Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 2748–2751, May 1983
Medical Sciences

Cerebral metabolic studies *in vivo* by ^{31}P NMR

(epilepsy/hypoxia/hypoglycemia)

JAMES W. PRICHARD*, JEFFRY R. ALGER†, KEVIN L. BEHAR†, OGNEN A. C. PETROFF*, AND ROBERT G. SHULMAN†

Departments of *Neurology and †Molecular Biophysics and Biochemistry, Yale University, 333 Cedar Street, New Haven, Connecticut 06510

Contributed by R. G. Shulman, January 24, 1983

ABSTRACT **^{31}P NMR studies on the brains of living rabbits were carried out at 32 MHz in a spectrometer having a 200-mm clear bore. Paralyzed pump-ventilated animals under nitrous oxide analgesia were inserted into the 1.89-T field and signals were focused in the brain by using a 4-cm surface coil. Several conventional physiological variables were monitored together with ^{31}P spectra during induction and reversal of insulin shock and hypoxic hypoxia sufficient to abolish the electroencephalogram and during status epilepticus. A reversible decrease in phosphocreatine stores accompanied by an increase in P_i was detected during hypoglycemia and hypoxia. Similar changes were observed in prolonged status epilepticus but were not reversed. ATP levels fell about 50% in hypoglycemia but only slightly in the other two metabolic stresses. Intracellular pH rose in hypoglycemia; in status epilepticus and hypoxia it fell, but only when cardiovascular function was severely impaired. From the measured NMR parameters and the assumptions (*i*) that creatine kinase was at equilibrium and (*ii*) that the creatine/phosphocreatine pool was constant, it was possible to calculate the relative changes in cytoplasmic ADP levels associated with these metabolic disturbances.**

High-resolution NMR spectroscopy of ^{31}P-containing compounds is a powerful tool for investigation of cerebral metabolism *in vivo* (1–6). The technique permits measurement of phosphate energy stores and P_i in undisturbed functioning tissue, while the chemical shift of the phosphate peak yields the most direct estimate of intracellular pH currently available. Because these measurements are noninvasive, they can be made repeatedly on the same subject and, because they are thought to be free of biological hazard (7), they can in principle be made on human subjects. Their actual sensitivity *in vivo* to various kinds of controlled metabolic stress is therefore important to establish. We followed changes in phosphate energy stores and intracellular pH by ^{31}P NMR in the brains of rabbits subjected to hypoglycemia, hypoxia, and status epilepticus while simultaneously recording an array of conventional physiological variables. The results show that the NMR measurements are sensitive to experimental cerebral metabolic changes in the range of pathophysiological interest and yield unique information about such changes.

METHODS

NMR measurements were made in an Oxford Research Systems TMR-32/200 spectrometer (clear bore, 200 mm; B_0 field, 1.89 T; ^{31}P resonant frequency, 32.5 MHz). Single-turn 4- or 4.25-cm surface coils placed against the animal's scalp were used for transmission and reception of radiofrequency signals. Shim current adjustment for maximum B_0 field homogeneity was guided by observation of the water proton signal. Spectra were produced by Fourier transformation of averaged free-induction decays; a broad peak from relatively immobile phosphate residues was removed mathematically by a convolution-difference procedure. All spectra from the same experiment were collected and processed identically. Excitation pulses were delivered every 0.6 sec to achieve an adequate signal/noise ratio during periods of reversible metabolic stress. Because the interpulse interval was short relative to the T_1 values of the particle ensembles contributing to the ^{31}P spectra, the latter were not fully relaxed. The intensity of each peak at different times accurately reflects changes in concentration of individual metabolites but comparison of different peaks in the same spectrum does not yield accurate concentration ratios. Chemical shifts were measured on a scale with 85% phosphoric acid at the origin and higher frequency resonances were given positive signs in accordance with the conventions of the International Union of Pure and Applied Chemistry. Intracellular pH (pH_i) was calculated from the relationship $pH = 6.66 + \log[(\text{shift P} - 0.729)/(3.22 - \text{shift P})]$, where shift P = the chemical shift of the P_i peak; the constants were determined by titration of phosphoric acid (unpublished data).

