Plant Physiol. (1994) 104: 581–589

Nucleotide Availability in Maize (*Zea mays* L.) Root Tips[1]

Estimation of Free and Protein-Bound Nucleotides Using ^{31}P-Nuclear Magnetic Resonance and a Novel Protein-Ligand-Binding Assay

Mark A. Hooks, Gregory C. Shearer, and Justin K. M. Roberts*

Department of Biochemistry, University of California, Riverside, California 92521

Sequestration of nucleotides in cells through protein binding could influence the availability of nucleotides and free energy for metabolic reactions and, therefore, affect rates of physiological processes. We have estimated the proportion of nucleotides bound to proteins in maize (*Zea mays* L.) root tips. Binding of nucleoside mono- and diphosphates to total root-tip protein was studied in vitro using high-performance liquid chromatography and a new ligand-binding technique. We estimate that approximately 40% of the ADP, 65% of the GDP, 50% of the AMP, and virtually all the GMP in aerobic cells are bound to proteins. In hypoxic cells, free concentrations of these nucleotides increase proportionately much more than total intracellular concentrations. Little or no binding of CDP, UDP, CMP, and UMP was observed in vitro. Binding of nucleoside triphosphate (NTP) to protein was estimated from in vivo ^{31}P-nuclear magnetic resonance relaxation measurements. In aerobic root tips most (approximately 70%) of the NTP is free, whereas under hypoxia NTP appears predominantly bound to protein. Our results indicate that binding of nucleotides to proteins in plant cells will significantly influence levels of free purine nucleotides available to drive and regulate respiration, protein synthesis, ion transport, and other physiological processes.

The interactions of nucleotides with hundreds of different proteins in living cells during individual catalytic and regulatory events have implications for cellular energy metabolism. Binding will reduce the free concentrations of nucleotides in cells and, therefore, influence both nucleotide availability and the free energy relations governing metabolic processes such as respiration (Dry et al., 1987; Raymond et al., 1987) and protein synthesis (Kurland, 1978; Hucul et al., 1985). Knowing the physical state of nucleotides in cells is, therefore, important for understanding the regulation of metabolism.

The existence of significant pools of nucleotides that are protein bound has been inferred through mass-action ratio analysis of near-equilibrium reactions (Veech et al., 1979; Bünger, 1986), differences in NTP/NDP ratios between organelles (Erecínska and Wilson, 1982; Stitt et al., 1982), and NMR invisibility of nucleotides in tissues versus tissue extracts (Costa et al., 1979; Ugurbil et al., 1979; Freeman et al., 1983; Hooks et al., 1989). In this study we have directly examined the extent of NMP and NDP binding by measuring the binding of these nucleotides to maize (*Zea mays* L.) root-tip proteins in vitro.

Although only part of the NDP in maize root tips can be observed by in vivo ^{31}P-NMR, virtually all intracellular NTP is NMR visible (Hooks et al., 1989). The relative contributions of signals from free and protein-bound NTP to in vivo ^{31}P-NMR spectra have not been ascertained (Gadian, 1982; Srivastava and Bernhard, 1986). Distinction between NMR signals from free and protein-bound nucleotides is possible in principle because the rate of relaxation of nucleotide ^{31}P-spins changes on binding to macromolecules, due to restriction of nucleotide mobility. Here we estimate the proportion of NTP bound to proteins from ^{31}P-NMR relaxation of nucleotides in vivo.

MATERIALS AND METHODS

Plant Material and Reagents

Maize seeds (*Zea mays* L., hybrid 4327, Germain Seeds, Los Angeles, CA) were soaked in flowing distilled water for 24 h and then germinated on moist paper towels in the dark for about 36 h. Chemicals were purchased from Sigma, United States Biochemical (Cleveland, OH), and Fisher (Los Angeles, CA). High-temperature silicone oil was from Aldrich (Milwaukee, WI). [^{3}H]ADP and [^{3}H]GDP were from DuPont-New England Nuclear; [^{3}H]CDP, [^{3}H]UDP, and ^{32}Pi were from Amersham; [^{3}H]AMP, [^{3}H]CMP, [^{3}H]GMP, and [^{3}H]-UMP were from Moravek Biochemicals Inc. (Brea, CA), at the highest specific activity available.

Isolation of Total Maize Root-Tip Protein

Root tips (2 mm long) were excised on dry ice and stored at −70°C. Frozen root tips (4 or 10 g) were ground to a fine powder at −20° with a mortar and pestle, combined with polyvinylpolypyrrolidone (10% by weight), and blended in

[1] This work was supported by U.S. Department of Agriculture grant 89-37264-4933.

* Corresponding author; fax 1-909-787-3590.

Abbreviations: AK, adenylate kinase; eIF-2, eukaryotic initiation factor 2; GEF, guanine nucleotide exchange factor; K_d, dissociation constant; K_{eq}, equilibrium constant; NDP, nucleoside diphosphate; NMP, nucleoside monophosphate, NTP, nucleoside triphosphate; PGK, phosphoglycerate kinase; T_1, longitudinal relaxation time; T_2, transverse relaxation time.

0.75 volume of buffer A (100 mM Hepes/KOH [pH 7.0], 25 mM KCl, 1 mM β-mercaptoethanol) containing 1% Triton X-100. Subsequent steps were performed at 4°C. After centrifugation at 27,000g for 15 min, supernatants were run on Sephadex G-25 (fine) columns in buffer A, with bed volumes of 80 mL (17 × 2.5 cm) or 13 mL (7.5 × 1.5 cm) for the 10- and 4-g homogenates, respectively, at 0.1 to 0.3 mL min^{-1}. Protein content was determined using the method of Bradford (1976) using dye reagent and IgG protein standard from Bio-Rad. Magnesium was determined using a Perkin-Elmer model 4000 atomic absorption spectrophotometer. In some experiments, eluates were combined and the protein was concentrated about 2-fold to about 20 mg mL^{-1} in a 50-mL Amicon (Danvers, MA) ultrafiltration apparatus with a PM-10 membrane. Nucleotides in 3 to 6 mL of protein solution and filtrate were extracted with TCA/ether and analyzed by HPLC (Hooks et al., 1989). For studies of the pH dependence of nucleotide binding, aliquots of protein solution were dialyzed for 1 h in column buffer at pHs 7, 7.6, and 8.

NDP- and NMP-Binding Assay

Free nucleotide in nucleotide-protein mixtures was determined in an assay based on the centrifugal filtration method used to analyze metabolite pools in organelles (Werkheiser and Bartely, 1957; Stitt et al., 1980), using gel filtration beads (Fig. 1). Centrifugation through silicone oil quickly separates Sephadex G-25 from the protein-NDP mixture. The G-25 contains only free ligand; less than 1% of the protein in the assay was carried by the G-25 through silicone oil into 25% glycerol. There are three advantages to this method for the study of the binding of labile metabolites. First, it is rapid; free ligand can be sampled within 15 s of ligand addition to the protein solution. This is as fast as the method related by Penefsky (1977), in which protein-ligand is centrifuged through Sephadex G-25. Second, it directly samples the free concentration of ligand, which is preferable for some binding systems (Siiteri, 1984). Third, it works with small sample volumes (<50 μL). This method can also be adapted to other types of binding studies, such as ligand binding to purified proteins or membrane components.

