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Estrogen-Induced Changes in High-Energy Phosphate Metabolism in Rat Uterus: ^{31}P NMR Studies†

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ABSTRACT: Changes in the concentrations of high-energy phosphate metabolites were measured by ^{31}P NMR spectroscopy of surviving rat uteri from 0–48 h following estrogen administration. Concentrations (millimoles per kilogram wet weight) of these metabolites in the untreated immature uterus, measured at 4 °C, were found to be the following: creatine phosphate (CP), 2.1 ± 0.2; nucleoside triphosphates, mainly adenosine 5′-triphosphate (ATP), 4.6 ± 0.4; phospho monoesters, primarily sugar phosphates (SP), 5.4 ± 0.7; and inorganic phosphate (P_i), 0.8 ± 0.4. Adenosine 5′-diphosphate (ADP) concentration was estimated to be approximately 40 μmol/kg wet weight from the assumed equilibrium of the creatine kinase reaction. The concentration of CP, and to lesser extent ATP and SP, declined within the first 1.5–3 h after injection of 17β-estradiol, returned to control values between 6 and 12 h, and then increased, reaching maximal concentrations at 24 h. From the fractions of the total soluble ATP in free and Mg^{2+}-bound forms, [free Mg^{2+}] in the untreated uterus was estimated to be 0.2–0.4 mmol/kg wet weight. An increase in [free Mg^{2+}] in the uterus was detected 1.5 h after estrogen injection. A subsequent parallel increase in the ratio of ATP to CP concentrations suggests that estrogen can also affect the apparent creatine kinase equilibrium by modulating [free Mg^{2+}].

Estrogen stimulates the immature or ovariectomized rat uterus in roughly two stages (Katzenellenbogen & Gorski, 1975); the first leads to an accumulation of RNA and protein and includes an early increase both in the rate of phosphate incorporation into phospholipids and in the wet weight, the latter due mainly to fluid imbibition (Astwood, 1938). In the second stage, the rate of DNA synthesis increases, and at approximately 24 h, cell division takes place in all uterine cell types (Kaye et al., 1972).

The discovery by Notides & Gorski (1966) of a uterine protein named the estrogen-induced protein (IP) provided a useful marker of estrogen activity due to (1) the rapidity of its induction both in vivo and in vitro (Barnea & Gorski, 1970; Katzenellenbogen & Gorski, 1972), (2) direct evidence for the rapid accumulation of translatable mRNA for IP (Walker & Kaye, 1981), and (3) the identification of IP as the BB isozyme of creatine kinase (Reiss & Kaye, 1981).

Creatine kinase (CK) regulates the intracellular concentration of adenosine 5′-triphosphate (ATP) by an energy "buffering" action (Jacobus & Lehninger, 1973). The high-energy utilization by uterine cells during the early response to estrogen could result in a reduction of energy stored as creatine phosphate (CP), or the increased glucose uptake and metabolism during this stage (Smith & Gorski, 1968) may provide an excess supply of energy, stored in the form of CP.

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To determine if the increased rate of synthesis of creatine kinase BB in the rat uterus after estrogen treatment is physiologically significant, changes in uterine high-energy phosphates were measured by ^{31}P NMR spectroscopy. Previous determinations of the concentrations of high-energy phosphates in rat uterus, and their changes following estrogen administration, were performed on perchloric acid extracts of frozen uteri (Walaas & Walaas, 1950; Volfin et al., 1961; Gorski & Mueller, 1963; Aaronson et al., 1965; Oliver & Kellie, 1970). The variations in the reported results could be due to the partial degradation of the labile compounds during extraction.

Recent advances in the application of ^{31}P NMR to living cells in excised tissues and in whole animals have enhanced its value in following details of metabolism of phosphate-containing molecules [see Shulman et al. (1979), Gadian & Radda (1981), Meyer et al. (1982), Barany & Glonek (1982), and references cited therein]. We present here results of the use of this noninvasive technique to follow estrogen-mediated changes in high-energy phosphate compounds in the immature rat uterus.

