Phosphorus-31 nuclear magnetic resonance spectroscopy of toad retina

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ABSTRACT Phosphorus-31 nuclear magnetic resonance (^{31}P-NMR) spectra were obtained from living toad retinae and toad retinal extracts at 4°C. Several phosphorus metabolites—nucleoside di- and triphosphates (NTP), phosphocreatine, phosphodiesters, inorganic phosphate, and phosphomonoesters—were identified from the spectra of whole retinae. The intracellular pH was determined to be 7.27 ± 0.06 at 4°C and the intracellular MgNTP/NTP ratio was at least 0.77. These results are consistent with those reported by other techniques, and they show that ^{31}P-NMR spectroscopy can be used for noninvasively and quantitatively studying the metabolism of living toad retinae, and for monitoring its changes over time.

INTRODUCTION

Phosphorus-31 nuclear magnetic resonance (^{31}P-NMR)[1] spectroscopy has been successfully used to study tissue metabolism (Avison et al., 1986), including that of various regions and preparations of brain (Prichard and Shulman, 1986). Retinal metabolism has not been studied extensively using in vivo ^{31}P-NMR spectroscopy, probably because of the small size of the retina and the difficulty involved with its isolation and maintenance. In a previous study, a spectrum of bovine retinae was reported (Chapman et al., 1982), but there are no studies of the smaller amphibian retina, upon which most metabolic and electrophysiological studies are done.

^{31}P-NMR spectroscopy can be used to simultaneously measure intracellular pH, intracellular Mg^{2+}, and phosphorus metabolites all of which are important to retinal bioenergetics and function. ^{31}P-NMR spectroscopy has the additional advantage that just the mobile pools (typically those free to participate in chemical reactions) of metabolites are measured, rather than both bound and unbound forms as with chemical analysis techniques (Dawson and Wilkie, 1984). In this paper we present the results of ^{31}P-NMR spectroscopic experiments on living whole toad retinae and on retinal extracts. A preliminary account of this work was presented at the 1988 Biophysical Society Meeting in Phoenix, AZ (Apte et al., 1988*a*).

[1] *Abbreviations used in this paper:* NTP, nucleoside triphosphate; PCr, phosphocreatine; PDE, phosphodiester; PME, phosphomonoester; ^{31}P-NMR, phosphorus-31 nuclear magnetic resonance.

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MATERIALS AND METHODS

Tissue and sample preparations

Toads (*Bufo marinus*) were fed meal worms once a day and kept on a 12-h-light/12-h-dark cycle for at least 3 d at room temperature before sacrifice. After a 12-h-dark adaptation, the toads were killed by double pithing. This and all subsequent tissue handling was done under dim red light. The eyes were enucleated then hemisected, and the retinae were isolated with minimal contamination from the pigment epithelium. Immediately they were placed in a Hepes buffer (*N*,2-hydroxyethylpiperazine-*N*'-2-ethane sulfonic acid; 10 mM Hepes, 105 mM NaCl 2.5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 5 mM $NaHCO_3$; pH adjusted to 7.8 with 1.0 N NaOH) prebubbled with O_2 for 1 h and precooled to 4°C. In <30 min, eight retinae were placed in a 12-mm NMR tube (precision glassware; Wilmad Glass Co., Inc., Buena, NJ) in the buffer, which was bubbled with O_2 throughout the experiment. Particular care was taken to keep any turbulence generated by bubbling O_2 to a minimum. The retinae were kept in the dark during data acquisition. The time from isolating the retinae to the beginning of data acquisition was <45 min.

Retinal extracts

Lyophilized extracts from retinae (dry weight is ~7 mg per retina), isolated as described above, were prepared according to the method of Glonek et al. (1982), modified for small tissue samples by using 1 ml each of 140 mM perchloric acid and methanol for the extraction. To obtain spectra, the lyophilate was resuspended in 2 ml of 20 mM Tris to which 20% D_2O was added to lock the field frequency. For pH titrations, the pH of each extract was adjusted by adding small volumes of 1.0 N HCl.

