

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

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Using ^{31}P NMR spectroscopy, we have measured the rate of ATP synthesis, and the free concentrations of ATP, ADP, cytoplasmic P_i , and H^+ in maize root tips under a wide range of conditions. We show that the ratio $[\text{ATP}]/[\text{ADP}]$ in normoxic root tips is >25 . We found no simple relationship between the concentration of ATP and the rate of ATP synthesis: when the rate of ATP synthesis decreases in response to different treatments, the concentration of ATP can increase, decrease, or remain unchanged. Clear relationships were obtained, however, when the rate of synthesis of ATP was plotted against the logarithm of the ratio ψ , defined as $[\text{ATP}]/[\text{ADP}][P_i][\text{H}^+]$. Two curves were obtained, depending on which of two situations pertained. First, if mitochondrial ATP synthesis was inhibited, e.g., by KCN or hypoxia, $\ln \psi$ decreased monotonically as rates of ATP synthesis decreased. The decrease in $\ln \psi$ may account for decreases in the rates of biosynthetic reactions dependent on ATP, such as protein synthesis, as they approach equilibrium. Second, if consumption of ATP for biosynthetic reactions was inhibited, by treatment with succinate, $\ln \psi$ increased as rates of ATP synthesis decreased. The increase in $\ln \psi$ may account for decreases in the rate of ATP synthesis, as oxidative phosphorylation approaches equilibrium.

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An important aspect of metabolic regulation concerns the correspondence between reactions that generate ATP and reduced nicotinamide adenine nucleotides, and reactions that consume them. It appears that the redox state of cytoplasmic NAD and NADP and the phosphorylation state of adenine nucleotides are linked *in vivo*, because of high activities of enzymes such as glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase (1). It is well established that the concentration of cytoplasmic ATP in cells varies only slightly over a large range of

metabolic activities (rates of ATP synthesis) (2). Thus, utilization of ATP and ATP synthesis are closely tuned.

In plants, attention has focused in particular on the possible role that the energy charge—defined as the ratio $([\text{ATP}] + 0.5[\text{ADP}])/([\text{ATP}] + [\text{ADP}] + [\text{AMP}])$ (3)—plays in regulating ATP-utilizing and ATP-regenerating pathways via allosteric interactions [see Refs. (2, 4, 5) for review]. In studies of energy metabolism in animals, the ratio $[\text{ATP}]/[\text{ADP}][P_i]$ has been considered to be more useful in many circumstances (1), particularly with respect to mechanisms of respiratory control [see Ref. (6) for review]. Under certain

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conditions there is a simple correlation between both these measures of the energy status in cells (7).

We have used saturation transfer ^{31}P NMR spectroscopy to measure rates of synthesis of ATP in maize root tips over a wide range of conditions (8). ^{31}P NMR can also be used to measure the concentrations of ATP, ADP, cytoplasmic P_i , and cytoplasmic pH, and so the Gibbs free energy for hydrolysis of ATP, in maize root tips (9). The measurements of concentrations and rates of synthesis of ATP can be made simultaneously, and on living tissue. In this paper we report such measurements on maize root tips in which either oxidative phosphorylation (ATP synthesis) is inhibited, or ATP consumption is inhibited. We discuss the results with respect to the role that mass action ratios might play in determining rates of ATP synthesis and ATP consumption.

MATERIALS AND METHODS

Maize (*Zea mays* L.) hybrid Funk 4323 (Germain's Seeds, Los Angeles, Calif.) were grown for 2 days in the dark, and 2-mm-long root tips were excised with a razor blade and stored in ice-cold 50 mM glucose plus 0.1 mM CaSO_4 . Normally 3-g samples were prepared for NMR experiments; perchloric acid extracts were made from ca. 10-g samples. Root tip samples were perfused as described previously (10) with oxygen-saturated 0.1 mM CaSO_4 at 50 ml/min, except where indicated under Results. Perchloric acid extracts of maize root tips were made as follows. The perfusion medium was drained (pumped) from the thin-walled glass tubing containing the root tips and, within 1 s, the sample was submerged in liquid nitrogen. The frozen sample, and pieces of glass tubing, were ground to a powder in liquid nitrogen using a mortar and pestle, and the powder (ca. 10 g) was quickly shaken into ca. 60 ml ice-cold 5% perchloric acid, contained in a 100-ml Waring blender vessel, in which it was homogenized. The resulting brei was centrifuged at 30,000*g* for 5 min at 0°C; the supernatant was neutralized on ice with cold, saturated KOH, and the KHCIO_4 precipitate was removed by a second centrifugation step; the resulting supernatant was frozen and lyophilized. Prior to analysis by NMR spectroscopy, the dry powder was dissolved in ca. 0.5 ml H_2O , and the insoluble material was removed by centrifugation at 6000*g* for 15 min; the viscous supernatant was diluted with H_2O to a total volume of 5 ml. After the ^{31}P -NMR spectrum had been obtained at Stanford, the extract was

lyophilized again and the dry powder was sent to Riverside for chromatographic analysis. There the powder was taken up in 1 ml of ice-cold water for each gram of fresh tissue; this solution was centrifuged and any residue was discarded. Nucleotides were freed from contaminants (11), separated, and determined by anion high-performance liquid chromatography (12).