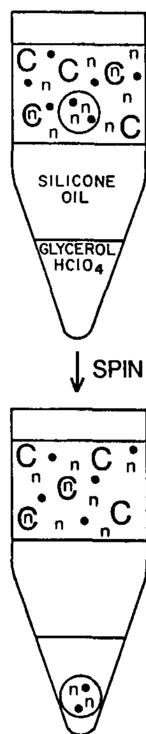

Figure 1. Method for sampling free ligand in protein-ligand mixtures using Sephadex G-25 and silicone oil. Symbols: ●, ^{32}Pi; n, [^{3}H]-NDP; C, protein; O, preswollen Sephadex G-25. Radiolabeled ligand is added to a mixture of root-tip protein and G-25; free ligand and ^{32}Pi diffuses into the G-25 beads; the beads are then centrifuged through the silicone oil and analyzed as described in "Materials and Methods."

Nucleotide stock solutions were prepared by adding different amounts of nonlabeled nucleotide to constant amounts of ^{3}H-labeled nucleotide. The binding assay was performed at 4°C. Fifty microliters of protein or column buffer (control) were added to a 0.5-mL tube containing 10 μL of preswollen Sephadex G-25 and 10 mM KPi labeled with ^{32}P. The tubes were chilled on ice for 10 min. Ten microliters of the appropriate nucleotide stock were added, and the solution was vigorously mixed; the mixture was then transferred to a 1.5-mL tube containing 400 μL of high-temperature silicone oil that covered 60 μL of 5% $HClO_4$/25% glycerol. The tube was spun at 13,000g for 20 s. Five microliters of the protein/nucleotide mixture remaining above the oil were removed to a tube containing 110 μL of the $HClO_4$/glycerol solution. The remaining protein solution and oil were aspirated off, the inside of the tube above the $HClO_4$/glycerol layer was wiped with a cotton swab, 50 μL more of $HClO_4$/glycerol were added, and the tubes were vortexed for at least 1 min. All of the tubes were spun at 13,000g for 2 min, and 80-μL aliquots were added to 3.5 mL of scintillation cocktail and counted. At least three assays were run at each nucleotide concentration.

The amount of nucleotide in each sample was determined from the cpm of an aliquot of the protein or control solution after the G-25 had been centrifuged through the oil and the specific activities of ^{3}H-nucleotide (cpm μmol^{-1}) and ^{32}Pi (cpm μL^{-1}). For the protein sample, the ^{3}H-nucleotide carried into $HClO_4$/glycerol layer represented the free nucleotide, and for the minus-protein control sample, the ^{3}H-nucleotide represented the total amount of nucleotide. At 10 mM total Pi, binding of the ^{32}Pi was negligible; therefore, the ^{32}P dpm in the lower layer served as a measure of the amount of solution removed by the Sephadex G-25 (Hammond et al., 1980). Normalized $^3H/^{32}P$ ratios were calculated by dividing the plus-protein sample $^3H/^{32}P$ cpm by the minus-protein $^3H/^{32}P$ cpm. Values less than 1 indicated binding. The particular concentrations at which significant binding occurred were determined by running Student's *t* tests of the normalized $^3H/^{32}P$ ratios against a control concentration of 1 mM (Hooks, 1992). For samples that showed binding, the normalized ratio was multiplied by the concentration of nucleotide added to the assay to give the $[nucleotide]_{free}$. Subtraction gave the $[nucleotide]_{bound}$.

Scatchard plots were generated and analyzed by the EBDA and Ligand radioligand-binding analysis programs (writ-

ten by P.J. Munson and D. Rodbard; adapted by G.A. McPherson; distributed by Elsevier-Biosoft, Cambridge, UK) (Munson and Rodbard, 1980). The lines represent the best-fit deconvolutions of the curves. Differentiation between linear and nonlinear curves was confirmed by tests performed on the residuals of the nonlinear least-squares fits ($P > 0.05$).

The accuracy of the binding method was checked by measuring the binding of ATP to the high-affinity site of yeast PGK. PGK (Boehringer-Mannheim, Indianapolis, IN) was used at 35 μM, determined spectroscopically (Nageswara Rao et al., 1978), and ATP was varied from 1 to 100 μM (no Mg^{2+} present). Both our new ligand-binding method and measurements using the ultrafiltration method (Sophianopoulos et al., 1978) using the Amicon Centricon-10s gave a K_d of 2 μM and one high-affinity site per PGK molecule, which is in agreement with the findings of Wrobel and Stinson (1978). Varying the incubation time of ATP with PGK and Sephadex G-25 between 15 s and a few minutes did not change the measured binding constants.

Two points can be noted regarding the accuracy of our NDP-binding measurements in total root-tip protein. First, there may have been a loss of binding activity over the time required for protein preparation. Second, any membrane- or protein-associated binding may have been disrupted by homogenization in Triton X-100. Both of these factors would lead to an underestimation of the amount of NDP or NMP that is bound to proteins in vivo.

Measurement of Nucleotide Purity and Breakdown in Protein Solutions

Nucleotide purity was checked by TLC using polyethyleneimine-cellulose plates developed in 1 M sodium formate (pH 7.0) (Randerath and Randerath, 1964). Spots were visualized under short-wave UV light using 0.002% fluorescein (free acid) in methanol (Mangold, 1969). Assays of nucleotide breakdown were performed using 20 μM nucleotide and run as described for the binding studies, except that stop tubes (1.5-mL tubes with 45 μL of 10% aqueous TCA) were prepared instead of the separation tubes. After protein was incubated with nucleotide (about 15 s), a small aliquot was removed to a stop tube and spun at 13,000*g* for 5 min, and the supernatants were transferred to 0.5-mL tubes. The supernatants were washed six times with double volumes of ether to remove the TCA, and 15 μL plus 15 to 20 nmol of the pure nonlabeled NDP and NMP were analyzed by TLC, as above. Spots were scraped from the plate, incubated in 0.5 mL of 1 M NaCl for approximately 8 h, and counted in 3.5 mL of scintillation cocktail. Breakdown was calculated as the ratio of nucleotide cpm from the plus-protein samples to the minus-protein control samples (breakdown in the controls was <1%). Breakdown of added NDP to NMP would bias our NDP-binding results if significant amounts of the corresponding labeled NMP also bound. Development of a rapid binding method was one way that we minimized this potential problem of breakdown. In samples containing free magnesium, less than 25% of ADP and GDP were hydrolyzed, and the AMP or GMP that did form contributed no more than 15% to protein-bound cpm (data not shown). The contribution from labeled NMP could be virtually eliminated by adding either EDTA, which reduced NDP breakdown to less than 5%, or a 10-fold excess of unlabeled NMP, to compete off labeled NMP. Neither addition affected NDP binding (data not shown). NMP-binding results were not influenced by NMP breakdown, because breakdown was negligible during the time course of binding (<5%, data not shown).