Phosphorus-31 nuclear magnetic resonance spectroscopy

A vertical, high-resolution, wide-bore spectrometer (GN300; General Electric Co., Medical Systems Group, Fremont, CA) operating at

0006-3495/89/09/447/06 $2.00

121.48 MHz for phosphorus was used. Spectra of whole retinae were obtained by averaging the data set of 5,000 one-pulse experiments with a pulse duration of 15 μs (tip angle, ~45°), a 256-ms interval between pulses, a data size of 4 K, and a sweep width of ±4,000 Hz (total time for one set, 22.5 min). Quadrature phase decoupling was used. The spectral peak of endogenous phosphocreatine (PCr), set to zero parts per million (1 ppm = 121.48 Hz), was used as a resonance frequency standard. Routine baseline correction procedures and 10 Hz line broadening were applied. For retinal extracts, usually 13 sets of 10,000 one-pulse experiments, with the same pulse duration but a pulse interval of 512 ms and a data size of 8 K, were averaged (total time for one set, 1.5 h).

RESULTS

Fig. 1 *A* shows a ^{31}P-NMR spectrum of eight whole toad retinae in oxygenated Hepes buffer at 4°C, representing an average of 40,000 scans (3 h). The presence of different phosphorus metabolites—nucleoside triphosphates (NTP), pyridine nucleotides (NAD and NADP), phosphocreatine (PCr), phosphodiesters (PDE), inorganic phosphate (P_i), and phosphomonoesters (PME)—can be identified on the basis of the chemical shifts of metabolites known to exist in neural tissues, and the pH dependence of these peaks in retinal extracts (Glonek et al., 1982; Petroff and Prichard, 1983; Petroff et al., 1985). Spectra of standard solutions containing GTP and ATP indicate that these triphosphates, both of which have high concentrations in the retina (de Azeredo et al., 1981; Salceda et al., 1982; Klethi et al., 1970), cannot be distinguished under our conditions. Consequently, they are referred to collectively as nucleoside triphosphates (NTP).

Because averaging broadens peaks that shift over time (noted the capped peaks, P_i and PME, in Fig. 1 *A*), spectra acquired over shorter periods (see Fig. 1 *B*) provide more accurate information about peak positions. Fig. 1 B is a spectrum averaged over 22.5 min (5,000 scans) under the same conditions as for Fig. 1 *A*. The τ-phosphate of nucleoside triphosphates (NTP) and the β-phosphate of nucleoside diphosphates (NDP) resonate at the same position (with respect to PCr), −2.62 ppm (linewidth at half height [$\Delta\nu_{1/2}$] = 1.00 ± 0.06 ppm); similarly, the α-phosphate of NTP and the α-phosphate of NDP are at the same position, −7.60 ppm ($\Delta\nu_{1/2}$ = 0.35 ± 0.02 ppm); the β-phosphate of NTP is at −16.66 ppm ($\Delta\nu_{1/2}$ = 1.77 ± 0.13 ppm). The shoulder of the α-NTP peak, at ~−7.9 ppm, is nicotinamide adenine dinucleotide (NAD) and/or nicotinamide adenine dinucleotide phosphate (NADP). There are at least three phosphodiester peaks at 3.60, 3.07, and 2.65 ppm ($\Delta\nu_{1/2}$ = 0.31 ± 0.03, 0.29 ± 0.02, 0.24 ± 0.01 ppm, respectively). Inorganic phosphate (Pi) resonates at 5.10 ppm ($\Delta\nu_{1/2}$ = 0.75 ± 0.06 ppm) and the phosphomonoester (PME) at 6.67 ppm ($\Delta\nu_{1/2}$ = 0.43 ± 0.03 ppm). Linewidths at half heights were determined by first fitting Lorentzian curves to the line shapes.

The pH titration of retinal extracts (Fig. 2) was done to aid in the identification of peaks whose chemical shifts are dependent on pH. Several titratable species can be identified: P_i, the PMEs, the PDEs (Burt et al., 1979; Pettegrew et al., 1986; Gyulai et al., 1984) and the NTP phosphates. Of the NTP peaks, τ-NTP is the most pH dependent, particularly in the pH range from 4 to 9, consistent with work by Cohn and Hughes (1960). The extract studies