Materials and Methods

Preparation of Uteri. Immature (25–28-day old) Wistar-derived female rats from the Hormone Research Department colony were given intraperitoneal (ip) injections of 0.1 mL of either 5% ethanol or 17β-estradiol (Organon, Oss, The Netherlands) in 5% ethanol at a dose of 1 μg/7 g body weight. The rats were killed by decapitation 0–48 h later. The uteri were rapidly excised, stripped of fat, and placed in an oxygenated (95% O_2–5% CO_2), phosphate-free, Krebs–Ringer buffer solution at pH 7.4 and 4 °C. The buffer solution, prepared with 30% (v/v) ^2H_2O, contained 140 mM NaCl, 5.6 mM KCl, 3.0 mM $CaCl_2$, 1.4 mM$MgSO_4$, 1 mg/mL glucose, and 20 mM *N*-(2-hydroxyethyl)piperiazine-*N'*-2-ethanesulfonic acid (Hepes) buffer. The uteri, together with ice-cold oxygenated buffer, were transferred to a 10-mm glass NMR tube. The tube contained a coaxial capillary, containing 10 μL of reference solution, held with a perforated Teflon plug at the same level as the height of the reference solution, that also fixed the position of the uteri, so that the same proportion of the reference and sample volume was sensed by the coil. During measurements at temperatures above 4 °C, oxygen was continuously bubbled through the solution, through a tube which ended with a perforated bulb that fitted into the bottom of the NMR tube, in place of the external reference.

Measurements of CK Activity. After NMR measurements, uteri were frozen and stored in liquid nitrogen for subsequent determination of CK activity. CK assays were performed on preparations of cytosols (Reiss & Kaye, 1981). CK activity was measured spectrophotometrically in a Gilford 250 recording spectrophotometer at 340 nm, by using a coupled assay in 1-mL volume containing 50 mM imidazole buffer, pH 6.7, 25 mM CP, 2 mM adenosine 5'-diphosphate (ADP), 10 mM magnesium acetate, 20 mM D-glucose, 2 mM NAD, 5 mM ethylenediaminetetraacetic acid (EDTA), 10 μg/mL bovine serum albumin, 50μM diadenosine pentaphosphate, 20 mM *N*-acetylcysteine, 2 mM dithiothreitol (DTT), 2.4 units of a glucose-6-phosphate dehydrogenase, and 1.6 units of hexokinase. Protein was determined by the Bradford (1976) method using bovine serum albumin as the protein standard.

NMR Measurements. ^{31}P NMR spectra were recorded on a Bruker WH-270 pulsed-FT spectrometer at 109.29 MHz. Temperature was maintained to within ±1 °C. The field was locked on internal ^2H_2O. Radio-frequency pulses at a 40° angle were used with an interval of 0.68 s between pulses. These conditions were found to be sufficient to avoid saturation of all signals. From 1200 to 3000 transients were accumulated for each spectrum. Chemical shifts were measured relative to a calibrated external reference solution which contained 10 μL of 1 M H_3PO_4, 1 M HCl, and 110 mM $PrCl_3$. Addition of $PrCl_3$ both shifted the ^{31}P signal downfield, away from the other signals, and enhanced the longitudinal relaxation rate, eliminating its saturation. The integrated intensity of each signal was measured by weighing the paper corresponding to its area from a copy of the spectrum; the variation was 15% of the mean value. The amount of each compound in millimoles per sample was calculated by dividing the area of each signal by the area of the reference phosphate signal (10 μmol). The amount of ADP, calculated by subtracting the area of the β-APT signal from that of the γ-ATP + β-ADP signal, was close to zero.

The fraction of metal ion free ATP with respect to total soluble ATP (ϕ) was calculated by the method of Gupta et al. (1978) which utilizes the difference in chemical shifts among the ATP peaks ($\delta_{\alpha\beta}$ and $\delta_{\gamma\beta}$). The values of the shifts for free ATP and MgATP, at the same ^{31}P NMR frequency and under conditions similar to those used in this study, were taken from Gupta & Moore (1980) by using their temperature correction factor for the shifts of MgATP of −0.4 Hz/°C. Differences in $\delta_{\alpha\beta}$ and $\delta_{\gamma\beta}$ ranging from 10 to 40 Hz were measured to a precision of <10 Hz.

