CrP were consistently present in saturating concentrations in all three tissues. On the other hand, ADP and Cr were present in concentrations that were at or below the range of dissociation constants, suggesting that these substrates are present in concentrations

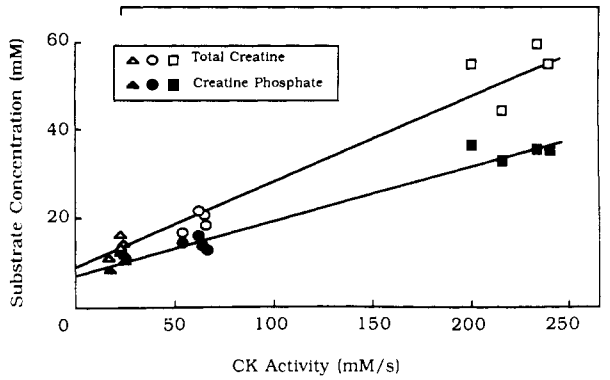

FIGURE 5: Total creatine and creatine phosphate concentration vs. creatine kinase activity. The creatine concentration was linearly related to the total enzyme activity in brain (△), heart (○), and skeletal muscle (□). The linear functions are given by total Cr = 0.20(activity) + 9.1 (r^2 = 0.96; p = 0.0001) and CrP = 0.12(activity) + 7.8 (r^2 = 0.98; p = 0.0001).

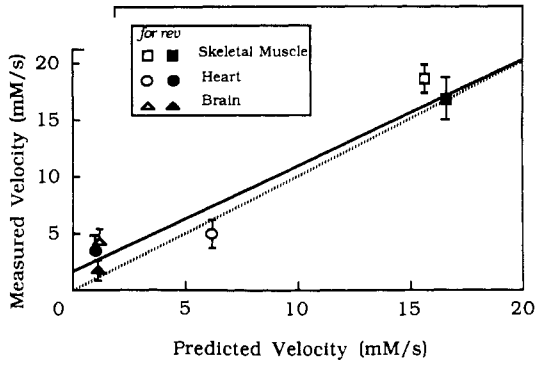

FIGURE 6: NMR-measured velocity vs. the predicted velocity from the creatine kinase rate equation. Measured vs. predicted velocity defined a linear function [solid line, measured = 0.91(predicted) + 1.75; r^2 = 0.98; $p < 0.001$] that was indistinguishable from the line of identity (dotted line).

that regulate reaction velocity. When the ratios of substrate concentration to dissociation constant are calculated as terms for the creatine kinase rate equation, the terms that contain values for [ADP] and [Cr] predominate (Table VI). Although the dissociation constants for each isozyme showed wide variation from study to study, the predicted velocities from the use of averaged constants in the rate equation agreed with the NMR-measured reaction velocities (Figure 6).

Discussion

We compared the NMR measurements of creatine kinase reaction velocity in vivo to the tissue content of creatine kinase activity, isozyme distribution, and metabolite concentrations in brain, heart, and skeletal muscle of living rat. We observed a close relationship between the reaction velocity, as measured by magnetization transfer, and the tissue content of creatine kinase activity. The findings suggest that the kinetics of magnetic transfer are related to the capacity of the tissue for chemical transfer via creatine kinase and, at least under basal conditions, are independent of isozyme composition and localization. The NMR measurements of reaction velocity are also in good agreement with predictions from the rate equation for the creatine kinase reaction (Morrison & Cleland, 1966; Kupriyanov et al., 1984). This rate equation analysis identifies the determinants of reaction velocity in vivo and provides independent validation for the accuracy of the NMR measurements of reaction velocity in tissue.

We observed a linear relationship between reaction velocity and substrate concentration in the three tissues. If V_{max} is held

Table VI: Predictions for Creatine Kinase Reaction Velocity from the Rate Equation[a]

	brain	heart	muscle
V_{max}(for) (mM/s)	23	62	224
V_{max}(rev) (mM/s)	6	16	56
[ADP][CrP]/K_m(ADP)K_i(CrP)	2.4	3.2	1.5
[ATP][Cr]/K_m(ATP)K_i(Cr)	8.9	1.3	6.5
[ADP][Cr]/K_m(ADP)K_i(Cr)	0.05	0.06	0.15
[ATP]/K_i(ATP)	25.6	10.3	26.7
[Cr]/K_i(Cr)	0.3	0.2	0.7
[ADP]/K_i(ADP)	0.3	0.2	0.1
[CrP]/K_i(CrP)	16.3	13.0	7.6
D	54.8	29.3	21.0
v(for), pred (mM/s)	1.0	6.8	17.3
v(for), measd (mM/s)	4.1	5.1	18.4
v(rev) pred (mM/s)	0.9	0.7	17.3
v(rev) measd (mM/s)	1.8	4.6	17.5

[a] The rate equation for creatine kinase (Morrison & Cleland, 1966; Kupriyanov et al., 1984; see Experimental Procedures) was used to calculate the predicted values for v(for) and v(rev) for comparison to NMR-measured values in brain, heart, and skeletal muscle. V_{max}(rev) was estimated from values of V_{max}(for) obtained from enzyme activities in tissue extracts (Rosalki, 1967; Jacobs & Kuby, 1970; Hall et al., 1979; Saks et al., 1975).

constant, the rate equation predicts a hyperbolic relationship between reaction velocity and substrate concentration. The observed linear relationship between velocity and substrate level is due to the fact that substrate concentration is directly proportional to enzyme activity in each sample.