In Vivo ^{31}P-NMR Relaxation Measurements

Root tips (2 mm) were perfused at about 10 mL min^{-1} as described previously (Roberts, 1986). All perfusion media contained 0.1 mM $CaSO_4$ and 50 mM Glc. Except where specifically noted, ^{31}P-NMR spectra were obtained with a GN-500 spectrometer operating at 202 MHz, containing a dedicated 10-mm ^{31}P probe. Spectra were obtained under nonsaturating conditions and without field-frequency locking, proton decoupling, or sample spinning. Magnetic field homogeneity was optimized by shimming on the sample ^1H water signal, observed through the ^{31}P coil. T_1 values of NTP were determined by the method of saturation recovery (Markley et al., 1971), in which signal intensity (peak height) is recorded at different times following a train of saturating pulses. T_2 values were determined in a Carr-Purcell-Meiboom-Gill experiment (Meiboom and Gill, 1958) using a train of 180° pulses at 1-ms intervals; scans were taken every 6 s. For each T_1 or T_2 measurement, 10 or 11 spectra were collected, each after different periods of relaxation. Each spectrum was obtained by adding together four or eight scan blocks, the spectrometer cycling through the progression of different settings (of delays or number of pulses) to control for any variation of NMR signals over time. Often, both T_1 and T_2 measurements were made on the same sample over the same time (2–10 h) by this interleaving of data acquisitions. Relaxation measurements for a given sample were reproducible to within 25%. The field dependence of ^{31}P-relaxation in NTP was examined by comparing relaxation data at 202 MHz with that obtained on an NT-300 spectrometer (^{31}P-resonance at 121 MHz) using a 20-mm probe. Relaxation of ATP in solutions of PGK was measured at pH 7 in 0.1 M Hepes/KOH.

Determination of the K_{eq} for the AK Reaction

The K_{eq} for the AK reaction depends strongly on pH (Noda, 1973) and Mg^{2+} (Blair, 1970; De Weer and Lowe, 1973). The K_{eq} for the AK reaction was found to be about 0.85 under conditions of Mg^{2+} and pH found in aerobic and anaerobic maize root tips, determined by ^{31}P-NMR (Gupta et al., 1984; Roberts, 1986; Roberts and Testa, 1988). AK (20 IU mL^{-1}) was added to 3 mM ADP in 0.1 M Hepes/KOH at pH 7 or 7.6; equilibration of adenylates occurred within 10 min. Mg^{2+} was titrated in 0.3 mM increments, and spectra were collected in successive 10-min blocks. The in vitro K_{eq}s of AK ($[ADP]^2/[ATP][AMP]$) were calculated from the peak areas. In vivo ^{31}P-NMR spectra of oxygenated root tips were obtained during 16 h in 1-h blocks. After 16 h the sample was switched to a nitrogen-saturated solution to make the sample anaerobic, and successive 1-min spectra were recorded for approximately 30 min. The differences in chemical shifts (|β-

ATP − γ-ATP| − |α-ATP − γ-ATP|) (Gupta et al., 1984; Roberts, 1986) and the intensities of the γ- and β-ATP signals were used to match in vitro with in vivo spectra with respect to free Mg^{2+}.

RESULTS AND DISCUSSION

NDP and NMP Binding to Maize Root-Tip Proteins in Vitro

We measured two components of NDP and NMP binding to total maize root-tip protein in vitro: the amount of endogenous NDP and NMP that remains bound to proteins during purification and the amount of added NDP and NMP that binds. These data were combined and extrapolated to provide estimates of the amounts of each nucleotide bound to protein in aerobic root tips.

Protein Isolated from Maize Root Tips Contains Tightly Bound Purine NDPs and NMPs

Extraction and HPLC analysis of total maize root-tip protein showed that significant amounts of NDPs and NMPs were not readily separated from protein by gel filtration. To determine whether these nucleotides were protein bound or present because of incomplete chromatographic separation, protein extracts were fractionated by ultrafiltration, and the resulting protein fractions and filtrates were analyzed by HPLC (Table I). We found that essentially all of the ADP and GDP remained in the protein solution following ultrafiltration, indicating tight binding. In contrast low levels of CDP and UDP were observed in both the protein solution and filtrate, indicating that no more than about 15% of cellular CDP and 2% of cellular UDP are bound tightly to protein. These upper limits are likely to be overestimates, because UDP and CDP in these extracts are probably breakdown remnants of UDP-Glc and an unidentified cytidine-containing compound, which are present in large amounts in whole-tissue extracts but not in protein extracts (data not shown).

Greater amounts of NMPs than NDPs were found in protein extracts after gel filtration, and much of this NMP could be separated from the proteins by ultrafiltration (Table I). To determine whether NMP retained in the protein fraction was protein bound, gel filtration chromatography of protein samples was performed for a second time, with subsequent analysis of eluates by HPLC. Coelution of 90% of the AMP and 70% of the GMP with protein indicated that both AMP and GMP were tightly bound to proteins (data not shown). In contrast, only 20% of the CMP and 25% of the UMP coeluted with protein, indicating little or no tight binding of these nucleotides (data not shown).

Table I. *NDP concentrations in maize root-tip protein preparations and ultrafiltrates determined by HPLC*

The concentrations of nucleotides in the protein (approximately 23 mg mL^{-1}) and filtrate solutions are the averages ± ranges of values from two ultrafiltration experiments.

Nucleotide	Protein	Filtrate
	nmol ml⁻¹	
ADP	0.60 ± 0.15	<0.09[a]
CDP	0.34 ± 0.01	0.32 ± 0.01
GDP	0.84 ± 0.14	<0.06[a]
UDP	0.25 ± 0.04	0.26 ± 0.03
AMP	1.03 ± 0.03	0.87 ± 0.21
CMP	4.33 ± 0.95	5.61 ± 0.68
GMP	4.09 ± 0.79	4.08 ± 0.23
UMP	2.66 ± 0.72	7.35 ± 2.95

[a] Levels less than detection limit.

Root-Tip Protein Extracts Can Bind Significant Amounts of Purine NDPs and NMPs

The capacity of maize root-tip proteins to bind exogenous NDPs and NMPs was determined by the ability of proteins to bind [^{3}H]NDP and [^{3}H]NMP and thereby prevent its diffusion into Sephadex beads, using the ligand-binding method described in "Materials and Methods." This binding method samples free nucleotides in nucleotide-protein mixtures on a time scale of about 15 s. Tightly bound nucleotides are unlikely to exchange during the time course of this rapid binding assay; ADP and GDP were not released in significant quantities during about 2 h of ultrafiltration, and AMP and GMP were not released during the approximate 25 min of gel filtration chromatography, described in the previous section.