Results

To study temperature effects independent of estrogen-induced changes, ^{31}P NMR spectra of surviving uteri of immature control rats were recorded sequentially at 4, 25, and 37 °C. The uteri were first scanned at 4 °C without continuous oxygenation. Then oxygenation was resumed, the temperature was gradually elevated to 25 °C, and a spectrum was recorded. The temperature was raised to 37 °C, and a final spectrum was obtained. Spectra of uteri at 4 °C without continuous oxygenation were stable for at least 1.5 h, spectra at 25 °C with continuous oxygenation were stable for at least 5 h, and spectra at 37 °C with continuous oxygenation were stable for 1 h only.

The NMR signals (Figure 1) were assigned by comparison with similar NMR data in other tissues (Hoult et al., 1974; Burt et al., 1976). The main signals are due to sugar phosphates (SP), inorganic phosphate (P_i), creatine phosphate (CP), and the nucleoside phosphates, primarily adenosine 5'-triphosphate (ATP). Although the uterus contains small amounts of free guanosine 5'-triphosphate (GTP) and inosine 5'-triphosphate (ITP) (Oliver & Kellie, 1970), the resolution of the spectra was not sufficient to clearly distinguish these signals from the ATP signals. The signals upfield of the α-ATP signal (not labeled in the figure) are tentatively assigned to the nicotinamide adenine dinucleotides (NAD and NADP) and to uridine nucleoside diphosphosugars.

The 4, 25, and 37 °C spectra in Figure 1 are generally similar although slight differences are observed: [P_i] increases up to 2 mmol/kg wet weight at 37 °C while the areas of the CP and ATP signals decrease slightly. These changes in the new steady state maintained at each temperature for at least 1 h are therefore not likely to be due to lack of oxygen. In order to slow down metabolic activity so that in vivo effects of estrogen were "frozen" during measurements, the following studies were performed at 4 °C.

Typical ^{31}P NMR spectra of uteri excised from immature rats, from 0 to 48 h after estrogen injection, are shown in Figure 2. The number of uteri in each sample was adjusted to compensate for the increase in uterine weight at later times. These spectra reveal that SP, CP, and ATP concentrations

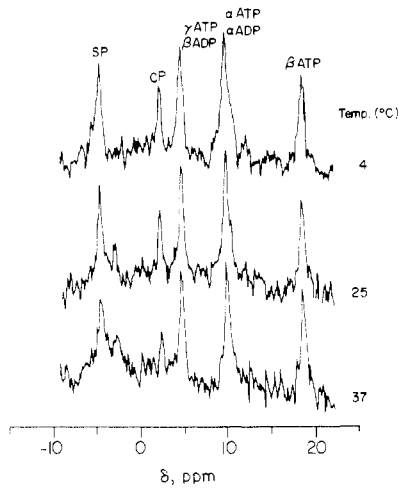

FIGURE 1: ^{31}P NMR spectra of surviving immature rat uteri (12) recorded at 4, 25, and 37 °C. 3000 transients were accumulated for each spectrum (see Materials and Methods) and recorded with a line broadening of 7 Hz. Continuous oxygenation (95% O_2–5% CO_2) was applied at 25 and 37 °C. SP represents phospho monoesters including sugar phosphates.

