Dynamic changes in the organophosphate profile upon treatment of the crystalline lens with dexamethasone

Jack V. Greiner, Stephen J. Kopp, and Thomas Glonek

Dynamic changes in organophosphate metabolites during incubation of the intact crystalline lens in a medium containing \(2 \times 10^{-3}\)M dexamethasone were studied using phosphorus-31 nuclear magnetic resonance intact-tissue spectroscopy. ATP, ADP, inorganic orthophosphate, \(\alpha\)-glycerophosphate, nicotinamide adenine dinucleotides, uridine diphosphorylgucose and diphasphorylgalactose, glycerol 3-phosphorylthanolamine and \(-\)phosphorylcholine, and an unidentified phosphorus-containing molecule at 6 ppm were quantitated. Time-dependent changes in lens metabolism involving these compounds were examined during a 24-hr incubation period with dexamethasone. The first event in the decline of the tissue after steroid incubation was the consumption of ATP, which was linear with time until the ATP was reduced to 10% of its initial value. The sugar phosphates, inorganic orthophosphate, and the nucleoside diphasphorylsugars increased linearly with time, whereas the dinucleotides decreased. The ADP concentration was unaffected by the steroid treatment, as was that of the other organophosphates in the intact tissue profile. The steroid-induced alterations in lens phosphate metabolite levels follow a distinctive pattern that differs substantially from that observed in response to experimental hypoglycistic or hyperglycistic conditions. These findings suggest that pharmacologic steroid doses may directly alter lens metabolism through a complex mechanism involving, at least in part, a steroid-mediated antagonism of lens glucose uptake and/or utilization. (INVEST OPHTHALMOL VIS SCI 23:14-22, 1982.)

Key words: steroids, organophosphates, crystalline lens, phosphorus-31 nuclear magnetic resonance spectroscopy, rabbit, dexamethasone

The metabolic basis for cataract formation associated with high doses of corticosteroids is unresolved. Recently, phosphorus-31 nuclear magnetic resonance spectroscopy (P-31 NMR) has been applied to the study of dynamic metabolic processes that occur in the intact mammalian crystalline lens in conjunction with various purported cataractogenic stimuli.1, 2 P-31 NMR nondestructive tissue analysis1–5 of the intact lens has yielded quantitative data regarding lens metabolite levels and has made possible the measurement of
intralenticular pH. Furthermore, unique dynamic information is obtained by this method concerning metabolic changes that occur during defined time increments in response to cataractogenic insults. Subsequent tissue perchloric acid (PCA) extracts prepared from these same lenses have been analyzed by this technique and have verified metabolite identifications and quantitated minor lens metabolite concentrations.

As a consequence of these initial studies, which demonstrated the feasibility of this method and its ability to detect changes in lens metabolism during hypoglycistic and hyperglycistic incubations, the present studies were conducted to evaluate time-dependent changes in lens metabolism during steroid-induced cataract formation.

Methods

Surgery. Albino rabbits, weighing 2 to 3 kg, were killed with injections of sodium pentobarbital, and the eyes were enucleated. Sagittal and frontal incisions were made at the posterior pole. With curved blunt scissors, these incisions were extended to the ora serrata. The lens was exposed by gently separating the vitreous humor with a glass spatula. The zonules were then cut with curved scissors, and lenses were removed with a glass lens loupe.

In vitro incubation. Freshly excised lenses were isolated, weighed, and placed together in a tared 12 mm NMR tube containing a volume of Earle’s buffer: 116.4 mM NaCl, 5.6 mM dextrose, 5.4 mM KCl, 1.8 mM CaCl₂, 1.4 mM MgSO₄, 0.9 mM NaH₂PO₄ • H₂O, 26.4 mM NaHCO₃, with an osmolarity of 295 mOsm, 37° C, pH 7.4. The experimental medium was modified Earle’s buffer with 2 x 10⁻³M dexamethasone added, and Earle’s buffer served as control medium. Earle’s buffer has been shown to maintain lens clarity and lens organophosphate metabolites for at least 24 hr of in vitro incubation at 37° C. Lenses were equilibrated for 2 hr in standard Earle’s buffer and then placed in the NMR sample tube and individual phosphorus NMR profiles were acquired at consecutive 1 hr intervals. Although useable data were obtained in as little as 5 min, continuous 1 hr signal-averaging periods were used to gather the phosphorus NMR data presented in this study.