Binding of pyrimidine NDPs and NMPs to root-tip protein was not detected (data not shown). In contrast, we observed hyperbolic isotherms for purine nucleotides (Fig. 2, insets). Binding was analyzed by Scatchard (1949) plots (Fig. 2) to obtain the number and affinity of binding sites for each nucleotide (Table II, lines B and C). For ADP binding, the simplest model that can account for the data assumes two different populations of binding sites whose parameters are defined by the limiting slopes of the deconvoluted curve at low and high degrees of saturation (Fig. 2A) (Dahlquist, 1978). In contrast, the binding data for GDP, GMP, and AMP are best described by single straight lines (Fig. 2, B–D). The small K_d values for purine NDP binding approximate our earlier prediction based on ^{31}P-NMR analysis of root tips (Hooks et al., 1989). The binding of NMP and NDP to root-tip proteins did not change when measured at pH 7, 7.6, and 8 and was not affected by Mg^{2+} between 0 and 5 mM (data not shown). We were unable to study NTP binding to root-tip protein in vitro because NTP is rapidly hydrolyzed by the protein preparations (data not shown).

Purine NDPs and NMPs Bind to Distinct Sites

The possibility that ADP, GDP, AMP, and GMP bind to the same sites was tested through competition experiments, in which binding of one nucleotide was performed in the presence of greater than a 10-fold excess of the other nucleotide. We were particularly interested in the possibility of competitive inhibition of GDP binding by ADP, because the ADP concentration in vivo is always approximately 3-fold greater than that of GDP (Hooks et al., 1989). The overlap of the binding profiles in the insets to Figure 2, A and B, indicated that ADP and GDP do not compete for binding sites. A competitive inhibition of NMP binding by NDP in

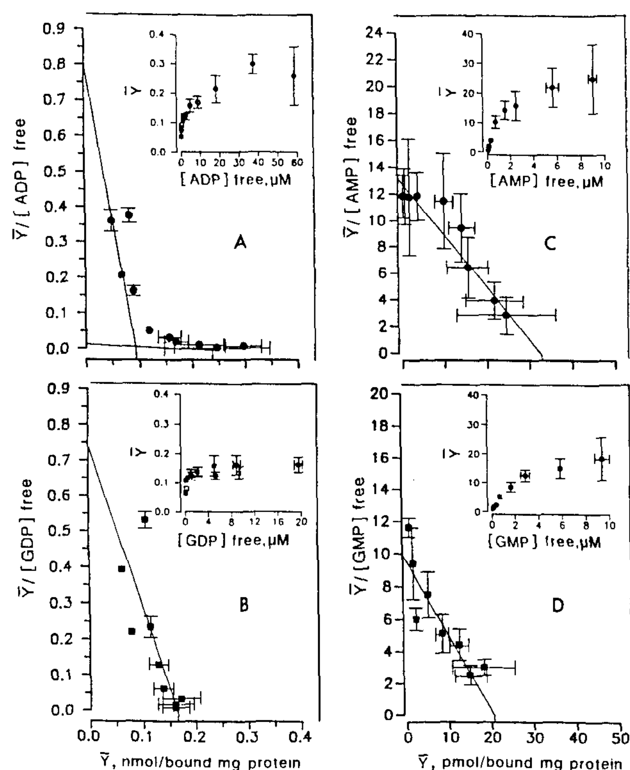

Figure 2. Scatchard plots and isotherms (insets) for the binding of ADP (A) GDP (B), AMP (C), and GMP (D) to maize proteins in vitro. The binding curves and Scatchard plots were generated as described in "Materials and Methods." Symbols: Ȳ, fractional saturation (Scatchard, 1949); ●, ADP and AMP; O, ADP + 100 μM GDP; ■, GDP and GMP; □, GDP + 100 μM ADP. In the classical Scatchard plot, Ȳ is dimensionless (number of binding sites occupied with ligand/total number of binding sites); in our experiments the mol wts and absolute amounts of the proteins responsible for binding were unknown. Hence, the fraction of nucleotide bound was expressed in units of pmol mg^{-1} of protein.

aerobic roots was also considered, because ADP/AMP and GDP/GMP ratios are about 5 in vivo (Table II, line E); NDP had no effect (data not shown).

Estimation of the Proportion of NDP and NMP Bound to Protein in Aerobic and Hypoxic Root Tips

The binding data suggest that a significant proportion of purine NDP and NMP present in aerobic root tips is protein bound (Table II, lines F and G). Pyrimidine nucleotides exhibited little or no binding (data not shown). Approximately 60 μM purine NDP is bound to proteins in vivo (Table II, line F). This estimate suggests that nucleotide binding can account for approximately 40% of the NDP in root tips that is NMR invisible (Hooks et al., 1989). The low apparent K_d values for ADP and GDP binding reported here indicate that the amount of NDP bound to these sites can only increase by approximately 12% (from 60–68 μM) if total cellular NDP increases, as occurs during hypoxia. Hence, when root tips become hypoxic, the concentration of free ADP and GDP will increase much more sharply than total cellular ADP and GDP. Approximately 50% of total cellular AMP in aerobic root tips is bound to proteins in vivo (Table II, line G). Any AMP formed during a stress such as hypoxia would exist primarily as the free species, because the amount of bound AMP can increase by no more than about 10%, based on the number and affinities of AMP-binding sites (Table II, lines B–D). GMP is unique in that its tissue concentration (about 6 μM) is much lower than the concentration of GMP bound to high-affinity sites in protein extracts (about 24 μM). This result presumably reflects breakdown of GDP and GTP to GMP during protein extraction, leading to near saturation of the high-affinity GMP-binding sites.

^{31}P-NMR Relaxation Measurements Indicate that Most of the NTP in Hypoxic Root Tips Is Protein Bound

The extent of NTP binding to protein was estimated from in vivo measurements of the NMR relaxation of ^{31}P in NTP and comparisons with ^{31}P relaxation of nucleotides in defined solutions containing various agents that influence relaxation.

T_2 relaxation is enhanced in molecules that tumble slowly and/or experience chemical exchange between bound and free states having different chemical shifts, such as nucleotides bound to proteins (Nageswara Rao, 1984). If nucleotides in root tips were associated with proteins, in vivo ^{31}P T_2 values of the NTPs would be shorter than T_2 values for NTP in free solution. Furthermore, T_2 values would be shortest in cells having lowest NTP, because the proportion of NTP bound to protein at any instant would be greatest. Our results conform to both these possibilities. First, T_2 values of NTP in root tips are severalfold shorter than the T_2 values of sugar nucleotides in vivo and NTP in simple solution (Fig. 3). Second, T_2 values of NTP in root tips decrease when NTP levels decrease (Fig. 4A), in a manner similar to that modeled in vitro with the ATP-binding protein PGK (Fig. 4B).