Rabbits weighing 2 to 3 kg were anesthetized with halothane for placement of a tracheal cannula, arterial and venous catheters, and subcutaneous wire leads on the left extremities and scalp for recording the electrocardiogram and electroencephalogram (EEG). The animal was then placed on a plastic restraining board that maintained the head in a fixed position, paralyzed with intravenous pancuronium bromide, and ventilated with a Harvard model 661 respirator. Board and animal were arranged in the bore of the magnet so as to center the cranial cavity on the volume of homogeneous magnetic field of the instrument. During experiments, the rabbits were ventilated with 20–30% oxygen in 70–80% nitrous oxide; they received additional pancuronium at the rate of 0.3–0.6 (mg/kg)/hr. Blood pressure was monitored with a Statham P50 transducer. Electrical variables were amplified and recorded with a Grass model 7 polygraph. Arterial pH, pCO_2, and pO_2 were measured with a Radiometer BGA3 blood gas analyzer. Blood glucose was measured on arterial samples with Dextrostix read in an Ames Glucometer.

RESULTS

The results of three major metabolic perturbations on the EEG and ^{31}P spectra of rabbit brain are shown in Fig. 1. The salient feature observed is the decrease in phosphocreatine and the coincident increase in P_i associated with EEG silence during hypoglycemia and the first period of hypoxia, with EEG slow waves during the second period of hypoxia, and with prolonged status epilepticus caused by intravenous bicucculine. Two other

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: pH_i, intracellular pH derived from the chemical shift of P_i; EEG, electroencephalogram.

features are worthy of notice. First, the nature and reversibility of the metabolic changes varied with the imposed stress. When the EEG had been abolished by insulin-induced hypoglycemia, there was a decrease in both high-energy phosphate and a large increase in P_i; intravenous glucose reversed these changes in part and restored activity, albeit abnormal, to the EEG. In contrast, two descents into severe hypoxia caused phosphocreatine to decrease without a clear change in ATP. After both descents, the EEG and spectrum very nearly regained their original appearance, showing that the changes illustrated were due specifically to the imposed metabolic stress, not to progressive energy failure of unknown origin. Status epilepticus induced with intravenous bicucculine caused a clear decrease in the phosphocreatine/P_i ratio, but the change was less than in hypoxia and hypoglycemia, as shown by the slopes of the dotted lines joining the two peaks. No effort was made to terminate the seizure state, and cardiovascular function collapsed after 40 min of it.

The quantitative course of each of the same three experiments is shown in Fig. 2. Insulin-induced hypoglycemia developed quite slowly but, when the blood sugar decreased to <26 mg% (1.41 on the logarithmic scale of the figure), EEG activity disappeared and there was an abrupt decrease in phosphocreatine, coincident with an increase in P_i. Over the same period, there was a less abrupt 50% decline in ATP levels, and the pH_i rose. All of these changes reversed in the hour following administration of glucose, but the brain did not completely return to its control state; pH_i remained low and P_i leveled off at a concentration higher than its original one.

Two descents into hypoxic hypoxia yielded similar reciprocal changes in phosphocreatine and P_i. However, in contrast to the hypoglycemia experiment, there was no clear reduction of ATP. During the first descent, the pH_i fell below the range of its earlier variation, and it recovered on restoration of the normoxic state. During the second descent, it did not depart from the slow acid drift also displayed by the pH of the arterial blood, despite changes in phosphocreatine and P_i comparable with those in the first descent. The reason for the difference is to be found in the measurements of heart rate and mean blood pressure. During the first descent, these fell to levels low enough to reduce cerebral blood flow to a critical level; during the second descent, they did not. Since the poorer regulation of pH_i occurred first, it cannot be attributed to general deterioration of the preparation.