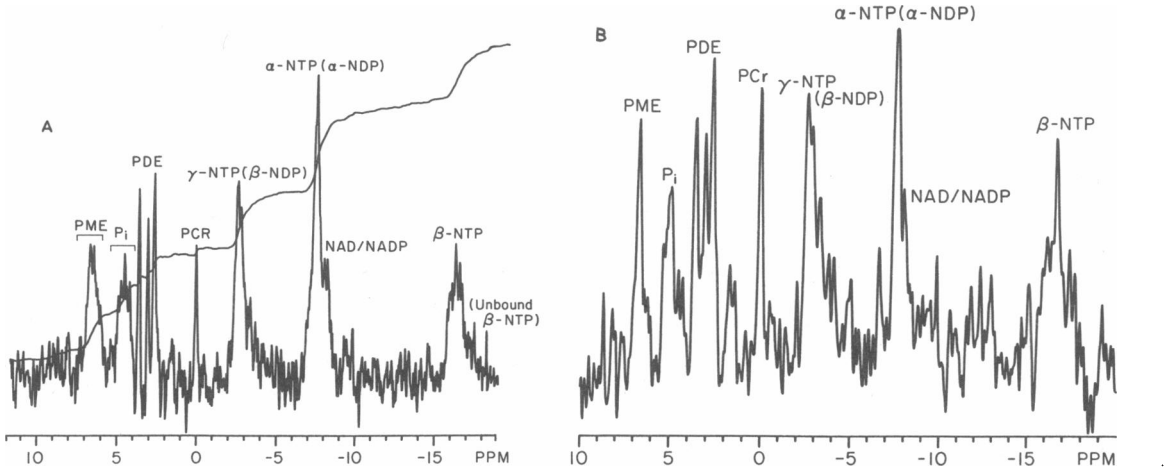

FIGURE 1 (*A*) In vivo ^{31}P-NMR spectrum of eight toad retinae in Hepes buffer bubbled with O_2 at 4°C, representing an average of 40,000 scans (3 h). Peak assignments are: NTP (nucleoside triphosphate; τ-, β-, α- peaks); NDP (nucleoside diphosphate; β-, α- peaks); PCr (phosphocreatine); PDE (phosphodiesters); P_i (inorganic phosphate); PME (phosphomonoesters). Superimposed is the integral of the spectrum. The wet weight of the total tissue is ~700 mg. (*B*) In vivo ^{31}P-NMR spectrum of eight toad retinae in oxygenated Hepes buffer at 4°C, representing an average of 5,000 scans (22.5 min). This is the initial signal averaging period for the experiment shown in *A*.

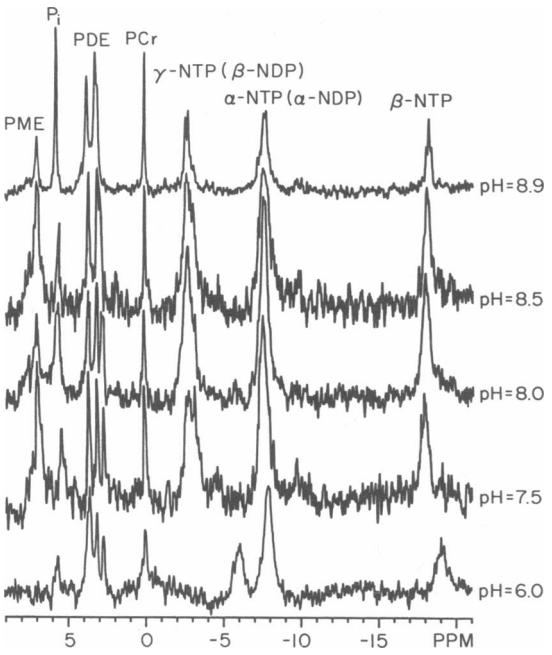

FIGURE 2 The pH dependence of the chemical shifts of phosphorus metabolites in ^{31}P-NMR spectra of extracts from eight toad retinae. Each spectrum represents an extract at a different pH (note the single P_i peak) and an average of 130,000 scans obtained over a 19.5-h period. Note that changes in pH primarily affect positions of PMEs, P_i, and τ-NTP.

also demonstrate that there is only a single peak in the P_i region of the spectrum; therefore, although in the living retinae the P_i peak is broad, it does not contain contributions from other metabolites.

The intracellular pH was calculated from the position of the P_i peak (Fig. 1 *B*) to be 7.27 ± 0.06 at 4.0°C on the basis that: $pH = pK + \log [(\delta - \delta_1)/(\delta_2 - \delta)]$; δ_1 and δ_2 are the chemical shifts of $H_2PO_4^-$ (3.29 ppm) and HPO_4^{2-} (5.68 ppm) with respect to PCr (0.00 ppm), δ is the position of the P_i peak of the sample, and pK = 6.77 (in the presence of 1.36 mM Mg and 155 mM KCl) (Petroff et al., 1985). Alterations of temperature, ionic strength, monovalent or divalent cation concentrations within the physiological range in the titrating solution (Elliott and Dawson, 1988) have little effect on this pH determination.