Short T_2 can arise from a variety of phenomena other than binding to macromolecules, including chemical shift anisotropy, MgNTP-NTP chemical exchange, viscosity, and paramagnetic ions (Gadian, 1982). However, none of these phenomena can account for the relaxation behavior observed in vivo for the following reasons: (a) Unlike other relaxation mechanisms, relaxation due to chemical shift anisotropy is strongly dependent on the applied magnetic field strength (McCain, 1987). This mechanism was excluded because relaxation of NTP signals was not dependent on magnetic field strength. At 121 MHz, T_2 values of γ- and α-NTP in maize root tips were found to range from 13 to 18 ms in oxygenated root tips, whereas in KCN-treated tissue (in which NTP levels are reduced by about 40%) values ranging from 8 to 15 ms were obtained. These T_2 values are similar in magnitude to those observed at 202 MHz (Fig. 4A). (b) Relaxation due primarily to MgNTP-NTP chemical exchange was excluded by comparing the relaxation of γ- and α-phosphates of NTP. In vitro, subsaturating Mg^{2+} concentrations that reduce the T_2 of γ-ATP to about 10 ms, as in vivo, concomitantly give an α-ATP T_2 of about 89 ms, which is much larger than observed in vivo (Fig. 4A). (c) Transverse relaxation is enhanced (T_2s are shortened) in viscous solutions (Bloembergen et al., 1948). In the presence of more than 75% (w/w) glycerol, T_2 values for γ-NTP and α-NTP are reduced to 12

Table II. *Protein-bound ADP, GDP, AMP, and GMP in root-tip protein extracts (lines A–C) and in vivo (lines D–G)*

	ADP	GDP	AMP	GMP
A. Endogenous bound (μM)[a]	3.5	4.9	6.3	~6
B. Apparent K_d (μM)[b]				
High affinity	0.12 (0.07–0.27)	0.22 (0.18–0.28)	<0.1	<0.1
Low affinity	17.1 (8.9–46.5)	—[c]	2.5 (2.1–3.0)	2.1 (1.8–2.7)
C. In vitro [site][b]				
High affinity	0.10 (0.08–0.11)	0.16 (0.16–0.17)	46 (35–57)	152 (96–216)
Low affinity	0.24 (0.20–0.28)	—	33 (29–38)	21 (18–24)
D. In vivo [site] (μM)[d,e]				
High affinity	13.4 (10.5–16.6)	22.1 (19.0–24.5)	6.3 (4.5–8.3)	21 (12–31)
Low affinity	32.5 (25.2–40.6)	—	4.5 (3.7–5.5)	2.9 (2.3–3.4)
E. In vivo $[\text{nucleotide}]_{total}$[e,f] (μM ± SD)	94 ± 8	34 ± 11	20 ± 6	6 ± 1
F. In vivo $[\text{nucleotide}]_{bound}$ (μM)[f,g]	38.3 (26.1–49.3)	21.7 (19.7–21.7)	9.9 (7.7–11.9)	~6
G. % bound (F/E × 100)	41 (26–57)	64 (44–94)	50 (29–86)	~100

[a] From HPLC data such as in Table I. [b] Determined from Scatchard plots (see Fig. 2). Units are nmol mg^{-1} of protein for NDPs and pmol mg^{-1} of protein for NMPs. The high-affinity values for ADP and GDP reflect both the endogenous and added nucleotides that are bound to high-affinity sites. For AMP and GMP, high and low affinity refer to endogenous and added components, respectively. Values are best estimates (Scatchard plot intercepts) and ranges. [c] Nonsymmetrical ranges. [d] The tissue protein content was determined to be 88.8 ± 5.8 mg g^{-1} of frozen weight (n = 19). In vivo [site] = (nmol sites mg^{-1} of protein) × (88.8 mg mL^{-1}). Values are best estimates and ranges. [e] Assumes entire extravacuolar volume is accessible (1 g of tissue ≃ 0.65 mL of cytoplasm; Steer, 1981). [f] Determined by HPLC analysis of tissue extracts. The tissue used for these experiments was aerobic and gave extract ATP/ADP ratios of 7.5 ± 1.2 (n = 12). Concentrations of CDP, UDP, CMP, and UMP were <12 μM, 68 ± 18, 3.8 ± 0.6, and 55 ± 7, respectively, assuming each occupies 65% of the tissue volume (Steer, 1981) (n = 12 for ADP and AMP; n = 7 for GDP, UDP, and UMP; n = 3 for GMP; n = 2 for CMP). Values are averages and SD. [g] $[NXP]_{free}$ (NXP, nucleotide) was calculated from the tissue $[NXP]_{total}$, the in vivo [site], and the apparent K_d for that population of sites, using the equation:

$$K_{d_{app}} = \frac{[[\text{site}] - ([NXP]_{total} - [NXP]_{free})]\,([NXP]_{free})}{[NXP]_{total} - [NXP]_{free}}.$$

$[NXP]_{bound}$ was determined by subtraction. For ADP, the $[ADP]_{bound}$ concentration was first determined for the high-affinity sites, which were essentially fully saturated, and the $[ADP]_{total}$ reduced by this amount for calculation of the $[ADP]_{bound}$ at the low-affinity sites; both were summed to give the total $[ADP]_{bound}$. Values are best estimates and ranges. Ranges represent limits obtained using extreme values. The best estimate for $[GDP]_{bound}$ also represents the maximum bound. All GMP is bound to high-affinity sites.

and 19 ms, respectively. However, cytoplasmic viscosity in root tips cannot be a major determinant of NTP T_2 values in vivo, because at more than 75% glycerol the T_2 of β-UDP-Glc is about 53 ms, far below the T_2 of the in vivo β-NDP-Glc signal (107 ms). (d) Paramagnetic ions can facilitate rapid T_2 and T_1 relaxation of ^{31}P-nuclei in metal-nucleotide complexes (Cohn and Hughes, 1962; Sternlicht et al., 1965). Trace amounts of paramagnetics in the cytoplasm of root tips could account for the in vivo T_1 values for γ- and α-NTP (300–400 ms), because these are slightly shorter than the approximate 700 ms T_1 values observed in MgATP solutions in vitro. However, paramagnetics cannot be the principal determinants of NTP T_2 values in vivo. Mn^{2+} concentrations (about 0.5 μM) sufficient to reduce γ- and α-ATP T_1s to values observed in vivo (300–400 ms) give T_2 values (18 ms for γ-ATP and 84 ms for α-ATP) that are significantly higher than those in vivo (Fig. 4A). Conversely, at Mn^{2+} concentrations (about 3 μM) sufficient to reduce the T_2 values of γ- (13 ms) and α-ATP (37 ms) to values observed in vivo, T_1s are shortened to values far below T_1s in vivo (138 ms for γ-ATP and 113 ms for α-ATP).

The above results indicate that interaction of NTP with macromolecules is the principal phenomenon responsible for the short T_2 values of NTP in vivo. In aerobic root tips, where NTP levels are highest, T_2 values for γ- and α-NTP are similar to those of ATP in solutions in which the total amount of ATP greatly exceeds the number of ATP-binding sites (Fig. 4); here, T_2s appear to be reduced primarily through chemical exchange effects, due to chemical shift differences between free and protein-bound nucleotides (Nageswara Rao, 1984). In hypoxic root tips, where NTP is much lower, NTP relaxation is similar to that of the ATP-PGK complex, judging from the absolute values of T_2 and the convergence of T_2 values for both γ- and α-phosphates (Fig. 4). We conclude that proteins in root tips can bind approximately 2.5 × 10^{-7} mol of NTP per g of tissue (the NTP content in hypoxically stressed plant cells; Fig. 4) such that in aerobic root tips approximately 30% of total NTP is protein bound.