Lens PCA extracts. After the incubation period, lenses were weighed, frozen in liquid nitrogen, and prepared for PCA extraction. Lens PCA extract preparation procedures and P-31 NMR calibrations and analyses were performed according to previously described lens tissue extract protocol.

P-31 NMR spectroscopy. A Nicolet NT-200 system equipped with deuterium stabilization, variable temperature, and Fourier-transform capabilities and operating at 80.997663 MHz for P-31 was used in this study. A wide-bore (99 mm) superconducting magnet (4.7 Tesla) was interfaced to the Nicolet system. Intact lenses were analyzed in 12 mm sample tubes under nonspinning proton-
Table I. Lens P-31 NMR data

<table>
<thead>
<tr>
<th>Phosphatic compound</th>
<th>Chemical shift (ppm)</th>
<th>Amount (as % of the total P detected)</th>
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<tbody>
<tr>
<td></td>
<td>Intact lens</td>
<td>PCA extract</td>
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| Unknown              | D                   | 18.05, 18.00, 17.81                  | 0.5                                   | 0.8
| Unknown              | D                   | 10.69                                | 0.03                                  | 0.5
| Unknown              | D                   | 5.90                                 | 1.2                                   | 1.1
| Trioses             | D                   | 3.67                                 | 8.7                                   | 7.3
| Hexoses             | D                   | 3.78                                 | 7.2                                   | 5.9
| P-ethanolamine      | D                   | 3.50                                 | 0.2                                   | 0.2
| Pi                  | 1                   | 3.33                                 | K                                     | 1.5
| Glu-l-P             | 1.63                | 2.75, 11.1                           | 11.1                                  | 7.3
| GPE                 | D                   | 0.40                                 | 1.3                                   | 0.9
| GPC                 | D                   | 0.13                                 | 2.1                                   | 2.0
| P-Cr                | D                   | -3.10                                | 0.05                                  | 0.1
| Unknown              | D                   | -5.36                                | 0.05                                  | 0.1
| ATP                 | α, -10.65; β, -19.24; γ, -5.62 | 46.8, 52.2                           | 51.3                                  | 1.9
| ADP                 | α, -10.65; β, -6.66 | 3.8                                 | 5.4                                   | 4.7
| DN                  | -11.34              | 13.6                                 | 12.5                                  | 16.2
| NS                  | -12.89              | -12.63                               | 2.2                                   | 1.7

*ANADP-2'-P indicates the 2' phosphate of nicotinamide adenine dinucleotide phosphate; Pi, inorganic orthophosphate; Glu-l-P, glucose-1-phosphate; GPE, glycerol 3-phosphorylethanolamine; GPC, glycerol 3-phosphorylcholine; P-Cr, phosphocreatine; DN, dinucleotides; NS, nucleoside diphosphosugars.

Field-independent nuclear magnetic resonance units of parts per million relative to the shift position of the 85% inorganic orthophosphoric acid reference phosphate at 25°.

Compound, as of this writing, is not identified with any known phosphorus-containing biomolecule.

Minor component not detectable in this intact tissue.

These separate resonance signals are detected in control (see F below).

Five signals are present: 18.650 ppm, 0.04%; 18.002, 0.4%; 17.959, 0.1%; 17.899, 0.1%; 17.813, 0.2%.

Two signals are present: 10.690 ppm, 0.30%; 9.969 ppm, 0.10%.

J(P-H[phosphate-hydrogen]) = 10.69 Hz.

Complex resonance band, the principal resonance signals of which come from the α-GP triplet; J(POCH) = 6.68 Hz.

Hexose and pentose phosphates, principally the resonance triplet of inosine monophosphate; J(POCH) = 3.81 Hz.

Cannot be determined in the intact tissue as a separate resonance band but is combined with the Pi signal.

J([POCH]) for the triplet = 3.63 Hz.

J([POP], αβ) = 19.44 Hz; J([POP], βγ) = 19.44 Hz.

J([POP], αβ) = 22.44 Hz.

Principal resonance signals of this band arise from the P,P'-diesterified pyrophosphate residues of NAD and NADH. The signals comprise two NMR ab multiplets.