^{31}P NMR spectra were obtained using a modified Bruker HX-360 spectrometer, operating at 145.7 MHz in the Fourier transform mode. The rate of synthesis of ATP in maize root tips was determined by saturation transfer ^{31}P NMR, as described in detail elsewhere (8), with the exception of rates in hypoxic root tips, in which the rate of synthesis was taken to equal the rate of production of ethanol, the principal fermentation end-product of this tissue (13). Spectra not involving saturation transfer were obtained using a 65° exciting pulse; the spectral width was ± 4000 Hz, and 4K data points were collected. A 12-mm probe, built at Stanford, permitting access of tubing to the sample from both above and below the detecting/receiving coil (i.e., permitting perfusion) was used. Magnetic field homogeneity was optimized by shimming on a 12-mm NMR tube filled with saturated KH_2PO_4 , prior to each experiment. Spectra were taken without field-frequency locking. Spectral signal-to-noise ratio was increased by exponential multiplication of the free induction decay prior to Fourier transformation (25 Hz line-broadening). Chemical shifts are reported relative to methylene diphosphonate (MDP, 0.5 M, at pH 8.9 in Tris, contained in a coaxial capillary) at 0 ppm. Cytoplasmic pH was estimated from the chemical shift of the cytoplasmic P_i resonance as previously described (9, 14). Tissue concentrations of NTP, NDP, and cytoplasmic P_i were estimated from the intensities (peak areas) of the appropriate spectral lines, after correction for saturation effects, and calibration of spectrometer sensitivity (8). Relative peak areas were determined from the weights of the respective peaks. Peak assignments are made as described elsewhere (14). Data on the rapid kinetics of changes in phosphate concentrations in root tips subjected to normoxic-hypoxic transitions (Fig. 3) were obtained by averaging spectra for a particular time period in the treatment from five experiments. Each experiment was judged to be equivalent based on the similarity of the kinetics of cytoplasmic pH, and the rate of ethanol production [cf. (13)].

In all of these measurements, quantitative uncertainties arise principally from systematic errors or uncertainties that most probably affect all the measurements equally. Hence, the ranking of rates of ATP synthesis and metabolite concentrations given here are more reliable—as well as being more important—than the absolute values. Concerning the accuracy of absolute measurements, uncertainties in

the measurements of rates of ATP synthesis are smaller than the differences between the various treatments (8); uncertainties in the absolute tissue concentrations of cytoplasmic P_i are ± 0.1 mM (8); NTP, ± 0.05 mM (15); pH, ± 0.1 pH unit (10). Only an upper limit for the concentration of NDP can be estimated in oxygenated root tips (see Results); in anaerobic roots NDP concentrations are accurate to ± 0.04 mM (Fig. 3).

RESULTS

[ATP]/[ADP] Ratios in Normoxic and Hypoxic Maize Root Tips

We have been able to observe NTP in plant tissues such as maize and pea root tips, immature maize cobs, etiolated pea stem, beam leaf, and carrot storage tissue (9) by ^{31}P NMR. However, in plant tissues perfused with oxygen-saturated 0.1 mM CaSO_4 (with or without sugar) NDP cannot be detected. Here we put an upper limit on the concentration of free NDP present in normoxic maize root tips.

Figure 1 shows a ^{31}P NMR spectrum of oxygenated maize root tips. The spectrum is the sum of spectra taken from five separate root tip samples, in order to increase the signal-to-noise ratio. From

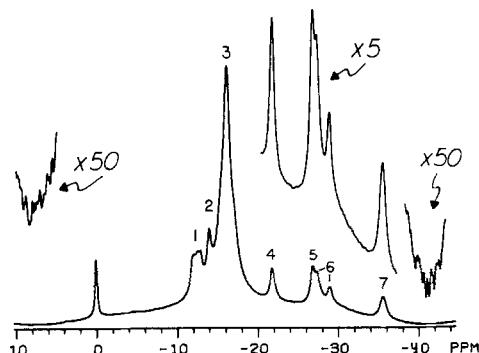


FIG. 1. ^{31}P NMR spectra of normoxic maize root tips perfused with oxygen-saturated 0.1 mM CaSO_4 . The spectrum is the sum of several spectra obtained from five separate samples; it is the result of 205,000 scans, taken over a total time period of 43 h. Peak assignments: (1) monophosphate esters; (2) cytoplasmic P_i ; (3) vacuolar P_i ; (4) γ -NTP; (5) α -NTP; (6) UDP-glucose; (7) β -NTP. The peak at 0 ppm is that of the reference compound, MDP. The vertically expanded plots allow comparison of intensity of the NTP signal to the background noise.