The integral of a peak (such as that superimposed upon the spectrum in Fig. 1 *A*) is related to the metabolite concentration. However, for an accurate calculation of the concentration of a metabolite, it is necessary to correct for saturation due to incomplete longitudinal relaxation. Because saturation factors were unobtainable for the retinae, rough estimates of these were made using T_1 (longitudinal relaxation time constant) values for the phosphorus metabolites (except phosphodiesters) from frog muscle at the same temperature as in our experiments (in seconds): PME (1.19), P_i (7.52), PCr (2.34), τ-NTP and β-NDP (1.23), α-NTP and α-NDP (0.90), β-NTP (1.01) (Roman, B. B., D. K. McFarlane, and M. J. Dawson, unpublished data). The relative ratios of peak areas, approximately corrected for saturation, are reported. The uncorrected values are in parentheses: PCr/PME = 0.55 (0.34); PCr/Pi = 0.24 (0.42); PCr/(τ-NTP + β-NDP) = 0.49 (0.31); PCr/(α-NTP + α-NDP + NAD/NADP) = 0.29 (0.15); PCr/(Mg^{2+}-bound β-NTP) = 0.76 (0.43); PCr/(Mg^{2+}-unbound β-NTP) ≥ 2.58 (1.47); PCr/PDE = (0.41). The NDP/NTP ratio was estimated to be ~0.54.

Fig. 3 shows six individual spectra, each an average of 5,000 scans acquired over consecutive 22.5-min intervals (I–V), except the last spectrum (*X*), which was acquired at the end of 3 h. Over time, the metabolic decay of NTP and PCr is shown by the disappearance of the β-NTP and PCr peaks. In comparing spectra II and III, note that during the time interval in which PCr has disappeared, β-NTP remains resolvable (*solid arrows*). Spectrum II demonstrates the presence of both PCr and NTP; Spectrum III demonstrates the disappearance of PCr, but the

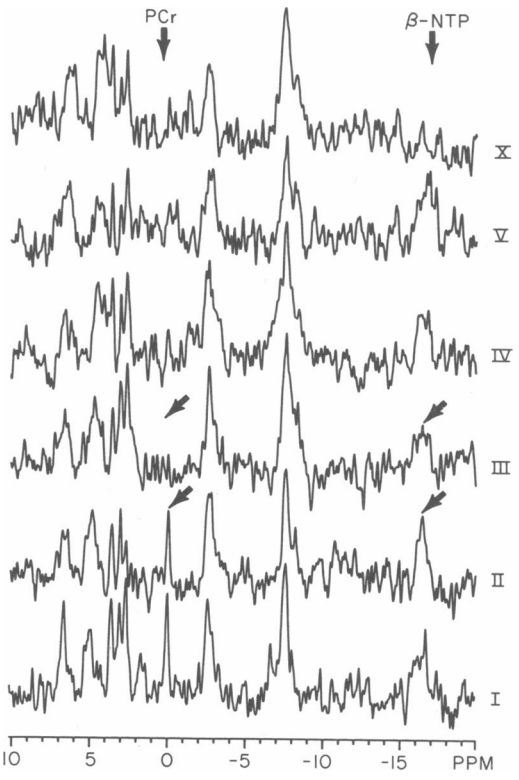

FIGURE 3 A series of ^{31}P-NMR spectra of eight retinae of 5,000 scans each at 22.5-min intervals apart (I–V); the last spectrum (*X*) is at the end of 3 h. Solid arrows point to PCr or β-NTP peaks.

presence of NTP. This suggests that PCr functions as an NTP buffer in the retinae as it does in muscle and brain (Dawson and Wilkie, 1984). Moreover, this experiment shows directly that under our experimental conditions, the breakdown of high-energy metabolites begins soon after dissection and continues until a complete loss of PCr and NTP occurs, within 3 h (the last spectrum, X, shows only NDP, PDE, PME, and P_i in an acidic environment of pH <6.8).