In the seizure experiment, reciprocal changes in phosphocreatine and P_i were evident well before cardiovascular function was sufficiently impaired to affect them. ATP and pH_i did not change appreciably in the first 20 min of status epilepticus, while phosphocreatine decreased. Over the last hour of the animal's life, acidosis developed in the brain and systemically, despite efforts to retard it by administration of intravenous sodium bicarbonate. These changes, the associated decrease in phosphate energy stores, and the instability leading to collapse of blood pressure most probably reflect failure of homeostatic mechanisms in the face of overwhelming metabolic demand from continuous seizure discharge and attendant disruption of neurally mediated cardiovascular reflexes. Neurogenic pulmonary edema has been shown to complicate analysis of cerebral metabolic changes during bicucculine-induced status epilepticus (8), but it cannot have been responsible for the changes in phosphocreatine, P_i, and pH_i shown in this figure. There was no period of marked hypertension, possibly because blood had been removed in hope of preventing it, and blood gas tensions reflected adequate pulmonary gas exchange until just before cardiovascular collapse. The transient decrease in pCO_2 is unexplained but not artifactual; it happened in three other experiments and has been reported previously in paralyzed mechani-

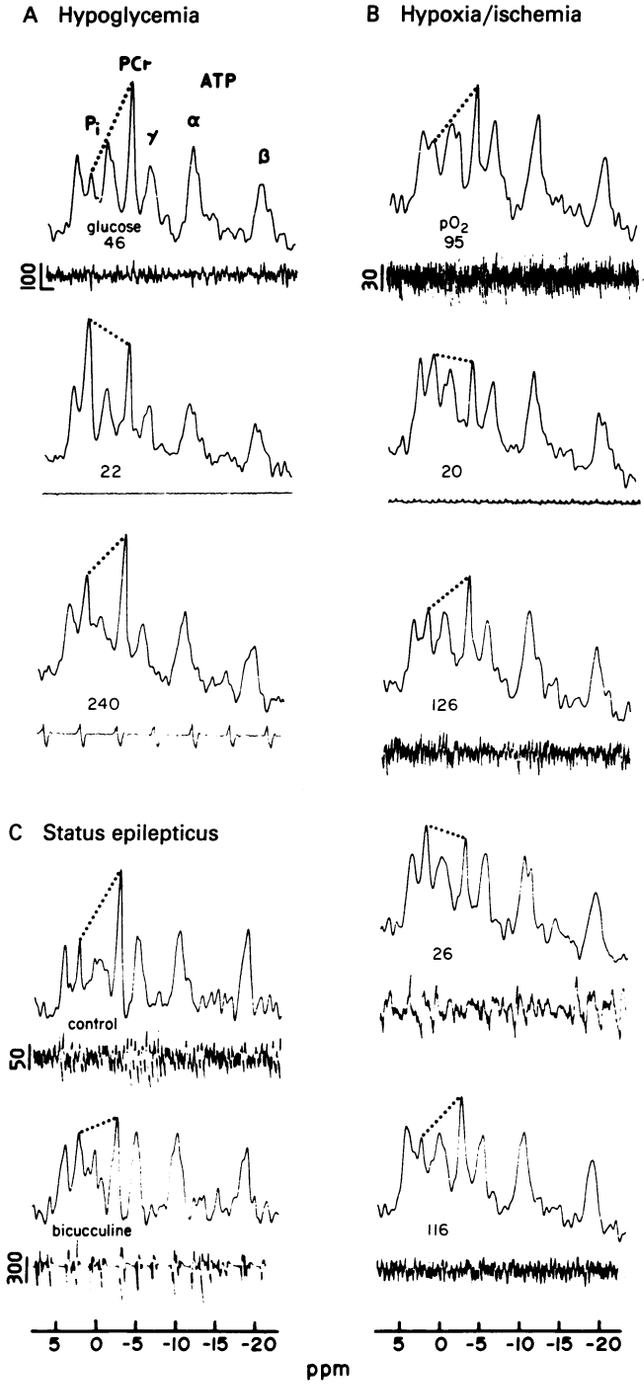

FIG. 1. Changes in ^{31}P spectra and EEGs during three kinds of metabolic stress. (*A*) Hypoglycemia. (*B*) Hypoxia/ischemia. (*C*) Status epilepticus. The P_i and phosphocreatine (PCr) resonances, which are joined by dotted lines in all spectra, and those of the γ, α, and β phosphate residues of ATP are indicated on the upper trace in *A*. Unlabeled peaks to the left and right of the P_i peak are currently attributed to sugar phosphate and phosphodiester residues, respectively. A large broad peak from phosphate residues in less mobile molecules was reduced by a convolution-difference technique. Spectra shown in *A* were made from the sum of 2,048 scans, others were from 1,024 scans; the interscan interval was always 0.6 sec for total accumulation times of 20 and 10 min, respectively. Associated data given beneath the spectra are blood glucose concentrations (mg/dl) (*A*) and arterial oxygen tension (torr; 1 torr = 133 Pa) (*B*). All EEG traces are 20 sec long; a 1-sec time calibration bar is shown below the upper trace in *A*. All traces were recorded during accumulation of the spectra above them. Vertical calibration bars are in μV.