Table I: Average Concentrations (±SEM)[a] of High-Energy Phosphate Compounds in the Uterus after Estrogen Treatment, Measured by ^{31}P NMR at 4 °C

time after estrogen treatment (h)	no. of expts	[CP]	[ATP]	[SP]	uterine wet wt[b] (mg)
0 (untreated)	14	2.1 ± 0.2	4.6 ± 0.4	5.4 ± 0.7	47
0.5	3	2.0 ± 0.1	5.7 ± 0.6	6.7 ± 0.7	47
2	3	1.3 ± 0.1	3.5 ± 0.2	3.9 ± 0.7	58
3	7	1.0 ± 0.1	2.9 ± 0.4	3.2 ± 0.4	63
6	4	0.9 ± 0.1	3.2 ± 0.6	2.9 ± 0.3	71
12	2	2.2 ± 0.2	4.9 ± 0.1	4.9 ± 0.5	78
24	4	1.7 ± 0.2	5.4 ± 0.5	6.6 ± 0.7	83
48	2	2.1 ± 0.2	5.9 ± 0.3	5.3 ± 0.2	74

[a] Concentrations are given in millimoles per kilogram wet weight. [b] The values represent the average of two to four determinations made prior to the NMR recording.

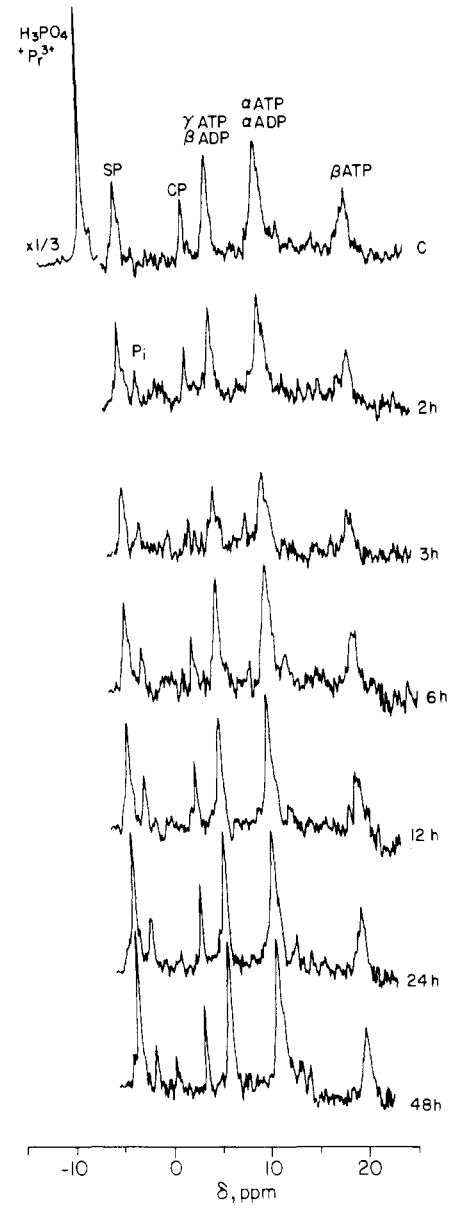

FIGURE 2: ^{31}P NMR spectra of immature rat uteri at 0–48 h after estrogen administration. The number of uteri in each sample was as follows: zero time (C) to 6 h, 12 uteri; 12 h, 10 uteri; 24 and 48 h, 8 uteri. Spectra were recorded (see Materials and Methods) at 4 °C. 0 ppm corresponds to the ^{31}P signal of 1 M H_3PO_4 which is shifted downfield by 88.2 Hz from 85% H_3PO_4. The reference signal is presented at one-third of its height.

decline to minima at 3 h and then rise to exceed control concentrations at 12 h. ADP was undetectable. The concentration of P_i in untreated uteri was relatively low (0.8 ± 0.4 mmol/kg wet weight); the fluctuations in [P_i] following estrogen administration were within experimental error. No pH variations were detected from changes in the chemical shift of the P_i signal.

The changes in CP, ATP, and SP content per uterus, after estrogen treatment (Figure 3), were calculated as percent changes with respect to their corresponding control values and then expressed in nanomoles per uterus by using the average value of the controls. The concentrations of CP, ATP, and SP (Table I) were estimated by dividing the average value in nanomols per uterus by the average uterine weight at each time point (Figure 3). Therefore, the data in concentration units include the effect of water imbibition during the first 6 h after estrogen treatment (Astwood, 1938), enhancing the extent of the decline in concentrations of high-energy phosphates during this period.