Equilibration of Adenylates by AK

AK is thought to equilibrate adenylates in plants, because of high in vivo activity and because the mass action ratio $[ADP]^2/[ATP][AMP]$ for total cellular nucleotides approximates K_{eq} for the AK reaction (Bomsel and Pradet, 1968; Raymond et al., 1987). This inference assumes that all adenylates in the cell are free and accessible to AK. Raymond et al. (1987) pointed out that reports of significant pools of immobilized ADP (Roberts et al., 1985; Hooks et al., 1989), sequestered from AK, may be reconciled with near equilib-

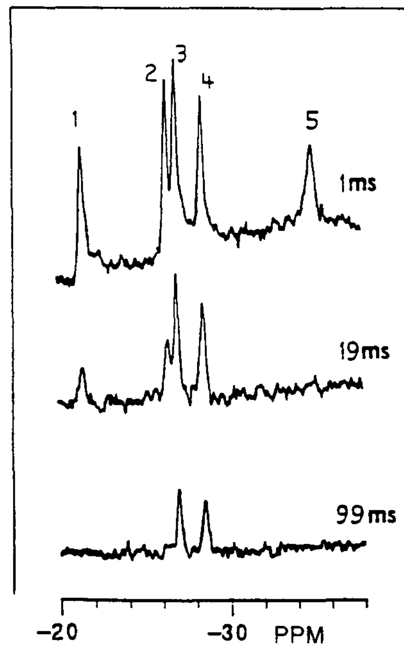

Figure 3. Rapid transverse relaxation of NTP ^{31}P-spins in maize root tips. ^{31}P-NMR partial spectra of maize root tips, perfused with oxygenated 0.1 mM $CaSO_4$. Spectra are from the same sample, acquired over the same period (total time approximately 7 h), each collected after the indicated period of T_2 relaxation. Peak assignments: 1, γ-NTP; 2, α-NTP; 3, α-NDP-Glc (together with a small contribution from pyridine nucleotides); 4, β-NDP-Glc; 5, β-NTP. Chemical shifts are referenced to methylene diphosphonate at 0 ppm. These data gave T_2 values of 16 ms for γ-NTP, 22 ms for α-NTP, and 107 ms for β-NDP-Glc. Respective T_2 values for a solution of 1 mM ATP, 1 mM UDP-Glc, and 1.8 mM $MgCl_2$ at pH 7 were 52, 142, and 202 ms.

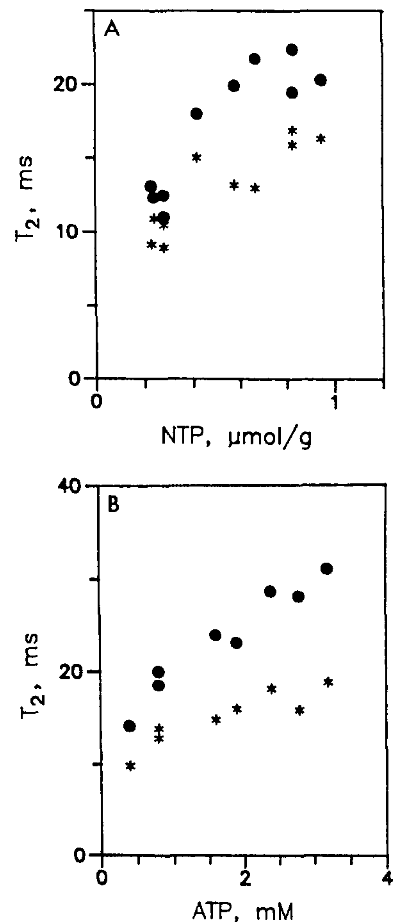

Figure 4. Dependence of NTP T_2 values on nucleotide/protein ratios in vivo and in vitro. A, T_2s of the γ-NTP (*) and α-NTP (●) in maize root tips under different conditions. NTP was lowered either by decreasing the oxygen tension of the perfusion medium or by inclusion of KCN (Roberts et al., 1985). Each pair of symbols represents a different sample, except for two of the low NTP points, which were each derived by adding spectra from three different samples. B, T_2s of ATP (symbols as in A) in the presence of 0.8 mM PGK and 0.3 µM $MnCl_2$. The data come from two titrations, one in which the total Mg^{2+} concentration was held constant at 3 mM and the other in which the total Mg^{2+} concentration was 1.2 mM when [ATP] was less than or equal to 1 mM and 0.2 mM higher than the concentration of ATP when [ATP] was more than 1 mM. According to Wrobel and Stinson (1978), PGK has a high-affinity site for ATP (K_d, 1.9 µM), and can also weakly bind Mg-ATP (K_d, 2.7 mM); Scopes (1978) described two MgATP-binding sites having K_d values of less than 0.15 and 4 mM. Hence, under our titration conditions, at low concentrations of ATP essentially all of the ATP is bound to PGK, whereas added ATP at concentrations greater than 0.8 mM remains mostly free (for example, at 3.9 mM ATP, no more than 1.2 mM ATP would be bound if the second MgATP-binding site has a K_d of 2.7 mM). Chemical exchange between free ATP and magnesium and/or enzyme-bound forms results in an averaged, often broad, NMR signal (Nageswara Rao, 1984).

rium of the AK reaction if AMP was also sequestered. Our results support this hypothesis. If all adenylates in aerobic root tips are assumed to be free, we obtain a mass action ratio of 0.72 ± 0.31 (mean ± SD; calculated for each of 12 samples), similar to the K_{eq} of AK, which is 0.85 under conditions matching those in vivo (see "Materials and Methods"). An identical value is obtained if we recalculate the mass action ratio using the best estimates for free ATP (70%) (assuming the proportion of free NTP and ATP are the same), ADP (59%), and AMP (50%). Thus, our nucleotide-protein binding data are consistent with near equilibrium of the AK reaction.

Implications for Metabolic Regulation

In Figure 5 we schematically describe the physical state of the adenylates in aerobic and anaerobic root tips; the pattern for guanine nucleotides is similar. It is apparent that ATP-protein binding will have a minor effect on the availability of ATP in aerobic cells but would certainly become more significant in limiting rates of ATP-dependent functions, such as ion transport, as cells become hypoxic (cf. Atkinson et al., 1966; Spanswick, 1981; Roberts, 1993). In contrast, ADP and AMP are most limiting in aerobic cells, because protein

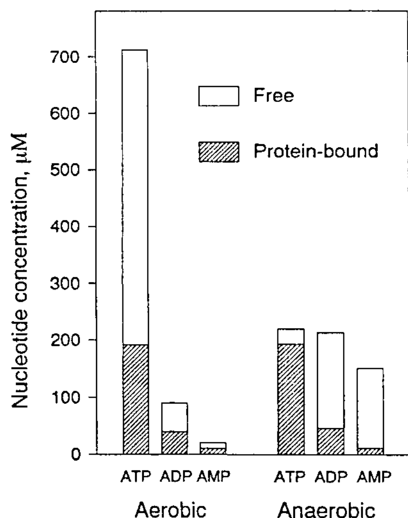

Figure 5. The physical state of adenylates in aerobic and anaerobic maize root tips. Total adenylate concentrations were determined by HPLC analysis of root-tip extracts (Hooks et al., 1989), assuming that the cytoplasm occupies 65% of the tissue volume (Steer, 1981). Aerobic values are from the samples used to give data in Table II; anaerobic values are taken from Hooks et al. (1989). The protein-bound ADP and AMP values are best estimates from in vitro binding data; protein-bound ATP values are estimated from in vivo ^{31}P-relaxation data, assuming that the proportion of protein-bound NTP and ATP are the same (see "Results and Discussion").