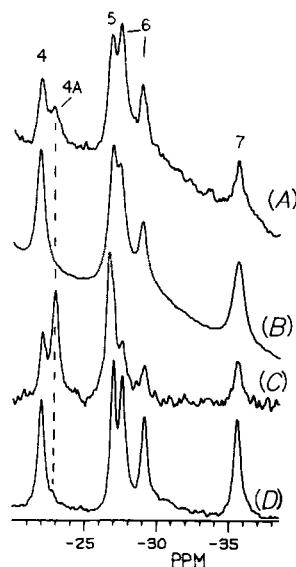


FIG. 2. ^{31}P NMR partial spectra of the nucleotide region of (A) hypoxic maize root tips, perfused with nitrogen-saturated 50 mM glucose plus 0.1 mM CaSO_4 at 4 ml/min (9600 scans over 2 h); (B) normoxic maize root tips (from same spectrum as in Fig. 1); (C) extract of normoxic maize root tips to which commercial ADP had been added (600 scans in 15 min); (D) extract of normoxic maize root tips prior to addition of ADP (15,000 scans in 6.25 h). Peak assignments as in Fig. 1 except 4A, β -NDP (peak 5 will include a contribution from α -NDP, in addition to α -NTP).

the expansions of the baseline signal, it is apparent that the ratio of the NTP signal to the background noise is approximately 50:1. No signal attributable to NDP is apparent. This point is emphasized in Fig. 2, in which the nucleotide regions of ^{31}P NMR spectra of root tips, and root tip extracts, are shown. Figure 2B is the spectrum obtained from oxygenated tissues, which contrasts with that from hypoxic root tips (Fig. 2A) in which an NDP signal is readily seen. These results indicate that whereas the free NTP/NDP ratio is close to unity in hypoxic maize root tips, the value of this ratio is >50 in normoxic tissue.

To test these conclusions further we performed experiments to see if our spectrometer was capable of detecting a signal at least 25-fold smaller than that from NTP in maize root tips. Conceivably, such

small signals might be lost as a result of dynamic range problems (digitization of a weak signal in the presence of a much stronger signal, such as the vacuolar P_i and MDP reference signal), or instrument noise. We found that a solution of 1 mM KH_2PO_4 gave a signal-to-noise ratio of ca. 4 in 100 scans (1.5-s pulse interval). In accord with this sensitivity was our ability to observe 23 μM KH_2PO_4 (in the presence of the usual, strong MDP reference signal) with a signal-to-noise ratio of 1-2 in 40,000 scans. The expected proportionality between peak intensity and concentration therefore appears to hold in practice down to the limit of detection. One complication concerning the sensitivity test just described relates to the fact that the linewidth of the signal from phosphate in simple solution is normally much narrower than the signal from phosphates inside tissues. Thus, one might argue that our sensitivity test exaggerates the lower limit of detection of phosphates *in vivo*, because sharp signals are more easily detected than broad signals. This problem can be considered negligible because, while the intrinsic linewidths observed during these experiments was significantly less than the NTP or NDP signals observed *in vivo* (ca. 15 Hz versus 60-70 Hz for the γ -NTP resonance), this difference is offset by the much shorter longitudinal relaxation times of the nucleotide ^{31}P resonances *in vivo*—ca. 0.4 s for γ -ATP (8) versus 5 s or longer for simple solutions of phosphates. This shorter relaxation time permits more rapid pulsing, and so a signal of a given intensity can be obtained in a shorter period of time. Hence, because (a) we can observe phosphate at a concentration of at least 23 μM , (b) the concentration of NTP in normoxic maize root tips is ca. 0.6 mM (8, 15), and (c) no NDP is detected in such maize root tips (Figs. 1 and 2), we conclude that the NTP/NDP ratio is at least 25.

The results of chromatographic analyses of nucleotides in perchloric acid extracts of normoxic maize root tips are given in Table I. The table shows that adenine nucleotides comprise approximately 60% of the total nucleotide pool. The values

TABLE I
RELATIVE CONCENTRATIONS OF NUCLEOTIDES IN
EXTRACTS OF MAIZE ROOT TIPS

Nucleotide	Experiment			
	I	II	III	IV
ATP	1	1	1	1
ADP	0.84	0.27	0.50	0.59
AMP	0.18	0.05	0.04	0.06
UTP	0.25	0.27	0.17	0.31
UDP	0.37	0.33	0.11	0.12
UMP	0.48	0.06	0.06	0.14
CTP	0.33	0.24	0.15	0.23
CDP	0.16	0.13	0.75	0.44
GTP	0.37	0.14	0.17	0.16
GDP	0.13	0.03	0.04	0.06
Nucleotide ratio				
ATP + ADP/				
NTP + NDP	0.55	0.52	0.68	0.63
ATP/ADP	1.2	3.75	2.0	1.7
NTP/NDP	1.3	2.17	2.04	2.1

Note. Concentrations are expressed relative to ATP.

for the NTP/NDP ratios in Table I are much lower than those evident from ^{31}P NMR data just described. Part of the reason for this can be ascribed to breakdown of NTPs during the transfer of samples from Stanford to Riverside. However, even ^{31}P NMR spectra of fresh extracts indicated NTP/NDP ratios always less than 7, as are the ratios in extracts of aerobic plant tissues reported elsewhere [e.g. (16-18)]. From Table I it is clear that ATP/ADP ratios are similar in magnitude to NTP/NDP ratios, a result indicating that the nucleoside diphosphokinase reaction is near equilibrium. And so, although we are unable to distinguish ^{31}P NMR signals of ATP and ADP from those of other nucleotides, the value of the ATP/ADP ratio *in vivo* appears to be similar to that of the NTP/NDP ratio.