Comparing the dissociation constants (Table V) to substrate concentrations (Table II), we observed that only Cr and MgADP are present in concentrations at or below the published K_m and K_i values for the isozymes in all tissues, supporting a regulatory role for Cr and MgADP. The regulatory role of MgADP has been evaluated in the heart in the anesthetized, open-chest animal (Bittl et al., 1987a,b). In these studies, the NMR-measured velocity for the creatine kinase reaction increased with estimates for [MgADP] under all normoxic conditions, consistent with a regulatory role for ADP. Matthews et al. (1982) proposed that [ADP] regulates the velocity of the creatine kinase reaction in the isolated heart. Comparing the beating, glucose-supplied heart to the arrested, acetate-perfused heart, they observed that the creatine kinase reaction velocity in the direction of ATP synthesis increased 1.9-fold as the estimate for [ADP] rose 5.5-fold. However, when Matthews et al. (1982) studied only glucose-supplied hearts over a 3.1-fold range of oxygen consumption, they observed only a 2.3-fold increase in [ADP] and an insignificant 26% increase in reaction velocity in the direction of ATP synthesis. This study can be compared to that of Bittl and Ingwall (1985), who studied glucose-supplied hearts over a 7.8-fold range of oxygen consumption and observed a 3.8-fold increase in reaction velocity for creatine kinase. These investigators observed that creatine kinase reaction velocity is related to the level of myocardial oxygen consumption and proposed that both oxygen consumption and creatine kinase reaction velocity are regulated by the same metabolites. Interestingly, the relationship between reaction velocity and ADP has not been observed under nonphysiologic conditions, such as hypoxia in the open-chest rat (Bittl et al., 1987b) or during KCl arrest in the isolated heart (Bittl & Ingwall, 1985, 1986). The regulatory role of Cr can be evaluated by reviewing the work of Shoubridge et al. (1985), who observed a 4-fold reduction in creatine kinase reaction velocity when Cr was depleted 4-fold in hearts from rats fed β-guanidinopropionic acid (β-GPA). These workers also observed a 10-fold reduction in CrP after β-GPA feeding; CrP levels were thus reduced to

the values near K_m and K_i for CrP in heart (Table V) and may have also limited the reaction velocity. In this study, the values for MgATP and creatine phosphate exceed the published K_m values for these substrates for all isozymes, suggesting that the total cellular content of the high-energy substrates is saturating for creatine kinase. It has been noted, however, that local concentrations of MgATP, for example, at the mitochondrial isozyme may regulate the creatine kinase reaction under conditions of physiologic stimulation (Jacobus & Lehninger, 1973; Saks et al., 1975; Kupriyanov et al., 1984; Bittl & Ingwall, 1985). The relationship between the guanidino substrates and creatine kinase activity is nonetheless of interest because creatine is not synthesized in brain, heart, or skeletal muscle but is transported from the bloodstream by an unknown mechanism. During fetal and neonatal cardiac development, tissue contents of creatine and creatine kinase activity increase coordinately (Ingwall et al., 1980).

The relationship between reaction velocity and pH was not directly investigated by this study because the inorganic phosphate line could not be reliably discriminated from 2,3-diphosphoglycerate lines in blood-perfused heart and brain. Nevertheless, the NMR measurements of creatine kinase reaction velocity were probably made in the three samples under similar pH conditions. Intracellular pH, as measured by ^{31}P NMR methods, varies over the small range from 6.98 to 7.20 for various tissues (Ingwall, 1982; Shoubridge et al., 1984). It is unlikely that small changes in pH among the three tissues contribute to the 10-fold range of reaction velocities measured in this study for the various isozymes since the in vitro reaction velocities for both beef heart mitochondrial creatine kinase (Hall et al., 1979) and rabbit muscle creatine kinase (Cook et al., 1981) show a similar dependence on pH, with the direction for MgATP synthesis having a pH optimum of about 6.7 and the opposite direction a pH optimum of 8.0.

The NMR methods used here are intrinsically insensitive. By optimizing the signal-to-noise ratio and averaging multiple observations, we were nonetheless able to measure reaction velocity precisely (with 15% standard deviation in heart and skeletal muscle and 25% standard deviation in brain). We were also able to measure reaction velocity accurately, as proved by the close agreement between the NMR measurements and the predicted values from the creatine kinase rate equation. [The NMR measurement did not agree with the rate equation prediction only for v(rev) for heart. Using a ratio of 1.1:1.0, as reported by Hall et al. (1979) for V_{max}(for):V_{max}(rev) for beef heart mitochondrial creatine kinase, instead of a ratio of 4:1, would have decreased the discrepancy.]