binding reduces free ADP and AMP to approximately half of the total. Reduced availability of ADP for oxidative phosphorylation to ATP may limit respiration in aerobic cells (Dry et al., 1987). When cells become hypoxic, the concentrations of free ADP and AMP increase more sharply than total concentrations, because binding sites for these nucleotides are nearly saturated in aerobic cells. Enzymes influenced by AMP will experience the most dynamic change in free levels when tissues switch between aerobic and anaerobic states (Denton and Pogson, 1976). A corollary to these conclusions is that the free energy available to drive metabolism in aerobic cells is significantly higher than that deduced from measurements of total cellular nucleotides, whereas the reverse is the case in hypoxic cells.

The time scales of nucleotide-protein interactions described here should be noted. Binding and dissociation of most intracellular NTP and much purine NDP appear to be much faster than the physiological time scale over which levels of these nucleotides change in response to various stimuli. Hence, these protein-bound nucleotides are not an inert pool, except to the extent that binding lowers the effective nucleotide concentration sensed by nucleotide-dependent processes. In contrast, small amounts of purine NDPs and significant proportions of purine NMPs in aerobic root tips do not exchange with free nucleotides over times of tens of minutes to hours (Tables I and II). Although such nucleotides may be involved in important regulatory functions, they are inert in the sense that they appear not to be able to respond to (e.g. buffer) changes in total intracellular levels of these nucleotides.

The observation that approximately 65% of GDP is protein bound in aerobic root tips is consistent with the presence in eukaryotic cells of a large number of diverse guanine nucleotide-binding proteins, such as membrane-associated G proteins (Blum et al., 1988), tubulin (Dawson and Lloyd, 1987), and initiation factors involved in protein synthesis (Proud, 1986). The metabolic consequences of GDP-protein binding can be expected to be numerous. Our results indicate that GDP-protein binding increases the free GTP/GDP ratio in cells, thus facilitating processes such as signal transduction (Kaziro, 1989), microtubule formation (Carlier and Pantaloni, 1989), and error-free translation (Kurland, 1978). Regarding the initiation of protein synthesis, significant reductions in free GDP due to binding by other proteins would increase the physiological GTP/GDP ratio and enhance GTP binding to the eIF-2·GEF complex (Proud, 1986; Panniers et al., 1988). Panniers et al. (1988) found that a GTP/GDP ratio of 10:1 (extracts of aerobic maize root tips have GTP/GDP ratios no greater than 3 [Hooks et al., 1989]) greatly decreased the binding of GDP to eIF-2. When root tips become hypoxic, levels of GDP increase significantly as GTP/GDP declines to 1 (Hooks et al., 1989). Our GDP-binding data indicate that most of this increased GDP will be free and, therefore, available to bind to eIF-2·GEF and inhibit initiation of translation (Hucul et al., 1985; Panniers et al., 1988). Such inhibition of eIF-2·GEF·GTP formation by GDP may contribute to the dramatic inhibition of initiation reactions in plant roots following the onset of hypoxia (Lin and Key, 1967).

Received June 1, 1993; accepted September 28, 1993.
Copyright Clearance Center: 0032-0889/94/104/0581/09.

LITERATURE CITED

Atkinson MR, Eckermann G, Grant M, Robertson RN (1966) Salt accumulation and adenosine triphosphate in carrot xylem tissue. Proc Natl Acad Sci USA **55:** 560–564

Blair JM (1970) Magnesium, potassium, and the adenylate kinase equilibrium. Eur J Biochem **13:** 384–390

Bloembergen N, Purcell EM, Pound EV (1948) Relaxation effects in nuclear magnetic resonance absorption. Phys Rev **73:** 679–712

Blum W, Hinsch K-D, Schultz G, Weiler EM (1988) Identification of GTP binding proteins in the plasma membrane of higher plants. Biochem Biophys Res Commun **156:** 954–959

Bomsel J-L, Pradet A (1968) Study of 5′-mono-, di-, and triphosphates in plant tissues. Regulation of the level of nucleotides, *in vivo*, by adenylate kinase: theoretical and experimental study. Biochim Biophys Acta **162:** 230–242

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248

Bünger R (1986) Thermodynamic state of cytosolic adenylates in guinea pig myocardium. Energy-linked adaptive changes in free adenylates and purine nucleoside release. *In* E Gerlach, B Becker, eds, Topics and Perspectives in Adenosine Research. Springer-Verlag, New York, pp 223–234

Carlier M-F, Pantaloni D (1989) Tubulin as a G-protein: regulation of tubulin-tubulin interactions by GTP hydrolysis. *In* L Bosch LB Kraal, A Parmeggiani, eds, The Guanine-Nucleotide Binding Proteins. Plenum Press, New York, pp 379–384

Cohn M, Hughes TR (1962) Nuclear magnetic resonance spectra of adenosine di- and triphosphate. J Biol Chem **237:** 176–181

Costa JL, Dobson CM, Kirk KL, Poulsen FM, Valeri CR, Vecchione JJ (1979) Studies of human platelets by ^{19}F and ^{31}P NMR. FEBS Lett **99:** 141–146

Dahlquist FW (1978) The meaning of Scatchard and Hill plots. Methods Enzymol **48:** 270–299

Dawson PJ, Lloyd CW (1987) Comparative biochemistry of plant and animal tubulins. *In* D Davies, ed, The Biochemistry of Plants, Vol 12. Academic Press, New York, pp 3–49

Denton RM, Pogson CI (1976) Outline Studies in Biology: Metabolic Regulation. John Wiley and Sons, New York

De Weer P, Lowe AG (1973) Myokinase equilibrium. J Biol Chem **248:** 2829–2835

Dry IB, Bryce JH, Wiskich JT (1987) Regulation of mitochondrial respiration. *In* D Davies, ed, The Biochemistry of Plants, Vol 11. Academic Press, New York, pp 213–252

Erecińska M, Wilson DF (1982) Regulation of cellular energy metabolism. J Membr Biol **70:** 1–14

Freeman D, Bartlett S, Radda G, Ross B (1983) Energetics of sodium transport in the kidney. Saturation ^{31}P-NMR. Biochim Biophys Acta **762:** 325–336

Gadian DG (1982) Nuclear Magnetic Resonance and Its Application to Living Systems. Oxford University Press, Oxford, UK

Gupta RK, Gupta P, Moore RD (1984) NMR studies of intracellular metal ions in intact cells and tissues. Annu Rev Biophys Bioeng **13:** 221–246