Complex resonance band composed of the three sets of overlapping, [31P,31P, ab NMR multiplets from UDP-glucose, -galactose, and -mannose.

Decoupled conditions. PCA extract samples were analyzed in NMR microcell assemblies with and without proton and phosphorus decoupling and were spun to enhance signal resolution for quantification of the minor lens metabolites. All samples were analyzed at 37° C.

Because the spectrometer analysis conditions used in this study were somewhat different for PCA extract and intact lens samples, the instrumental parameters are listed, as follows, for the tissue extract samples and, when different, the parameters for the intact lens analysis are presented in parentheses: pulse sequence, 1 pulse; pulse width, 9 μsec; 45° spin-flip angle; acquisition delay, 200 μsec; acquisition time, 1.64 sec (0.819 sec); number of data points per free induction decoupling.
cay, 16,384 (8192); sweep width, ±2500 Hz; number of scans, 30,000 (4096). Data reductions, including peak area integrations and chemical shift measurements, were obtained with the spectrometer’s computer. A filter time-constant introducing 0.6 (10) Hz line broadening was also applied to enhance signal-to-noise ratios.

The standard of 85% inorganic orthophosphoric acid was used for determining and reporting the chemical shift data. The primary internal chemical shift standard used was the natural glycerol 3-phosphorylcholine (GPC) resonance of the lenses. GPC is a compound with a relatively constant chemical shift for a phosphate (−0.13 ppm), which is not influenced by variable physiologic pH, ionic strength, or countercation conditions.

P-31 NMR spectroscopic analysis of the intact lens generates a spectrum in which each peak corresponds to a single phosphorus-containing functional group having a discrete resonance shift position. The identity of each resonance shift position is based on physical and chemical criteria described previously so that each peak is a physicochemical marker for individual organophosphate metabolites present in the crystalline lens.

Mathematical analysis of dynamic changes in the organophosphate profile. A least-squares regression analysis using a linear expression of the form $y = Ax + B$ was employed to fit time-course data for the incubation period, so that the sum of the squared deviations about the line is a minimum. The first degree expression was adequate for approximation of the real time-course data obtained from the intact lens tissue. Five time-course determinations were obtained; variation was ±10% at any given time in the kinetic run.

Results

Fig. 1 is a P-31 NMR spectrum obtained from intact rabbit lenses before incubation in media containing $2 \times 10^{-3}$M dexamethasone. The summarized data obtained from this and other spectra obtained in the course of this study are presented in Table I. The resonances from downfield (left) to upfield (right) in the control spectrum are as follows: the resonance indicated by the arrow, at 6.0

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Fig. 2. P-31 NMR spectra of the intact rabbit lens from six time points during incubation in control Earle’s buffer and in Earle’s buffer with $2 \times 10^{-3}$M dexamethasone at pH 7.4, 37° C.
Fig. 3. Time-course for the changes in the lens phosphate profile when two lenses are incubated in Earle's buffer containing $2 \times 10^{-3}$M dexamethasone. Experimental data points for one time-course experiment are indicated in the figure. The curves were derived from the least-squares analysis presented in Table II. SP, Sugar phosphates; DN, dinucleotides; NS, nucleoside diphosphosugars and related organophosphates.

Table II. Coefficients for the expression $y = Ax + B$, obtained in the linear regression analysis of the time course for lens phosphates after incubation of rabbit lenses in $2 \times 10^{-3}$M dexamethasone*

<table>
<thead>
<tr>
<th>Component of time course</th>
<th>Coefficients</th>
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<tr>
<td></td>
<td>$A$</td>
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</table>
| 6 ppm resonance         | 0.01 ± 0.013 | 1.898 ± 0.138  
| GPC                     | 0.00 ± 0.010 | 2.217 ± 0.092  
| ADP                     | -0.05 ± 0.056 | 5.975 ± 0.700  
| DN                      | -0.20 ± 0.023 | 15.07 ± 0.285  
| NS                      | 0.21 ± 0.031 | 2.431 ± 0.389  
| SP                      | 0.56 ± 0.030 | 19.78 ± 0.321  
| Pi                      | 1.82 ± 0.017 | 6.966 ± 0.175  
| ATP                     | -2.39 ± 0.076 | 45.96 ± 0.801  