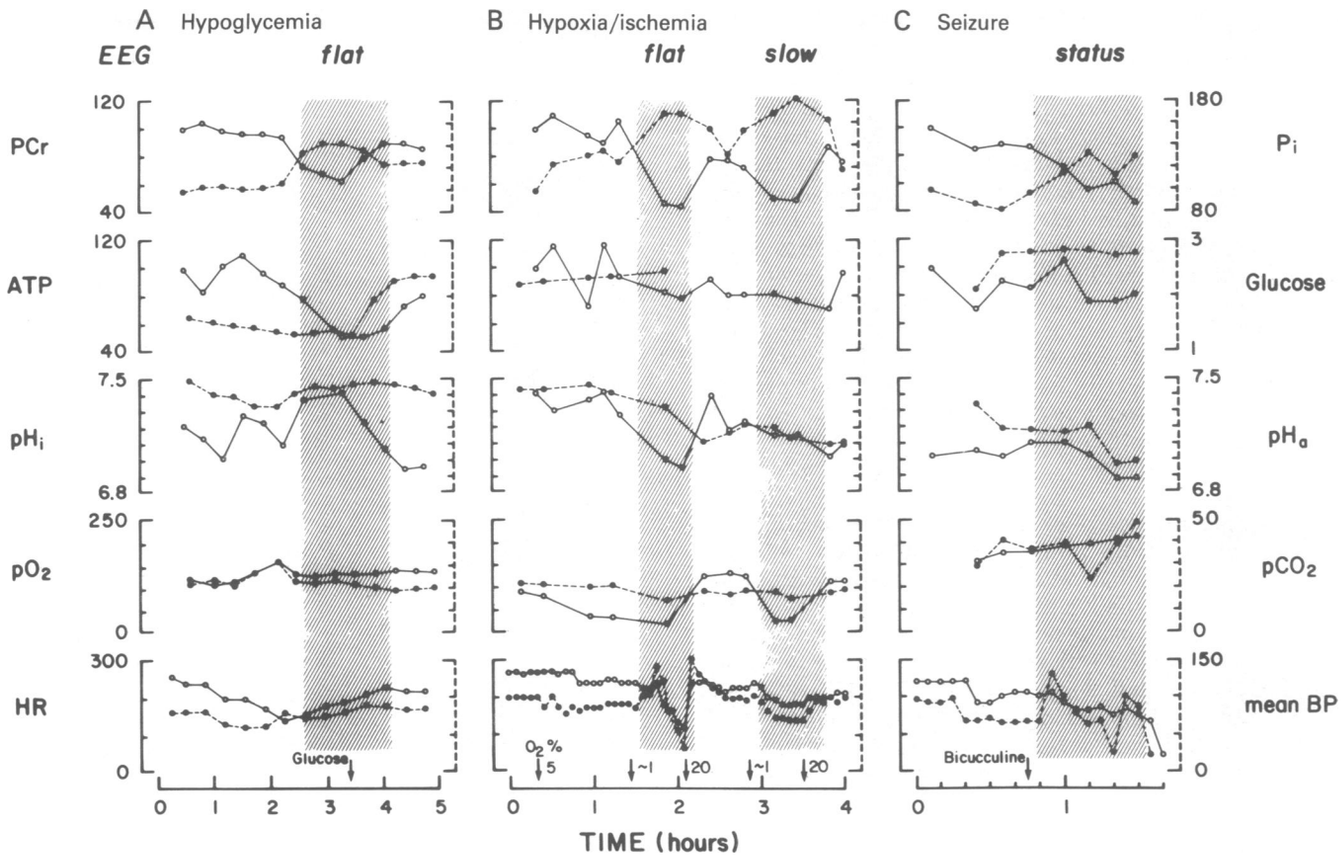

FIG. 2. Quantitative physiological and NMR data during metabolic stress. (*A*) Hypoglycemia. (*B*) Hypoxia/ischemia. (*C*) Seizure. Ten variables are plotted on identical scales for each experiment shown in Fig. 1. The solid lines are associated with the axes and labels at left; they show the course of phosphocreatine (PCr; % control), the β peak of ATP (% control), pH_i, arterial oxygen tension (pO_2; mm Hg) and heart rate (HR; beats per min). Dotted lines (right axes and labels) show the course of P_i (% control) and serum glucose (log mg%), pH, pCO_2 (mm Hg), and mean pressure of arterial blood (BP; mm Hg). Points on the phosphocreatine, P_i, and ATP curves are derived from peak heights relative to a baseline drawn through the signal-free portions of the first spectrum. Phosphocreatine, ATP, and P_i are plotted as percent change from the first point shown. Before normalization, phosphocreatine and ATP values from spectra begun within 5–10 min of blood pressure collapse were subtracted to remove the contribution to these signals of paralyzed muscle under the edges of the surface coil. Shaded areas indicate duration of maximum EEG changes caused by each stress; flat, an EEG like the second traces of both columns of Fig. 1; slow, an EEG like the fourth trace of the right column; status, an EEG like the bottom trace of the left column. Times of intravenous administration of glucose and bicucculine and changes of inspired oxygen concentration are indicated by arrows on the abscissas. Blood sugar was above the 400 mg% limit of the Glucometer during the second half of the hypoxia experiment.

cally ventilated rats early in bicucculine-induced status epilepticus (9). Increased pulmonary blood flow is the most likely mechanism.

DISCUSSION