Hammond GL, Nisker JA, Jones LA, Siiteri PK (1980) Estimation of the percentage of free steroid in undiluted serum by centrifugal ultrafiltration-dialysis. J Biol Chem **255:** 5023–5026

Hooks MA (1992) Compartmentation of nucleoside phosphates in maize root tips. PhD thesis. University of California, Riverside

Hooks MA, Clark RA, Nieman RH, Roberts JKM (1989) Compartmentation of nucleotides in corn root tips studied by ^{31}P-NMR and HPLC. Plant Physiol **89:** 963–969

Hucul JA, Henshaw EC, Young DA (1985) Nucleoside diphosphate regulation of overall rates of protein synthesis acting at the level of initiation. J Biol Chem **260:** 15585–15591

Kaziro Y (1989) Structure and function of G proteins from mammalian and yeast cells. *In* L Bosch, B Kraal, A Parmeggiani, eds, The Guanine-Nucleotide Binding Proteins. Plenum Press, New York, pp 291–304

Kurland CG (1978) The role of guanine nucleotides in protein biosynthesis. Biophys J **22:** 373–392

Lin CY, Key JL (1967) Dissociation and reassembly of polyribosomes in relation to protein synthesis in the soybean root. J Mol Biol **26:** 237–247

Mangold HK (1969) Nucleic acids and nucleotides. *In* E Stahl, ed, Thin-Layer Chromatography. Springer-Verlag, New York, pp 786–806

Markley JL, Horsley WJ, Klein MP (1971) Spin-lattice relaxation measurements in slowly relaxing complex spectra. J Chem Phys **55:** 3604–3605.

McCain DC (1987) ^{31}P nuclear spin relaxation. *In* CT Burt, ed, Phosphorus NMR in Biology. CRC Press, Boca Raton, FL, pp 25–61

Meiboom S, Gill D (1958) Modified spin-echo method for measuring nuclear relaxation times. Rev Sci Instrum **29:** 688–691

Munson PJ, Rodbard D (1980) Ligand: a versatile computerized approach for characterization of ligand-binding systems. Anal Biochem **107:** 220–239

Nageswara Rao BDN (1984) Phosphorus-31 NMR of enzyme complexes. *In* D Gorenstein, ed, Phosphorus-31 NMR. Academic Press, New York, pp 57–103

Nageswara Rao BDN, Cohn M, Scopes RK (1978) ^{31}P NMR study of bound reactants and products of yeast 3-phosphoglycerate kinase at equilibrium and the effect of sulfate ion. J Biol Chem **253:** 8056–8060

Noda L (1973) Adenylate kinase. *In* PD Boyer, ed, The Enzymes, Ed 3, Vol 8. Academic Press, New York, pp 279–305

Panniers R, Rowlands AG, Henshaw EC (1988) The effect of Mg^{2+} and guanine nucleotide exchange factor on the binding of guanine nucleotides to eukaryotic initiation factor 2. J Biol Chem **263:** 5519–5525

Penefsky HS (1977) Reversible binding of Pi by beef heart mitochondrial adenosine triphosphatase. J Biol Chem **252:** 2891–2899

Proud CG (1986) Guanine nucleotides, protein phosphorylation and the control of translation. Trends Biochem Sci **11:** 73–77

Randerath E, Randerath A (1964) Ion-exchange chromatography of nucleotides on poly-(ethyleneimine)-cellulose thin layers. J Chromatogr **16:** 126–129

Raymond P, Gidrol X, Salon C, Pradet A (1987) Control involving adenine and pyridine nucleotides. *In* D Davies, ed, The Biochemistry of Plants, Vol 11. Academic Press, New York, pp 129–176

Roberts JKM (1986) Determination of the energy status of plant cells by ^{31}P-nuclear magnetic resonance spectroscopy. *In* HF Linskins, JF Jackson, eds, Modern Methods of Plant Analysis, Vol 2. Springer-Verlag, New York, pp 43–59

Roberts JKM (1993) Interaction between cytoplasmic fermentation reactions and transport of protons between cytoplasm and vacuoles in maize root tips studied *in vivo* by NMR spectroscopy. *In* PW Hochachka, PL Lutz, T Sick, M Rosenthal, G van den Thillart, eds, Surviving Hypoxia: Mechanisms of Control and Adaptation. CRC Press, Boca Raton, FL, pp 187–199

Roberts JKM, Lane AN, Clark RA, Nieman RH (1985) Relationships between the rate of synthesis of ATP and the concentrations of reactants and products of ATP hydrolysis in maize root tips, determined by ^{31}P nuclear magnetic resonance. Arch Biochem Biophys **240:** 712–722

Roberts JKM, Testa MP (1988) ^{31}P-NMR spectroscopy of roots of intact corn seedlings. Plant Physiol **86:** 1127–1130

Scatchard G (1949) The attractions of proteins for small molecules and ions. Ann NY Acad Sci **51:** 660–672

Scopes RK (1978) Binding of substrates and other anions to yeast phosphoglycerate kinase. Eur J Biochem **91:** 119–129

Siiteri PK (1984) Receptor binding studies. Science **223:** 191–193

Sophianopoulos JA, Durham SJ, Sophianopoulos AJ, Ragsdale HL, Cropper WP (1978) Ultrafiltration is theoretically equivalent to equilibrium dialysis but much simpler to carry out. Arch Biochem Biophys **187:** 132–137

Spanswick RM (1981) Electrogenic ion pumps. Annu Rev Plant Physiol **32:** 267–289

Srivastava DK, Bernhard SA (1986) Enzyme-enzyme interactions and the regulation of metabolic reaction pathways. Curr Top Cell Regul **28:** 1–68

Steer MW (1981) Understanding Cell Structure. Cambridge University Press, Cambridge, UK

Sternlicht H, Shulman RG, Anderson EW (1965) Nuclear magnetic resonance study of metal-ion binding to adenosine triphosphate. J Chem Phys **43:** 3123–3132

Stitt M, Lilley RM, Heldt HW (1982) Adenine nucleotide levels in the cytosol, chloroplasts, and mitochondria of wheat leaf protoplasts. Plant Physiol **70:** 971–977

Stitt M, Wirtz W, Heldt HW (1980) Metabolite levels during induction in the chloroplast and extrachloroplast compartments of spinach protoplasts. Biochim Biophys Acta **593:** 85–102

Ugurbil K, Holmsen H, Shulman RG (1979) Adenine nucleotide storage and secretion in platelets as studied by ^{31}P nuclear magnetic resonance. Proc Natl Acad Sci USA **76:** 2227–2231

Veech RL, Randolph-Lawson JW, Cornell NW, Krebs HA (1979) Cytoplasmic phosphorylation potential. J Biol Chem **254:** 6538–6547

Werkheiser WC, Bartley W (1957) The study of steady-state concentrations of internal solutes of mitochondria by a rapid centrifugal transfer to a fixation medium. Biochem J **66:** 79–91

Wrobel JA, Stinson RA (1978) Anion binding to yeast phosphoglycerate kinase. Eur J Biochem **85:** 345–350