Our measurements of substrate concentrations, T_1 values, and the unidirectional rate constants for intact tissues agree fairly well with values in the literature. The absolute and relative values of brain creatine phosphate and ATP measured in this study agreed with those of Veech et al. (1979), Shoubridge et al. (1982), and Balaban et al. (1983). Using ^{31}P NMR, we measured higher brain creatine phosphate:creatine ratios than Chapman et al. (1977) and Veech et al. (1979), who used spectrophotometric methods and may have seen a mild degree of creatine phosphate hydrolysis. In heart, we measured and estimated substrate contents that agreed well with those in the literature (Ingwall, 1982; Matthews et al., 1982; Kupriyanov et al., 1984; Degani et al., 1985; Bittl & Ingwall, 1985). In skeletal muscle, our absolute and relative values for substrate content also agreed with published values (Fitch et al., 1974; Beis & Newsholme, 1975; Veech et al., 1979; Balaban et al., 1983; Shoubridge et al., 1984). Most authors have repoted that, in the absence of exchange, T_1 values for CrP are in the range 1.8–2.3 s and the T_1 values for [γ-P]ATP are in the range 0.6–1.3 s for brain (Balaban et al., 1983), heart (Nunnally & Hollis, 1979; Bittl & Ingwall, 1985), and skeletal muscle (Balaban et al., 1983). When other investigators calculated T_1 values from saturation recovery methods, results ouside this range were observed (Shoubridge et al., 1982; Matthews et al., 1982). In anesthetized brain, we obtained values for rate constants that differed from those of Shoubridge et al. (1982) by a factor of 2. The discrepancy may be due to different T_1 values, which were calculated by a diferent method and differed from our values by about a factor of 2. The calculated rate constants for the heart in the open-chest animal agree with those in the literature isolated hearts that are arrested or at very low workload (Matthews et al., 1982; Kupriyanov et al., 1984; Bittl & Ingwall, 1985). We calculated k in skeletal muscle to be 0.52 s^{-1}. This agrees well with the value of 0.40 s^{-1} calculated from the work of Balaban et al. (1983) but is twice as high as the value from Meyer et al. (1982), who performed experiments at 28 °C.

Although no investigators have compared the rate equation predictions to NMR measurements of reaction velocity in tissue, we can compare our values for creatine kinase reaction velocity to NMR measurements in the literature. Like Shoubridge et al. (1982), we observed a 2.4:1 ratio between forward and reverse flux in the anesthetized brain of living rat. Our results for creatine kinase reaction velocity in brain are in close agreement with those of Balaban et al. (1983), who obtained a value of 2 μmol (g wet weight)$^{-1}$ s^{-1} (which is equivalent to 3.1 mM/s) for the reaction velocity in the direction of MgATP synthesis. For skeletal muscle, Balaban et al. (1983) used two-dimensional Fourier transform NMR and measured the velocity for the creatine kinase reaction to be 13 μmol (g wet weight)$^{-1}$ s^{-1} (equivalent to 20 mM/s), similar to our value of 18 mM/s.

Consistent with our previous work (Bittl & Ingwall, 1985), we could detect no difference between the creatine kinase reaction velocities for MgATP or MgADP synthesis in hearts at very low workloads in this study. Apparent discrepancies between v(for) and v(rev) have been reported at higher levels of oxygen consumption when the NMR measurements are analyzed by a two-site chemical exchange model (Shoubridge et al., 1982; Bittl & Ingwall, 1985; Ugurbil et al., 1986). Estimates for oxygen consumption for each of the three samples can be obtained from literature values. Despite depression by halothane anesthesia, oxygen consumption in the rat brain (Haegerdal et al., 1975; Harp et al., 1976) and in the rat heart (Neely et al., 1967) was 20–30 times higher than in resting skeletal muscle (Crow & Kushmerick, 1982; Bockman, 1983; Thompson & Mohrman, 1983). Assuming that the stoichiometry of ATP synthesis to oxygen consumption is 6:1, we can calculate ATP synthesis rates from oxidative phosphorylation of 0.59, 0.49, and 0.02 mM/s for brain, heart, and skeletal muscle, respectively. Thus, the velocity of the creatine kinase reaction exceeded the estimates for mitochondrial ATP synthesis by factors of 5, 10, and 100 for brain, heart, and skeletal muscle, respectively. Thus, the apparent discrepancy between v(for) and v(rev) in brain can be attributed to the participation of ATP in other reactions that are not considered by the two-site chemical exchange model of Forsen and Hoffman (1963).

In summary, this study combines the method of NMR saturation transfer with traditional enzymatic analysis in biologic tissue to define the factors that regulate creatine kinase reaction velocity in vivo. The results suggest that under basal

conditions the velocity of the creatine kinase reaction is determined by the tissue activity of the enzyme and the substrate concentration in a manner similar to that observed in vitro, as predicted by the rate equation. Isozyme composition and intracellular localization, as well as substrate control from MgADP and Cr, may contribute to the regulation of the reaction velocity during physiologic stimulation.

Registry No. CK, 9001-15-4; Cr, 57-00-1; CrP, 67-07-2; MgADP, 7384-99-8; MgATP, 1476-84-2; ADP, 58-64-0; ATP, 56-65-5.

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