Responses of Concentrations of Phosphates in Root Tips to Changes in Oxygen Tension

Although metabolic changes are largely complete after 30 min of hypoxia (13, 16-

20), there are no published data on the concentrations of phosphates involved in energy metabolism with more than three time points in this period. Such data for maize root tips are shown in Fig. 3. Decreases in NTP and increases in NDP are essentially complete within 2 min of the onset of hypoxia (Fig. 3). This result is consistent with a rapid inhibition of oxidative phosphorylation under these conditions (13), and an average lifetime of ATP in normoxic maize root tips of ~8 s (8). Figure 3 also shows that the concentration of cytoplasmic P_i increases immediately after the onset of hypoxia, the increase being complete within 20 min—

the time course for changes in cytoplasmic P_i being very similar to those for the decrease in cytoplasmic pH (8). These changes in NTP, NDP, and cytoplasmic P_i are reversed equally rapidly when hypoxic root tips are supplied with oxygen (Fig. 3).

Variation of the Concentrations of NTP, NDP, Cytoplasmic P_i and Cytoplasmic H^+ with the Rate of Synthesis of ATP in Maize Root Tips

The variation of the tissue concentrations of NTP, NDP, cytoplasmic P_i , and cytoplasmic H^+ with the rate of synthesis of ATP in maize root tips is shown in Fig. 4. Each letter in Fig. 4 represents a different experimental condition. Letters A to E are from root tips in which ATP synthesis was inhibited to various degrees, relative to glucose-fed, normoxic root tips (letter F). Letters U to Z are from root tips exposed to various concentrations of succinate. No simple relationship between the rate of ATP synthesis and the concentrations of these metabolites is apparent, particularly at the lower rates of ATP synthesis. And so, while certain trends of concentration versus rate are apparent, there is no evidence for an absolute dependence of the concentration of NTP, cytoplasmic P_i , or cytoplasmic H^+ on the rate of ATP synthesis. Note that for most of the treatments we are unable to put more than an upper limit on the concentration of NDP ($20 \mu M$) because we cannot observe NDP in oxygenated maize root tips (described above).

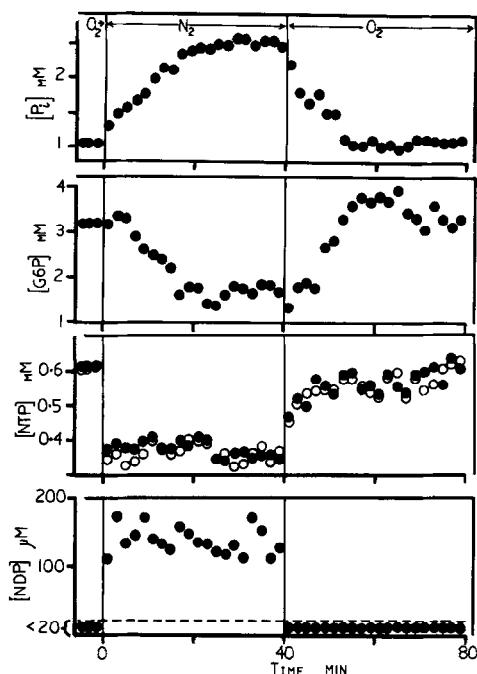


FIG. 3. Changes in concentrations of cytoplasmic P_i , glucose 6-phosphate, NTP, and NDP in maize root tips in response to hypoxic or normoxic conditions, determined as described under Materials and Methods. Initially roots were perfused at $40 \text{ ml}/\text{min}$ with oxygen-saturated 50 mM glucose, 0.1 mM CaSO_4 . At 0 min the roots were perfused with the same solution saturated with nitrogen. At 40 min, the roots were returned to the oxygen-saturated solution. In the NTP panel the symbol \circ refers to concentrations determined from the intensity of the β -ATP resonance, while \bullet refers to concentrations determined from the intensity of the γ -ATP resonance.

Variation of the Gibbs Free Energy for Hydrolysis of ATP with the Rate of Synthesis of ATP in Maize Root Tips

The data in Fig. 4 can be combined and plotted as $298R \cdot \ln ([\text{ATP}] \times 10^{-7} / [\text{ADP}] \cdot [P_i][H^+])$, giving Fig. 5. This quantity represents the amount of useful work that can be done by hydrolysis of ATP in excess of the standard Gibbs free energy, ΔG° . The experimental points in Fig. 5 fall into two groups. Group A to E gives an increase in free energy for hydrolysis of ATP with increasing rate of synthesis