CP, ATP, and SP concentrations decrease during the first 3 h following estrogen administration when expressed either per uterus (Figure 3) or per kilogram of tissue (Table I). A full recovery of ATP and SP concentrations to control values, but only partial recovery of CP concentration, occurs by 6 h after estrogen administration; during the first 6 h, the activity of creatine kinase (CK) does not change (Figure 3). From 6 to 24 h after estrogen injection, there is a marked increase in uterine CP, ATP, and SP content as well as in CK activity (Figure 3). The corresponding increase in uterine weight (maximal at 24 h) can be measured as an increase in dry weight (Astwood, 1938) and therefore reflects true uterine growth.

The average fraction (ϕ) of ATP in the form of monoprotonated, metal ion free acid as compared to total soluble ATP (including ATP metal ion complexes, mainly MgATP) calculated from changes in the chemical shifts ($\delta_{\alpha\beta}$ and $\delta_{\gamma\beta}$) was found to be 0.27 ± 0.03 in control uteri. Following estrogen injection, ϕ declined to 0.20 ± 0.03 between 1.5 and 6 h and then returned to the control value (0.26 ± 0.03) at 12 h followed by a second decrease at 24–48 h to 0.18 ± 0.02. These data were used (see Discussion) to calculate [free Mg^{2+}] in the uterus according to Gupta et al. (1978).

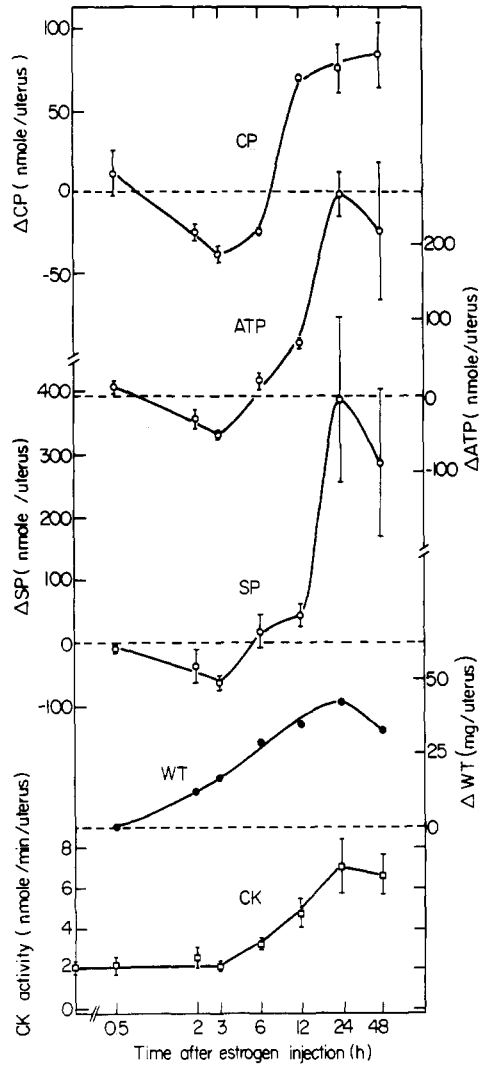

FIGURE 3: Changes in uterine creatine phosphate (CP), adenosine 5′-triphosphate (ATP), sugar phosphates (SP), wet weight (WT), and creatine kinase (CK) activity following ip injection of estrogen (1 μg/7 g body weight). For the phosphate compounds and CK activity, the number of measurements for each time point is the same as in Table I. The dashed lines are the averages of 14 independent control experiments, with the following values in nanomoles per uterus: CP, 100 ± 10; ATP, 215 ± 20; SP, 255 ± 35. Error bars represent the SEM. Changes in weight are the average of two to four independent measurements performed prior to the NMR measurements. The average weight of an untreated uterus was 47 mg. CK was assayed as described under Materials and Methods. The average specific activity of CK (in micromoles per minute per milligram of protein) was 4.6 ± 0.4 for untreated uteri and 7.7 ± 0.5 at 24 h after estrogen injection.