DN = dinucleotides; NS = nucleoside diphosphosugars; SP = sugar phosphates.
*The ordinate is the percent of total phosphorus detected and the abscissa is time in hours.

ppm, arises from a phosphatic molecule of unknown nature. Immediately upfield is a resonance band labeled $\alpha$-GP ($\alpha$-glycerophosphate) and SP (sugar phosphates). There are two principal components to this resonance band: the downfield signal arises from the triose phosphates, the principal molecule of which is $\alpha$-GP; the upfield resonance arises from the pentose and hexose phosphates, one of which is inosine monophosphate. The next prominent signal upfield is that of inorganic orthophosphate (Pi), followed by the resonance band of glycerol 3-phosphorylethanolamine (GPE) and GPC. The next upfield resonance band is that from the ionized end group phosphates, which begins at $-5.6$ ppm. The $\gamma$-phosphate resonance of ATP and the $\beta$-phosphate resonance of ADP are located in this region. Upfield from the ionized end-group phosphates are the esterified end-group phosphates, the $\alpha$-phosphate resonances of both ATP and ADP and the resonance band of the dinucleotides, nicotinamide adenine dinucleotide (NAD), and nicotinamide adenine dinucleotide phosphate (NADP). The next upfield resonance, labeled NS, arises from the nucleoside diphosphorylsugars; the primary nucleoside diphosphorylsugars in the lens are uridine diphosphorylgucose (UDP-
glucose) and uridine diphosphoryl galactose (UDP-galactose), with a minor contribution from uridine diphosphoryl mannose (UDP-mannose). The $\beta$-phosphate resonance of ATP is the most upfield signal in the spectrum.

Fig. 2 shows spectra taken during the time course of the incubation of rabbit lens in $2 \times 10^{-3}$M dexamethasone; corresponding spectra taken during a control time-course are also presented for comparison. The spectra of Fig. 2 illustrate the types of changes exhibited by the NMR data during lens incubation in the steroid-containing media. Integration of the respective spectral resonance areas yields quantitative data that can be employed to plot a multicomponent time course, as shown in Fig. 3.

Examination of the time-course data reveals that when rabbit lenses are exposed to buffer containing $2 \times 10^{-3}$M dexamethasone, the relative lens ATP content undergoes a linear decline with time until the ATP concentration approaches undetectable levels. Lenticular ADP levels, however, change little with time, similar to ADP levels in lenses exposed to elevated sugars; however, this lack of change in ADP levels is in contrast to the metabolic changes induced by glucose deprivation where the ADP levels are elevated. $\Pi$, on the other hand, undergoes a linear increase with time. The sugar phosphates and nucleoside diphosphorylsugars also are observed to increase with time, whereas the dinucleotides decrease. The other phosphorus resonances in the spectrum, 6 ppm and GPC, exhibit little or no change during this time course. Observable cataract formation was not seen to occur during the 22 hr time course until approximately 18 hr had elapsed, after which time cataract formation in the subcapsular and superficial cortical regions was visible. No changes were observed in the phosphate profiles of the control lens except for a slight elevation in ATP and $\alpha$-GP levels and a slight decrease in $\Pi$ levels. In addition, cataract formation was not observed during the 22 hr control time course.

The linear segments of the Fig. 3 time course were fitted by least-squares regression analysis to the linear function, $y = Ax + B$, for the ordinate in percent of total phosphorus and the abscissa in hours; the results are presented in Table II and plotted as straight line segments in Fig. 3. Note that the slopes of the 6 ppm resonance, GPC, and ADP are not significantly different from zero. The rate of dinucleotide loss equals the rate of nucleoside diphosphorylsugar accumulation, the rate of change over 22 hr being $4.5\%$ of the total phosphorus extracted. The rate of ATP loss equals the combined rates of $\Pi$ and sugar phosphate accumulation. Over 22 hr the extrapolated ATP loss amounts to $52.6\%$ of the total phosphorus, which is equivalent to a $114\%$ loss; that is, after about 22 hr, lens ATP levels are essentially exhausted. The time-course data of the control values exhibit no change except for a slight increase in ATP and decrease in $\Pi$.