These experiments show that ^{31}P NMR at a magnetic field strength of 1.89 T is a practical method for *in vivo* study of three kinds of cerebral metabolic disturbance. Simultaneous recording of conventional physiological variables including the EEG need not interfere with such NMR measurements, despite the presence of catheters and wires in the bore of the spectrometer, some of them quite near the volume of tissue from which the NMR signal is obtained. These facts suggest that there will be no important technical restriction on further detailed correlation of NMR and physiological measurements at 1.89 T in animals and man, including patients who require continuous monitoring of vital signs. Spectrometers large enough for human studies are now available.

Our data on hypoglycemia, hypoxia, and status epilepticus agree well with many aspects of *in vitro* work on the same metabolic stresses. In hypoglycemia, the decrease of both phosphocreatine and ATP, the failure of phosphocreatine and P_i to recover fully (Fig. 2), and the poor recovery of the EEG (Fig. 1) closely parallel experiments showing persistent metabolic abnormalities in the brains of rats unable to recover normal behavior after long periods of EEG silence caused by hypoglycemia (10). The rise of brain pH_i during severe hypoglycemia confirms the detection by indirect means of similar changes in normo- and hypocapnic rat brain, due possibly to reduced accumulation of glucose-derived metabolic acids and movement of bicarbonate ion into cells (11). Throughout the hypoglycemic period, mean arterial blood pressure, heart rate, pO_2, and pCO_2 remained within ranges that, in our other experiments, did not alter phosphate energy stores or pH_i. In contrast to the hypoglycemia experiment, during hypoxia and status epilepticus, the decrease in phosphocreatine was not accompanied by a clear change in ATP, in accordance with results obtained by several workers using conventional techniques (9, 12). The agreement of new with older methods of observation encourages confidence in both, as is most readily appreciated if one reflects on how perplexing the opposite result would have been.