The β-phosphate peak represents purely triphosphates, and its position can be used to determine the free intracellular Mg^{2+}, because its chemical shift depends on Mg^{2+}-binding (Son et al., 1975; Cohn and Hughes, 1962; Gupta and Moore, 1980). However, the calculation of free intracellular Mg^{2+} is complicated by the evidence for slow chemical exchange between bound and unbound forms of ATP found in standard solutions mimicking intracellular conditions observed at 4°C and also observed at 10°C (Misawa et al., 1982). The slow exchange is reflected by the two β-ATP peaks (rather than one in the case of fast exchange) in Fig. 4 which shows spectra of a standard solution to which differing amounts of Mg^{2+} have been added. Note that as the MgATP/ATP ratio increases, the contribution from MgATP (the downfield or left) peak increases. Because the two peaks do not merge in solution at 4°C, it may not be appropriate to use the chemical shift of the β-NTP peak (Gupta and Moore, 1980) to determine the amount of Mg^{2+}-bound ATP or NTP at 4.0°C from in vivo spectra. A comparison of the relative areas can be used, however, because our preliminary results indicate that the T_1s for the magnesium-bound and -unbound β-ATP are the same. Thus, based on the ratio of peak areas, the estimated MgNTP/total NTP, using corrected values (1.32/[1.32 + 0.39]), is at least 0.77. The chemical shift of the β-NTP peak in whole retinae is close to the position for fully Mg^{2+}-bound NTP; therefore, if fast exchange occurs inside the cells at this low temperature, the estimated MgNTP/total NTP ratio is even higher (>0.90).

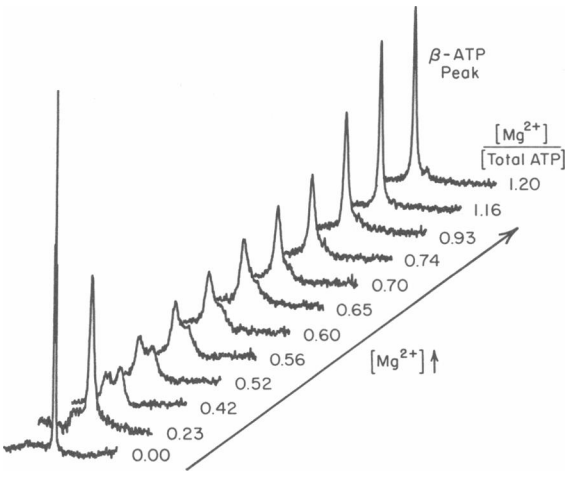

FIGURE 4 A stacked plot of ^{31}P-NMR spectra at 4°C representing titrations of magnesium with adenosine triphosphate (ATP) in solutions containing 20 mM PIPES (Piperazine-*N*,*N*′/-bis(2-ethane-sulfonic acid) buffer adjusted to pH 7.16 with 100 mM KCl at 4°C with 4.5 mM ATP and from 0.0 to 5.0 mM $MgCl_2$. Ratios are of [MgATP]/[ATP].

DISCUSSION

A comparison of the peak intensity ratios with chemical measurements is useful. de Azeredo et al. (1981) report values of ATP, GTP, and PCr in layers of the retina such that the PCr/ATP ratio may be estimated to be ~1.4. This is larger than our estimate of 0.76 and may mean that PCr is being depleted in our preparation. Caution is needed in interpreting the results from extraction experiments (de Azeredo et al., 1981; Salceda et al., 1982; Klethi et al., 1970; Robinson and Hagins, 1979) because extraction procedures can liberate tightly bound metabolites as well as destroy cellular compartmentation. However, preliminary studies comparing integrals of spectra from extracts and intact retinae indicate that all signal from PCr and NTP observed in the extracts is also apparent in the in vivo spectra. This suggests that the NTP and PCr are free and mobile and not compartmentalized in the living tissue.

Reciprocally, care must be taken in interpreting our results because our measurements do not distinguish between layers of the retinae: The PCr is mostly in the inner layers, and a greater portion of NTP may arise from the photoreceptor cells. The average of GDP/GTP ratios from different layers of dark-adapted frog retinae, 0.43 (de Azeredo et al., 1981), is consistent with our estimate of the NDP/NTP ratio of 0.54 from toad retinae. An important metabolite, cyclic guanosine monophosphate, could not be observed in our present spectra; its measurement remains an important objective.