Table I contrasts the metabolite levels of PCA extracts prepared from 24 hr incubated control and dexamethasone lenses. Four minor metabolites, the hexose band, GPE, dinucleotides NAD and NADP, and the nucleoside diphosphorylsugars undergo concentration changes after incubation of the lenses in dexamethasone. The hexose band increases to $284\%$ of its initial value; the phosphodiestert GPE decreases to $60\%$ of its initial value. The dinucleotides were decreased to $75\%$, whereas the nucleoside diphosphorylsugars were elevated $270\%$. Lens PCA extract signals exhibit line widths less than 0.5 Hz, and under such conditions the multiplet structures of UDP-glucose, -galactose, and -mannose are resolved to the spectral baseline. The unidentified phosphorus resonance band at 18 and 10 ppm are also well resolved in PCA extracts, the 18 ppm band being resolved into five separate resonance signals and the 10 ppm band being resolved into two separate resonance signals. Proton-phosphorus and phosphorus-phosphorus spin-decoupling experiments showed that these signals were not components of $^{31}$P-$^{31}$P spin-multiplets; thus, each signal represents a unique phosphatic molecule. The appearance of five 18 ppm resonance signals in extracts of lenses treated with steroid is a new finding, which demonstrates that the molecules giving rise
Fig. 4. P-31 NMR pH-titration data of prominent lens phosphates. Representative data points are indicated on the Pi titration curve. Rib-5-P, Ribose 5-phosphate; Fru-6-P, fructose 6-phosphate; Gal-6-P, galactose 6-phosphate.

to these resonances are influenced by the steroid incubation. Extracts from control incubations show only the 18.050, 18.002, and 17.813 ppm peaks, and their relative amounts are about the same as those seen in extracts from steroid-treated lenses. The 17.959 and 17.899 ppm resonances appear after treatment of lenses with steroid. What is known of the chemical nature of the molecules giving rise to these resonance signals does not correspond to any phosphorus-containing molecules presently identified as constituents of biological systems.\textsuperscript{1, 10}

**Intralenticular pH measurements.** Chemical shift values of organophosphates exhibit marked dependence on the pH environment of phosphorus atoms. Phosphorus-NMR-pH-titration data are given in Fig. 4 for several lens phosphates in water with potassium as the counter-cation at 37° C.\textsuperscript{9} Regarding the specific phosphates measured in Fig. 5, the α-GP, Pi, and 6 ppm resonance signals, to a first approximation, undergo similar time course behavior. The intercept and control values (Table III) agree well for Pi and 6 ppm resonance signals, to a first approximation, undergo similar time course behavior. The intercept and control values (Table III) agree well for Pi and 6 ppm but are different for α-GP. Determination of intralenticular pH is accomplished with titration data such as those presented in Fig. 4 in conjunction with intact tissue NMR spectra. As stated previously,\textsuperscript{7} the resonance signals from α-GP, Pi, and 6 ppm resonances have been useful for pH determination in the mammalian lens.\textsuperscript{11} Intralenticular pH decreased with time in this study to approximately 6.4. In contrast, control and galactose-treated lenses did not exhibit a pH change (pH 6.9) over a similar 22 hr incubation.\textsuperscript{1, 2}

The apparent pH differences indicated by the zero-time pH values shown for the three phosphates in Fig. 5 are well within the precision of measurement obtainable by NMR spectroscopy. Whether these apparent pH differences actually reflect only hydrogen ion concentration differences has not been established with certainty. Solvent effects, counter-cation effects, and NMR bulk-susceptibilities could account for the small differences in apparent pH indicated by the shift values of the three phosphates in Fig. 5, however, the accumulating data from all intact tissue resonance studies performed in this and a number of other laboratories strongly indicate that for the very low-molecular weight inorganic phosphate and phosphomonoester anions, only hydrogen ion concentration has any effect on the shift value in living tissue at 37° C. Thus we interpret the zero-time differences in pH to reflect the existence of differing hydrogen ion environments within the intact tissue.\textsuperscript{12}
Table III. Coefficients for the expression \( y = Ax + B \), obtained in the linear regression analysis of intralenticular pH time-course data for three phosphorus resonance signals observed in intact lens tissue spectra

<table>
<thead>
<tr>
<th>Component of time course</th>
<th>Coefficients</th>
<th>Control pH value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha )-GP</td>
<td>(-0.016 \pm 0.0002)</td>
<td>6.62 ± 0.002</td>
</tr>
<tr>
<td>Pi</td>
<td>(-0.013 \pm 0.0006)</td>
<td>6.67 ± 0.007</td>
</tr>
<tr>
<td>6 ppm resonance</td>
<td>(-0.011 \pm 0.0005)</td>
<td>6.51 ± 0.006</td>
</tr>
</tbody>
</table>

*The ordinate is the percent of total phosphorus detected and the abscissa is time in hours.