Beyond confirmation *in vivo* of earlier *in vitro* work, our data adumbrate three kinds of special NMR capability in cerebral metabolic studies. First, measurement of pH_i: the behavior of the pH_i in the hypoxia experiment demonstrates the utility of being able to monitor this variable throughout an experiment. The acid shift during the first descent into hypoxia—which caused critically low heart rate and blood pressure—contrasted with

the stability during the second descent, which did not, points to three conclusions. (*i*) Cerebral blood flow was adequate to carry away the acid products of metabolism during the second descent but not during the first. (*ii*) Either the changes in phosphocreatine and P_i during the second descent were due to hypoxia without ischemia or the creatine kinase reaction was more sensitive than the pH_i to ischemia. (*iii*) In the second descent, an increase in H^+ activity cannot have been the event leading to maintenance of nearly normal ATP levels at the expense of phosphocreatine. Repeated noninvasive measurement of the pH_i—a unique capability of *in vivo* ^{31}P NMR—was essential for each of these conclusions. The last of them is in accord with the view of Norberg and Siesjö (12), based on indirect estimates of intracellular pH, that acidosis was not the major factor regulating the creatine kinase reaction in early hypoxia.

Next, the behavior of the phosphodiester peak in hypoglycemia: in the data from that experiment shown in Fig. 1, the progressive fall in the peak at 0.5 to 0.6 ppm (between P_i and phosphocreatine) may reflect generation of energy from unusual sources by the glucose-starved brain. This peak fell sharply in another deep hypoglycemia experiment from which there was no metabolic recovery; we have seen no consistent change in it during other equally prolonged metabolic stresses. In perchloric acid extracts of brain, the largest resonances in this region are from the choline and ethanolamine esters of glycerol 3-phosphate (13). Evidence exists for consumption of phospholipids, amino acids, and ketone bodies by the brain during hypoglycemia (14). If peaks in the phosphodiester region are indeed sensitive to emergency mobilization of alternative fuel sources by the brain, their behavior can be exploited for *in vivo* study of metabolic adaptation processes that are not well understood.

Finally, NMR observations suggest rather strongly that cerebral ADP levels *in vivo* are much lower than the values obtained from killed brain by the most rapid fixation procedures available. ADP does not contribute to the ^{31}P spectrum directly, but its concentration can be estimated from detectable quantities if certain plausible assumptions are made. These are the following. (*i*) The creatine kinase reaction was at equilibrium. For the control periods of our experiments, there can be little doubt that this was true, given the large safety factor the reaction appears to have (15). (The maximum demands of the metabolic stresses we imposed may have disturbed the equilibrium, but that possibility is beyond the scope of this discussion.) (*ii*) The sum of the concentrations of phosphocreatine and creatine is stable in living tissue and can be assigned a value. Several groups of workers have documented a total near 10 mM for the two substances under a variety of conditions (11, 16, 17). (*iii*) Concentrations of phosphocreatine and ATP *in vivo* can be estimated. Evidence from many sources places these at about 5 and 3 mM, respectively (18).

With these assumptions, the equilibrium expression for the creatine kinase reaction,

$$K = [ADP][PCr][H^+]/[ATP][creatine],$$

where PCr = phosphocreatine, can be rearranged to

$$[ADP] = K[ATP](0.01 - [PCr])/[PCr][H^+].$$

Since K is known to be about 3×10^{-10} M (19), a numerical estimate of ADP concentration can be obtained. For the control periods of our hypoglycemia, hypoxia, and seizure experiments, the values were 0.016, 0.025, and 0.01 mM, respectively. Determination of ADP levels in funnel-frozen cerebral tissue by enzymatic methods typically yields values in the range of 0.2–0.3 mM (11, 18); values obtained by other methods are higher still. Estimates of ADP concentration similar to ours have been made for frog muscle (20) and rat brain (21) from ^{31}P NMR measurements and for rat brain by conventional biochemical measurements (22). We suspect that agonal hydrolysis of ATP during tissue preparation cannot be avoided. If resting ADP levels in the brain are indeed in the 0.02 mM range, concepts of how reactions involving ADP behave *in vivo* may need revising.