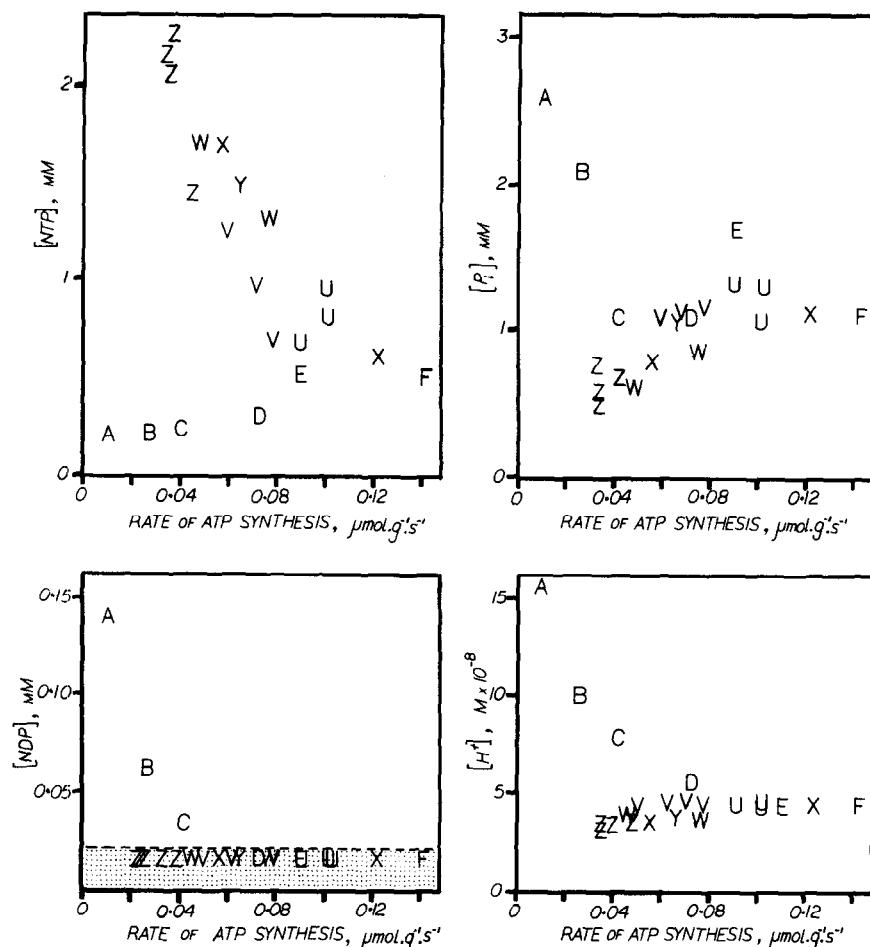


FIG. 4. The rates of synthesis of ATP and the concentrations of nucleotide diphosphate, nucleotide triphosphate, cytoplasmic P_i , and cytoplasmic H^+ in maize root tips under different metabolic conditions. Each letter corresponds to a different experimental condition. All conditions consisted of perfusion with oxygen-saturated solutions except (A). (A) N_2 -saturated 50 mM glucose; (B) 50 mM glucose, 0.5 mM KCN from 0–2 h; (C) 50 mM glucose, 0.5 mM KCN after 4 h; (D) 50 mM glucose, 0.05 mM KCN; (E) no glucose; (F) 50 mM glucose; (U) 10 mM succinate; (V) 20 mM succinate; (W) 30 mM succinate; (X) 40 mM succinate; (Y) 50 mM succinate; (Z) 80 mM succinate. Points A–F are the means from at least four replicates. Points U–Z are from individual experiments, obtained at different times after succinate exposure began. The rate of ATP synthesis given is the unidirectional rate. This rate is very close to the net rate of synthesis of ATP except for points Z, where the net rate is ~60% of the unidirectional rate (see text).

of ATP, while group U to Z gives a decrease. The two groups apparently intersect at a point occupied by F, that is, by the free energy and rate of ATP synthesis found in normal, glucose-supplied tissue. The phosphorylation potentials observed in succinate-fed root tips are similar in magnitude to maximum potentials reached

in rat liver mitochondria supplied with succinate (21).

P/O Ratios in Succinate-Fed Maize Root Tips

We have already reported the rates of oxygen consumption, together with rates

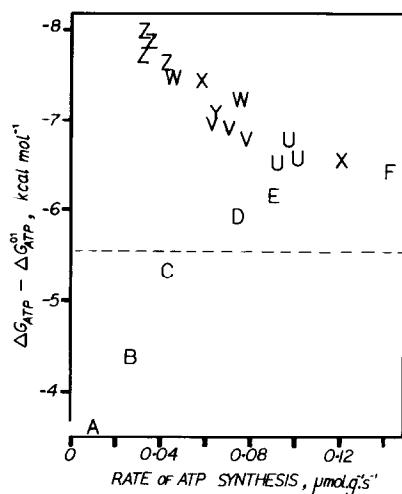


FIG. 5. The rates of synthesis of ATP and values for $RT \ln([ATP] \times 10^{-7}/[ADP][P_i]H^+)$ in maize root tips under different conditions. The data were determined from the data in Fig. 4. Each letter corresponds to a different experimental condition, as given in Fig. 4. The rate of ATP synthesis given is the unidirectional rate. This rate is very close to the net rate of synthesis of ATP except for points Z, where the net rate is $\sim 60\%$ of the unidirectional rate (see text). Points above the dashed line were obtained assuming $[ADP] = 20 \mu M$, the upper limit for the concentration of ADP under these conditions (see text).

of ATP synthesis, in maize root tips for most of the conditions described here, viz., points A to F, and point Y (8). In those experiments, P/O ratios (moles ATP generated per 0 atom consumed) of approximately 3 were found for tissue respiration on sugar, approximately 2 for tissue supplied with 50 mM succinate, and approximately 1 for glucose-fed tissue treated with 0.5 mM KCN (8). There was little evidence that the mitochondrial ATPase was responsible for a sizeable proportion of the ATP consumption, i.e., the mitochondrial ATPase appeared not to be freely reversible *in vivo* under these conditions (8). We noted that in root tips exposed to high concentrations of succinate, rates of respiration declined in a matter of hours (8).