Discussion

Glucocorticosteroid hormones (e.g., dexamethasone) antagonize the insulin-dependent glucose transport and uptake in peripheral tissues. The mechanistic basis for this action is unresolved; however, the rate-controlling steps for glucose uptake, active transmembrane transport, and subsequent phosphorylation processes, are purportedly suppressed in the presence of glucocorticosteroids. This depressed tissue glucose uptake is one of the earliest biochemical manifestations of the presence of glucocorticoid hormones. The presence of glucocorticosteroid-sensitive receptors has been demonstrated in the mammalian lens, and although the lens does not require insulin for glucose uptake in vitro, dexamethasone may antagonize lens glucose uptake and/or utilization so that the rate of ATP hydrolysis exceeds the rate of ATP synthesis. This rationale provides a plausible mechanistic interpretation that is consistent with the time-dependent catastrophic decrease in lens ATP observed in the present study.

The lack of changes in the concentration of ADP is an unexpected observation, which may simply reflect the efficiency of the adenyl kinase reaction in converting ADP to ATP. It should be noted, however, that in studies involving incubation of this same tissue in a glucose-depleted medium, ADP concentration does increase.

Harris and Gruber, in a study similar to ours involving steroid incubation at 37°C, showed that tissue water content was maintained even though \( K^+ \) levels were depressed and \( Na^+ \) levels were elevated. \( K^+ \) loss and \( Na^+ \) accumulation are often accompanied by tissue edema; therefore the presence of the steroid would appear to influence the distribution of water across the lens epithelium independent of changes in cellular \( K^+ \) and \( Na^+ \) levels. It appears that steroid treatment results in a more tightly controlled biological system even though ATP stores are eventually exhausted.

The accumulated biochemical knowledge of cellular metabolism does not provide any readily apparent reason for the steroid-induced catabolism of GPE and the pyridine nucleotides. The effect of GPE loss on the lens cannot be evaluated because the role of this phosphate in intermediate metabolism is unknown. The pyridine nucleotides are major cofactors in intermediate metabolism of the lens and function as carriers of the hydrogen-reducing potential for the oxidative phosphorylation and other redox processes of the cell. Thus the 25% decrease in lens pyridine dinucleotides (NAD and NADP) would have adverse consequences on the oxidative-reduction processes required for ATP production necessary to maintain tissue viability.

When lenses are incubated in glucose-deficient media, the intralenticular pH increases, approaching that of the supporting buffer. In our previous work, this pH value changed from 6.9 in control tissue to 7.4 in the glucose-depleted lens. In the present study, lens pH progressively decreased to a value of 6.4 after 22 hr. In both experimental investigations, ATP hydrolysis exceeded synthesis, thereby causing a net time-dependent decrease in lens ATP content and a concomi-
tant production of two equivalents of hydro-
onium ion per ATP, P-O-P bond hydrolyzed.
The rate of ATP loss in lenses incubated
without glucose was approximately threefold
greater than that in dexamethasone-treated
lenses. Thus we conclude that the pH of the
tissue after experimental manipulation re-
flects the state of the hydrogen ion pump ac-
tivity rather than the content of tissue ATP or
the quantity of hydrogen ion produced as a
result of high-energy phosphate hydrolysis.
Moreover, the low intralenticular pH result-
ing from steroid treatment may also reflect an
altered membrane potential brought about by
the relatively lowered K+ and elevated
Na+ ion concentrations.16
No studies to date have monitored the in-
fluence of corticosteroids on the organophos-
phate metabolites over a time course measur-
ing dynamic changes. The time course for
changes in phosphorus metabolite concen-
trations, which is zero order for all phos-
phtates undergoing change, indicates that the
control mechanism operating to regulate
phosphate metabolite concentrations is inde-
pendent of phosphate levels. The presence of
zero-order rate processes implies that a single
invariant parameter or set of parameters is
controlling all measured chemical reactions.

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