Discussion

It was important to demonstrate that during ^{31}P NMR measurements the concentrations of high-energy phosphates in immature rat uteri remained constant. Assuming that viability of the uteri in a buffered medium containing glucose depends on the oxygen supply, the available oxygen was reduced. Under these conditions, the CP signal decreased to zero, accompanied by an increase in the P_i signal. A reduction in the ATP signal was then seen with a concomitant further increase in the P_i signal. Therefore, uterine preparations were considered viable if a detectable CP signal, accompanied by a P_i signal less than 50 nmol/uterus, was present.

Since average NMR signals from whole immature uteri were reported, we cannot assign the data to either the endometrium (epithelial and stromal cells) or the myometrium (smooth muscle cells). The metabolic and biosynthetic processes induced by estrogen, and specifically the increase in the rate of CK synthesis, occur in all cell types of the uterus (Dupont-Mairesse & Galand, 1974; Mairesse & Galand, 1982) although there are gradients in the extent of the response and an increase in the relative mass of the myometrium with age. Therefore, the NMR data represent average values for processes that occur throughout the uterus.

The high concentrations of the high-energy phosphate compounds determined by NMR spectroscopy in this study are consistent with its ability to detect these compounds without loss due to hydrolysis during preparation of extracts for chemical analysis. The concentration of the soluble nucleoside triphosphates (primarily ATP) measured by NMR is 4.6 mmol/kg wet weight and is larger than values obtained previously; 3.0 (Gorski & Mueller, 1963) and 3.6 (Volfin et al., 1961) mmol/kg wet weight. The soluble nucleoside diphosphates (primarily ADP) were undetected by the ^{31}P NMR method. An upper limit of 0.4 mM for their concentration can be estimated by subtracting the area of the β-ATP peak from that of the γ-ATP peak. Another estimate, of approximately 40 μM, was calculated from the equilibrium constant of the CK reaction with the following assumptions: (1) The CK reaction in the uterus is close to equilibrium, as shown previously in brain and muscle (Veech et al., 1979; Gadian et al., 1981; Meyer et al., 1982; Shoubridge et al., 1982). (2) There is no compartmentation of reactants; therefore, the [soluble CP] and [soluble ATP] determined in this study, and [creatine] determined by Volfin et al. (1961), are the concentrations involved in the reaction. The apparent equilibrium constant for CK defined as

$$K_{CK} = [\Sigma ATP][creatine]/([\Sigma ADP][\Sigma CP])$$

is similar for brain and muscle isozymes (Eppenberger et al., 1967; Veech et al., 1979) and is approximately 52 at pH 7.4 and 4 °C (Noda et al., 1954; Gadian et al., 1981).

Both the above estimates for [free ADP] are lower than those previously reported (Volfin et al., 1961; Caroll & Graham, 1966). Similar discrepancies between [ADP] measured by NMR and by chemical analysis in other tissues (Gadian et al., 1981; Ackerman et al., 1980; Meyer et al., 1982) were attributed partially to the inability of the NMR method to detect ADP bound to actin and other macromolecules. However, in the immature rat uterus, the total concentration of nucleoside triphosphates plus diphosphates from chemical analysis is very close to that determined by NMR for nucleoside triphosphates. This suggests that during the extraction of tissue for chemical analysis, some of the triphosphates were degraded to diphosphates and that the concentration of free nucleoside diphosphates in immature uteri is indeed very low.

The concentration of CP by NMR is 2.1 mmol/kg wet weight. Chemically detected values are 0.3 (Walaas & Walaas, 1950) and 1.2 (Volfin et al., 1961). Again, the NMR value is larger, probably due to partial hydrolysis of the CP during preparation for chemical analysis. The total creatine concentration of 3 mmol/kg wet weight (Volfin et al., 1961) exceeds that of CP, as expected.