The results in Figs. 4 and 5 involving succinate-fed tissue (points U to Z) were

obtained over a period 6 to 20 h after exposure to succinate began—when respiratory rates were relatively stable. For root tips treated with 20 mM succinate, the rate of respiration was $\sim 1200 \mu l g^{-1} h^{-1}$, and the unidirectional rate of ATP synthesis was $\sim 0.07 \mu M g^{-1} s^{-1}$ (Fig. 4). These results yield a P/O ratio of ~ 2.3 , a value intermediate between the ratio observed in root tips respiring on endogenous sugars, ~ 3 , and the ratio observed in root tips supplied with 50 mM succinate, ~ 2 . This result contrasts with that obtained from root tips treated with 80 mM succinate. Here, rates of respiration were $\sim 450 \mu l g^{-1} h^{-1}$, and unidirectional rates of ATP synthesis were $\sim 0.038 \mu M g^{-1} s^{-1}$, giving a P/O ratio of ~ 3.5 . Such a P/O ratio with succinate as respiratory substrate is thermodynamically impossible (21). A simple explanation of this result is that, in root tips treated with 80 mM succinate, there is an ATPase-associated reaction which is both close to thermodynamic equilibrium, and occurs at a rate significant relative to the rate of oxygen consumption—such as the mitochondrial ATPase (22).

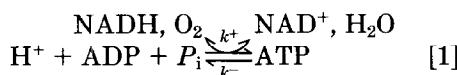
DISCUSSION

The results presented in Fig. 4 indicate that the concentrations of nucleotides and products of nucleotide breakdown do not respond in a simple way to variations in the rate of turnover of nucleotides. For example, NTP levels can either increase, decrease, or remain unchanged as the rate of synthesis of ATP decreases, depending on the treatment (Fig. 4). Hence, inferences about rates of production or consumption of ATP drawn solely from, for example, changes in the concentration of ATP in response to physiological changes must be made cautiously.

The scatter and, in some instances, apparently random disposition of data points in Fig. 4 contrasts with the clear division of data points into two groups in Fig. 5. Figure 5 can be interpreted as follows. In normal, aerobic maize root tips, provided with glucose as an energy and carbon source (point F in Fig. 5), synthesis and

utilization of ATP proceed at their maximal rates (determined by the concentrations of the relevant enzymes). If aerobic roots are fed succinate, biosynthesis (e.g., of cell walls) decreases as glucose 6-phosphate and UDP-glucose eventually disappear (8), and growth is poor compared to growth in glucose-fed root tips (data not shown). Utilization of ATP therefore becomes limited by the availability of glucose, and ATP accumulates, while the products of ATP hydrolysis will be depleted—the more so as carbon becomes scarcer. And eventually the rate of ATP synthesis must decline. This is evident from points U to Z in Fig. 5. The other possibility occurs when the ability of root tips to synthesize ATP becomes limited, while glucose for biosynthesis is available. In such circumstances ATP will be depleted, and the products of ATP hydrolysis will accumulate—the more so as ATP is made in lesser amounts. This is evident from points A to E in Fig. 5.

This interpretation can be continued in more quantitative terms with respect to the role that mass action ratios play in determining the disposition of data points in Fig. 5. Considering succinate-fed root tips first, the question arises: As the free energy for hydrolysis of ATP increases due to a lower rate of ATP consumption, how is the rate of ATP synthesis lowered to match consumption (so ATP levels reach a steady-state)? Oxidative phosphorylation is the principal ATP generating reaction in plant cells (23), and may be described as:



where k^+ and k^- are the apparent rate constants for the forward and reverse reactions, respectively. The net rate of synthesis of ATP via this reaction, J_{ATP} , can be described by simple kinetic equations [e.g., Ref. (24), pp. 96–97], extended to include both the forward and reverse reactions:

$$J_{\text{ATP}} = [E] \times \left(\frac{k^+[\text{ADP}][P_i][\text{H}^+]}{K_{\text{ADP}}K_{P_i}K_{\text{H}^+}} - \frac{k^-[\text{ATP}]}{K_{\text{ATP}}} \right). \quad [2]$$

Using the Haldane relationship, Eq. [2] becomes

$$J_{\text{ATP}} = \frac{[E]k^+}{K_{\text{ADP}}K_{P_i}K_{\text{H}^+}} [\text{ATP}] \times \left(\frac{[\text{ADP}][P_i][\text{H}^+]}{[\text{ATP}]} - \frac{1}{K_{eq}} \right), \quad [3]$$

where $[E]$ is the free concentration of the mitochondrial ATPase; K_{eq} is the equilibrium constant for the mitochondrial ATPase when coupled to electron transport (and implicitly includes the concentrations of NADH and O_2); and K_{ATP} , K_{ADP} , K_{P_i} , and K_{H^+} denote the apparent dissociation constants (either true dissociation constants or Michaelis constants) of the reactants ATP, ADP, P_i , and H^+ , respectively, to the mitochondrial ATPase.