By comparison with known chemical shifts for different PME and PDE species, the peaks may be tentatively assigned as follows. The 3.60 ppm and 3.07 ppm PDE peaks are assigned to glycerolphosphorylethanolamine and glycerolphosphorylcholine, respectively (Glonek et al., 1982), and on the basis of the pH titration (see Fig. 2) the 2.65-ppm PDE peak is likely to be serine phosphorylethanolamine (Burt, 1985). The PME peak has a very similar chemical shift to that of phosphorylethanolamine in dog brain (Gyulai et al., 1984). In contrast to bovine retinae (Chapman et al., 1982), there are high amounts of PDE in toad retinae, and indeed in toad tissues in general (Wilkie and Wray, 1986). Though their specific biological role in the toad is yet unclear, the PDEs are involved in membrane phospholipid degradation and they are phos-

pholipase inhibitors (Burt, 1985). In the toad (Wilkie and Wray, 1986), and in mammalian kidney (Bagnasco et al., 1986) they may also have a role in osmotic regulation.

The intracellular pH of 7.27 ± 0.06 of toad retinae at 4°C is more alkaline than the intracellular pH values reported for mammalian brain at 37°C, also measured by ^{31}P-NMR spectroscopy (Petroff et al., 1985). Hagins and Yoshikami (1977) found pH 6.8–7.1, for rat rods at 34–37°C, whereas Chapman et al. (1982) estimated 7.13 for bovine retinae at 37°C. This difference is largely due to the effect of temperature upon intracellular pH (Dawson and Elliott, 1984); our values do not vary significantly from values for frog muscle at the same temperature (Dawson and Elliott, 1984). In intact retinae (Fig. 1 *B*), as opposed to extracts (Fig. 2), the P_i region tends to show multiple peaks; this may be due to a slightly different pH milieu in the different cell types of the retinae.

The metabolic state of the retina has usually been judged by its ability to be excited by light and to undergo light adaptation. Fig. 3 shows that depletion of the mobile reserves of the high-energy metabolite phosphocreatine begins as early as 30 min after isolating the oxygenated retinae. Thus, the amount of NTP and PCr observed by ^{31}P-NMR spectroscopy could serve as a useful tool to evaluate tissue handling techniques.

Free intracellular Mg^{2+} has an important role in the GTP-hydrolytic cycle of rods (Yamazaki et al., 1987) and may directly control the conductance of rod membrane patches (Stern et al., 1987) as well as other functions. Also, trinucleotides involved as substrates for enzymes are almost always bound to Mg^{2+} to be active. Free intracellular Mg^{2+} also affects the free-energy change for ATP-hydrolysis, and thus the chemical driving force for all ATP-dependent reactions.

Although the free intracellular Mg^{2+} concentration in cells has been estimated to be from 0.2 to 2.0 mM (Maughan and Recchia, 1985), the intracellular Mg^{2+} for retinal cells is not known. ^{31}P-NMR spectroscopy studies of the brain, which use the dependence of the chemical shift of the β-NTP peak Mg^{2+} binding, show a MgNTP/NTP ratio of ~0.90 or greater, corresponding to a free intracellular Mg^{2+} of 1.5 mM or greater (Dawson and Wilkie, 1984). Our expreriments were done at 4°C to minimize the breakdown of metabolites. Hence, the peak areas of the bound NTP peak and unbound NTP region were used to determine a lower limit (the unbound-NTP region is lost in the noise) of MgNTP of ~75% of the total. If slow exchange is present in vivo, this lower limit is still consistent with the percentage of Mg^{2+} bound NTP. It is quite possible that the slower chemical exchange seen in solution is not present in vivo, in which case, the position of the β-NTP peak is consistent with a higher estimate of MgNTP/NTP, >0.9.

In conclusion, for studying the retina ^{31}P-NMR spectroscopy provides a wealth of information that is unique and complementary to other techniques. Where comparison is possible, our results agree reasonably well with those obtained by more conventional techniques. Using a specially designed horizontal probe, we have already shown that it is possible to make measurements like those on several retinae on a single toad retina (Apte et al., 1988*b*). This probe will facilitate the monitoring of retinal metabolism using ^{31}P-NMR spectroscopy by making the application of different experimental conditions easier.

We wish to thank C. Tyler Burt for helpful discussions and Brian B. Roman for the use of his unpublished results.

D. V. Apte was supported by a National Institutes of Health Molecular Biophysics Training Grant (PHSI T32GM082 76PH), the Kappa Kappa Gamma Fraternity Fellowship, and by the Hazel I. Craig Fellowship, University of Illinois, College of Medicine. This work was supported by National Institute of Health grants EYO1323 (to T. G. Ebrey) and the Servants United Foundation and the Research Board of the University of Illinois (to M. J. Dawson).

Received for publication 20 October 1988 and in final form 3 April 1989.

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