Table 1. Relative changes in ADP concentration during metabolic stress

	Hypoglycemia	Hypoxia/ischemia	Seizure
Stress/control	1.6	1.3	1.7
Recovery/control	0.63	0.84	—

With the further, perhaps adventurous, assumption that the creatine kinase reaction remained at equilibrium during the periods of metabolic stress in our experiments, it is possible to calculate relative changes in ADP levels that are independent of the actual concentrations assigned to ATP and phosphocreatine in assumption *iii* above. These are presented in Table 1. All three types of stress appeared to increase ADP levels by 30–70%. We are now in a position to investigate the effects of these concentration changes on metabolic activity, such as can be observed during respiratory and allosteric control, in the living brain.

This work was supported by Grant GM 30287 from the U.S. Public Health Service and a grant from the Esther A. and Joseph Klingenstein Foundation.

1. Chance, B., Nakase, Y., Bond, M., Leigh, J. S. & McDonald, G. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4925–4929.
2. Ackerman, J. J. H., Grove, T. H., Wong, G. G., Gadian, D. G. & Radda, G. K. (1980) *Nature (London)* **283**, 167–170.
3. Fossel, E. T. & Ingwall, J. S. (1980) in *Cerebrovascular Diseases: Twelfth Princeton Research Conference*, eds. Moossy, J. & Reinmuth, O. M. (Raven, New York), pp. 91–98.
4. Thulborn, K. R., du Boulay, G. H., Duchen, L. W. & Radda, G. K. (1982) *J. Cereb. Blood Flow Metab.* **2**, 299–306.
5. Shoubridge, E. A., Briggs, R. W. & Radda, G. K. (1982) *FEBS Lett.* **140**, 288–292.
6. Delpy, D. T., Gordon, R. E., Hope, P. L., Parker, D., Reynolds, E. O. R., Shaw, D. & Whitehead, M. D. (1982) *Pediatrics* **70**, 310–313.
7. Budinger, T. F. (1981) *J. Comput. Assist. Tomogr.* **5**, 800–811.
8. Kiessling, M., Hossmann, K.-A. & Kleihues, P. (1981) *Exp. Neurol.* **74**, 430–438.
9. Chapman, A. G., Meldrum, B. S. & Siesjö, B. K. (1977) *J. Neurochem.* **28**, 1025–1035.
10. Ghajar, J. B. G., Plum, F. & Duffy, T. E. (1982) *J. Neurochem.* **38**, 397–409.
11. Pelligrino, D. & Siesjö, B. K. (1981) *J. Cereb. Blood Flow Metab.* **1**, 85–96.
12. Norberg, K. & Siesjö, B. K. (1975) *Brain Res.* **86**, 31–44.
13. Pettegrew, J. W., Minshaw, N. J., Glonek, T., Kopp, S. J. & Cohen, M. M. (1982) *Neurology* **32**, A196.
14. Siesjö, B. K. (1978) *Brain Energy Metabolism* (Wiley, New York), pp. 125–130; 392–396.
15. Shoubridge, E. A., Briggs, R. W. & Radda, G. K. (1982) *FEBS Lett.* **140**, 288–292.
16. Hinzen, D. H & Mueller, U. (1971) *Pflueger's Arch.* **322**, 47–59.
17. Tarr, M., Brada, D. & Samson, F. E. (1962) *Am. J. Physiol.* **203**, 690–692.
18. Siesjö, B. K. (1978) *Brain Energy Metabolism* (Wiley, New York), pp. 229–230.
19. Watts, D. C. (1973) in *The Enzymes*, ed. Boyer, P. D. (Academic, New York), Vol. 8, pp. 428–431.
20. Gadian, D. G., Radda, G. K., Brown, T. R., Chance, E. M., Dawson, M. J. & Wilkie, D. R. (1981) *Biochem. J.* **194**, 215–228.
21. Ackerman, J. J. H., Grove, T. H., Wong, G. G., Gadian, D. G. & Radda, G. K. *Nature (London)* **283**, 167–170.
22. Veech, R. L., Lawson, J. W. R., Cornell, N. W. & Krebs, H. A. (1979) *J. Biol. Chem.* **254**, 6538–6547.