Equation [3] permits the effects of changes in the concentrations of reactants on the rate of ATP synthesis to be considered separately from possible kinetic effects (i.e., the dependence of J_{ATP} on $[E]k^+ / K_{\text{ADP}} \cdot K_{P_i} \cdot K_{\text{H}^+}$), as follows. The relative magnitude of the ratio $[\text{ADP}][P_i][\text{H}^+]/[\text{ATP}]$ to $1/K_{eq}$ will determine how sensitive J_{ATP} is to changes in the concentrations of reactants. The value of K_{eq} can be obtained from Fig. 5, by extrapolation of data points U to Z to the ordinate, so giving the value of $[\text{ATP}]/[\text{ADP}][P_i][\text{H}^+]$ at which net ATP synthesis is zero. Note that the saturation transfer NMR method measures the unidirectional rate of ATP synthesis, and so it is essential to know the extent to which ATP synthesized by the mitochondrial ATPase is also hydrolyzed by this enzyme (as opposed to hydrolysis by other ATPases in the cell). As described under Results [see also Ref. (8)], P/O ratios in maize root tips, determined by comparison of unidirectional rates of ATP synthesis with rates of oxygen consumption, indicate that ATP consumption by the mitochondrial ATPase is negligible under most circumstances. Only after

prolonged exposure to high concentrations of succinate (80 mM) do P/O ratios increase to thermodynamically impossible values, indicating cycling of ATP via the mitochondrial ATPase reaction, because it is close to equilibrium. And so, it appears that the unidirectional rate of ATP synthesis we estimate is equivalent to the net rate of ATP synthesis by the mitochondrial ATPase, except for root tips exposed to 80 mM succinate, where the net rate is ca. 60% (2/3.5) of the unidirectional rate. Using these arguments, points U to Z in Fig. 5 can be replotted in terms of net rate of ATP synthesis to give Fig. 6 (larger symbols). Extrapolation of the data to a rate of net ATP synthesis of zero gives a value for K_{eq} of ca. $1.6 \times 10^6 \text{ mol}^{-2}$.

In testing the possibility that respiratory control in succinate-fed root tips is due to changes in the concentrations of ATP and products of ATP hydrolysis, and not kinetic mechanisms, we assume that the value of the term $[E]k^+/K_{ADP} \cdot K_{Pi} \cdot K_{H^+}$

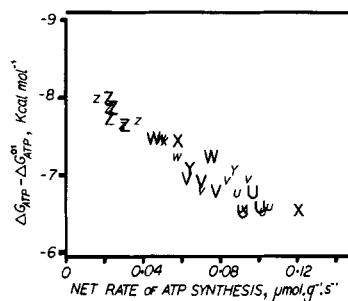


FIG. 6. The net rates of synthesis of ATP and values for $RT \ln([ATP] \times 10^{-7}/[ADP]P_i[H^+])$ in succinate-fed maize root tips. Each letter corresponds to a different experimental condition, as given in Fig. 4. The tissue concentration of ADP is assumed to be 20 μM (see text). The net rates of ATP synthesis represented by the large data points were estimated by saturation transfer ^{31}P NMR, and from rates of respiration, assuming that the P/O ratio was equal to 2. The net rates of ATP synthesis represented by the smaller, italicized letters were obtained by inserting the appropriate metabolite concentrations from Fig. 4 into Eq. [3], and solving for J_{ATP} , using a value for K_{eq} of $1.6 \times 10^6 \text{ mol}^{-2}$, and a value for $[E]k^+/K_{ADP} \cdot K_{Pi} \cdot K_{H^+}$ of $1.08 \times 10^7 \text{ mol}^{-2} 10^{-6} \text{ g}^{-1} \text{ s}^{-1}$ (see text).

$K_{Pi} \cdot K_{H^+}$ in Eq. [3] is constant. Based on this assumption, the value of this constant can be estimated using Eq. [2], from the data in Fig. 4. Thus, with 80 mM succinate, $J_{ATP} \sim 0.0217 \mu\text{M g}^{-1} \text{ s}^{-1}$, $[ATP]/[ADP]P_i[H^+] \sim 6.5 \times 10^5 \text{ mol}^{-2}$, and $[ATP] \sim 2.15 \text{ mM}$ ($[ATP] \sim [NTP]$ since, from Table I, $[ATP] \sim 0.6 [NTP]$), and root tips are $\sim 70\%$ cytoplasm (see Ref. (25)). This gives a value for $[E]k^+/K_{ADP} \cdot K_{Pi} \cdot K_{H^+}$ of ca. $1.08 \times 10^7 \text{ mol}^{-2} \times 10^{-6} \text{ g}^{-1} \text{ s}^{-1}$. Given these values for K_{eq} and $[E]k^+/K_{ADP} \cdot K_{Pi} \cdot K_{H^+}$, the concentrations of ATP, ADP, P_i , and H^+ in Fig. 4 can be plotted as a function of J_{ATP} , using Eq. [3], as shown in Fig. 6 (smaller symbols). It is apparent that the calculated rates of ATP synthesis lie on the same line as the rates of synthesis estimated by ^{31}P NMR. This result shows the plausibility of a mechanism of respiratory control in which the concentrations of ATP and products of ATP hydrolysis, and not kinetic parameters, are critical. Respiratory control by kinetic mechanisms cannot be ruled out until the values of $[E]k^+/K_{ADP} \cdot K_{Pi} \cdot K_{H^+}$ in root tips, under these conditions, are determined.