Effect of Estrogen on the Concentration of High-Energy Phosphates. Our NMR measurements confirm the decrease in [ATP] during the first 4 h after estrogen administration previously reported by Aaronson et al. (1965) and by Oliver & Kellie (1970) and demonstrate a comparable decrease in [SP] and a relatively larger and more prolonged decrease in [CP]. The increased utilization of ATP (assuming no efflux of high-energy phosphates) may be due to the increased rate of synthesis of RNA and phospholipids which occurs during the first 4 h after estrogen administration (Gorski & Nicolette,

1963; Barker & Warren, 1966; Spooner & Gorski, 1972; Aizawa & Mueller, 1961). CP can be consumed only via its conversion to ATP, catalyzed by CK. The high utilization of ATP early after estrogen treatment shifts the equilibrium of the CK reaction further toward the formation of ATP, preventing its too rapid depletion.

The SP signal is composite and may be due to several phospho monoesters including AMP. Oliver & Kellie (1970) observed a decrease in the uterine [AMP] approximately 4 h after estrogen treatment; their value for [AMP] (0.2 mmol/kg dry wt) is too low to be detected by NMR. However, the enhanced glucose oxidation, 1 h after estrogen administration (Smith & Gorski, 1968), which proceeds via phosphorylated sugar intermediates may lead to a temporary drainage of the sugar phosphate pool.

Although ATP is being used for continuing synthesis, processes seem to be initiated at 12–24 h after estrogen treatment which enhance the rate of ATP accumulation. This increase in ATP concentration can shift the reaction catalyzed by CK toward the formation of more CP, as evidenced by the subsequent increase in CP concentration at 12 and 24 h (Figure 3, Table I).

In the early phase of estrogen stimulation in which both ATP and CP are consumed, no increase in the activity of CK was observed in these experiments, although the rate of synthesis of this enzyme as measured by [^{35}S]methionine incorporation has increased (Reiss & Kaye, 1981). Perhaps, the constitutive level of CK is sufficient to minimize too rapid depletion of ATP, or compartmentation of the newly synthesized CK occurs. On the other hand, the increased activity of CK in the later stage of estrogen action (12–24 h) can help to convert the ATP formed to CP, minimizing inhibitory allosteric effects of excess ATP and simultaneously storing energy in the form of CP.

Effect of Estrogen on the Concentration of Free Mg^{2+}. The estrogen-induced changes in the chemical shifts of the ATP signals ($\delta_{\alpha\beta}$, $\delta_{\gamma\beta}$) were used to calculate changes in the ratio of metal ion free to total ATP concentration (ϕ) in the soluble fraction of the cell by using the method of Gupta & Moore (1980). Determination of ϕ provides a method for calculating [free Mg^{2+}] according to Gupta et al. (1978):

$$[\text{free } Mg^{2+}] = K_D(1/\phi - 1)$$

where K_D is the equilibrium dissociation constant for the reaction $MgATP \rightleftharpoons Mg^{2+} + ATP$. The value of K_D determines the absolute value of [free Mg^{2+}] but, assuming it remains constant, is not necessary for determining relative changes in [free Mg^{2+}]. Since different values for this K_D are available (Martell & Schwarzenbach, 1956; Nanninga, 1961a; Gupta et al., 1978; Gupta & Benovic, 1978), high and low values for [free Mg^{2+}] were calculated. A K_D at 4 °C of 68 μM was calculated by using the data of Gupta et al. (1978) and Gupta & Benovic (1978). A K_D^{app} of 140 μM at pH 7.4 and 4 °C was calculated by using the method and values of Naninga (1961a) (Wu et al., 1981), incorporating the data of Cole (1950) and Walaas (1950) for uterine ionic composition.

The values for K_D lead to an estimate of [free Mg^{2+}] in the rat uterus between 0.2 and 0.4 mM, substantially lower than values determined for [free Mg^{2+}] in skeletal muscle by the same method (Gupta & Moore, 1980; Wu et al., 1981). The low [free Mg^{2+}] is consistent with the possibility that small changes in [free Mg^{2+}] can regulate uterine metabolism. Indeed, 1.5 h after estrogen injection, a 25% increase in [free Mg^{2+}] is observed which persists up to 6 h and then declines toward the control value at 12 h. A comparable increase subsequently occurs at 24 and 48 h. Although these changes in [free Mg^{2+}] are relatively small, in this concentration range they can increase the apparent equilibrium constant for the CK reaction (Lawson & Veech, 1979) and can therefore increase the ratio of total soluble ATP to CP (provided the creatine to ADP concentration ratio and the pH remain unchanged). The ratio [ATP]/[CP], determined independently from the corresponding NMR signal areas (Table I), was 2.2 ± 0.3 for control uteri and increased to 3.1 ± 0.4 between 0.5 and 6 h after estrogen administration; it then declined at 12 h to 2.3 ± 0.2 and increased again at 24 and 48 h to 3.1 ± 0.5. These data support the proposal that estrogen-mediated changes in [free Mg^{2+}] provide a mechanism for shifting the apparent equilibrium of the CK reaction. The increase in [free Mg^{2+}] (50–100 μmol/kg wet weight) early after estrogen treatment may derive from the metabolic depletion of ATP (1.7 mmol/kg wet weight at 3 h) which is present intracellularly primarily as MgATP (approximately 75%).

Therefore, there may be two mechanisms whereby estrogen affects the reaction catalyzed by CK: (1) enhanced synthesis and accumulation of the enzyme and (2) modulation of the [free Mg^{2+}].

Registry No. ATP, 56-65-5; 17β-estradiol, 50-28-2; creatine phosphate, 67-07-2.

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Internal and External Alkali Ion Complexes of Enniatin B: An Empirical Force Field Analysis†

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ABSTRACT: The empirical force field method is used to calculate conformations and energies of the natural ion carrier enniatin B (EnB) and its alkali ion complexes. Solvent effects are circumvented by focusing the study on conformational characteristics and trends in ionophoric behavior, which do not require an evaluation of solvent interactions. A few calculated, low-energy conformations of the EnB ring are presented. The C_3-symmetric conformation of EnB is analyzed in detail. Its rotational isomeric states of the isopropyl side chains are found to interact strongly with its carbonyl and *N*-methyl groups, thus restricting significantly the flexibility of EnB's skeletal ring. Two kinds of 1:1 EnB–ion complexes are obtained: internal and external. In internal complexes, the ion is located at or near the center of an octahedral cavity formed by the six carbonyls of EnB and binds to all these carbonyls. The large strain energy imposed on the ligand by bending the carbonyls inward destabilizes the internal binding. In external complexes, the ion binds only to the three carbonyls of the hydroxyisovaleryl residues, but its binding energy can be the same or even stronger, due to the better dipole–ion alignment and lower strain energy. Li^+ is a poor binder, in that it binds only internally and is too small to bind simultaneously to all six carbonyls. Na^+ fits the molecular cavity well and is the best internal binder. It can also form stable external complexes and is expected to prefer such complexes in polar solvents, where it is partly solvated. K^+ is too big for the EnB cavity, but it squeezes in asymmetrically by distorting the molecule. Rb^+ can hardly fit internally and Cs^+ not at all. However, these three ions do bind quite well externally. External 1:1 EnB–ion complexes can bind a second EnB ligand to form sandwich 2:1 dimer complexes. The calculated energy of the second ligand binding is stronger than the 1:1 external complex energy for K^+, Rb^+, and Cs^+ but not for Na^+, implying that the 2:1 complex is more favored by the larger ions.

Enniatins are natural ion carriers whose biological functions are determined by their ability to transport cations across lipid bilayer membranes. Enniatins, and particularly enniatin B,[1] have been studied extensively and reviewed comprehensively (Ovchinnikov et al., 1974; Burgermeister & Winkler-Oswatitsch, 1977; Ovchinnikov & Ivanov, 1982). They are cyclic depsipeptides, composed of alternating D-hydroxyisovaleric acid

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[1] Abbreviations: EnB, enniatin B; $(LacAla)_3$, EnB methyl side chain analogue [(–D-lactyl-L-*N*-methylalanyl)$_3$]; Lac_6, EnB hexalactyl analogue [(–D-lactyl-L-lactyl)$_3$].

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