We now consider data from root tips in which the rate of ATP synthesis is decreased by treatment with inhibitors of oxidative phosphorylation, or lack of the substrates oxygen or glucose. In such root tips the demand for ATP exceeds supply, and so the question arises: How is the rate of ATP consumption lowered to match synthesis (so ATP levels reach a steady state)? It is not possible to address this question in a manner analogous to that employed above for succinate-fed root tips, in which the net flux through an ATP-dependent reaction is analyzed with respect to either kinetic components or concentration and equilibrium terms. For the dependence of rates of reactions such as protein synthesis, polysaccharide synthesis, and ion transport, on the rate of ATP synthesis is not known—beyond the knowledge that they are inhibited at low oxygen tensions. Furthermore, there is a lack of data on concentrations of reactants other than ATP and products of ATP hydrolysis, and values of equilibrium con-

stants (e.g., for many reactions in cell wall synthesis).

At present only ATP consumption due to protein synthesis can be analyzed in any detail. Specifically, it appears that the low free energy for hydrolysis of ATP in anaerobic root tips (Fig. 5) causes the aminoacyl tRNA synthetase reactions for the less abundant amino acids to become close to equilibrium. This will result in lower rates of protein synthesis, and so lower rates of ATP consumption. Thus, the ratio

$$\frac{[\text{AMP}][\text{PP}_i][\text{H}^+][\text{aminoacyl tRNA}]}{[\text{ATP}][\text{amino acid}][\text{tRNA}]} = 0.3 \quad [4]$$

at equilibrium (26). We can write

$$\psi = \frac{[\text{ATP}] \times 10^{-7}}{[\text{ADP}][\text{P}_i][\text{H}^+]} \quad [5]$$

and

$$\frac{[\text{ATP}] \times 10^{-7}}{[\text{AMP}][\text{PP}_i][\text{H}^+]} = \frac{\psi^2}{[\text{ATP}][\text{AMP}] \times [\text{PP}_i] \times 10^{-7}} \sim \frac{\psi^2}{20}, \quad [6]$$

$$\frac{[\text{ADP}]^2}{[\text{P}_i]^2[\text{H}^+]}$$

because the adenylate kinase reaction is close to equilibrium *in vivo* ($K_{eq} \sim 1$) [Table I; Ref. (27)], and the concentration of PP_i *in vivo* is $\sim 20 \mu\text{M}$ (28, 29). From these expressions, and using the data in Fig. 4, we can calculate that for net protein synthesis to occur in root tips, the value of the ratio $[\text{tRNA}][\text{amino acid}]/[\text{aminoacyl tRNA}]$ must be greater than $1.7 \times 10^{-8} \text{ M}$ under normoxia, and greater than $3.4 \times 10^{-4} \text{ M}$ under hypoxia. So, in normoxic root tips, 50% of a particular species of tRNA can be conjugated to amino acids ($[\text{tRNA}]/[\text{aminoacyl tRNA}] = 1$) at sub-micromolar concentrations of the amino acid. However, in anaerobic root tips, for a significant proportion of tRNA to be esterified to amino acids the amino acid must be present at concentrations of $\sim 0.3 \text{ mM}$ —a concentration not observed for some amino acids *in vivo* (30). We note

that methionine is invariably the least abundant amino acid in plant tissues (31–34), and so production of methionyl tRNA will be inhibited first when ATP production is inhibited. These observations may account for the inhibition of protein synthesis, and the dissociation of polysomes due to inhibition of ribosome attachment to mRNA and subsequent initiation of new peptide chains (35). The rapidity with which concentrations of ATP, ADP, P_i , and H^+ change in response to oxygen tension (Fig. 3) parallels changes in the rate of protein synthesis (35). We conclude that thermodynamic constraints can serve to slow down certain ATP-consuming reactions in root tips when oxidative phosphorylation is inhibited. This mechanism will allow, at least in part, ATP consumption to match ATP production. One can speculate that the magnitudes of the equilibrium constants for ATP-consuming reactions (36) provides the basis for the hierarchy among metabolic pathways when roots become hypoxic. The reactions in less immediately crucial pathways (e.g., charging of tRNAs for protein synthesis, $K_{eq} \sim 0.3$) are slowed down before reactions in more important pathways (e.g., glutamine synthetase, $K_{eq} \sim 440$; and hexokinase, $K_{eq} \sim 5000$) in hypoxic roots. Such a means of metabolic control is intrinsic to metabolism, in contrast to an evolved adaptive mechanism based on kinetic control, and in contrast to mechanisms of metabolic control in normal, oxygenated tissues.

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