Delayed ("Secondary") Cerebral Energy Failure after Acute Hypoxia-Ischemia in the Newborn Piglet: Continuous 48-Hour Studies by Phosphorus Magnetic Resonance Spectroscopy

ANN LOREK, Y. TAKEI, E. B. CADY, J. S. WYATT, JULIET PENRICE, A. D. EDWARDS, D. PEEBLES, MARZENA WYLEZINSKA, H. OWEN-REECE, V. KIRKBRIDE, C. E. COOPER, R. F. ALDRIDGE, S. C. ROTH, G. BROWN, D. T. DELPY, AND E. O. R. REYNOLDS

Departments of Paediatrics [A.L., Y.T., J.S.W., J.P., A.D.E., D.P., H.O.-R. V.K., C.E.C., S.C.R., E.O.R.], Medical Physics and Bioengineering [E.B.C., M.W., R.F.A., D.T.D.], and Biochemistry and Molecular Biology [G.B.], University College London, London, United Kingdom

ABSTRACT

Phosphorus (^{31}P) spectra from the brains of severely birth-asphyxiated human infants are commonly normal on the first day of life. Later, cerebral energy failure develops, which carries a serious prognosis. The main purpose of this study was to test the hypothesis that this delayed ("secondary") energy failure could be reproduced in the newborn piglet after a severe acute reversed cerebral hypoxic-ischemic insult. Twelve piglets were subjected to temporary occlusion of the common carotid arteries and hypoxemia [mean arterial P_{O_2} 3.1 (SD 0.6) kPa]. Mean cerebral phosphocreatine concentration [PCr]/inorganic orthophosphate concentration [Pi] decreased from 1.40 (SD 0.29) to 0.01 (SD 0.02), and nucleotide triphosphate concentration [NTP]/ exchangeable phosphate pool concentration [EPP] decreased from 0.19 (SD 0.02) to 0.06 (SD 0.04) ($p < 0.001$ for each decrease). On reperfusion and reoxygenation of the brain, mean [PCr]/[Pi] and [NTP]/[EPP] returned to baseline. Observations continuing for the next 48 h showed that [PCr]/[Pi] again decreased, in spite of normal arterial P_{O_2}, mean arterial blood pressure, and blood glucose, to 0.62 (SD 0.61) at 24 h ($p < 0.01$) and 0.49 (SD 0.37) at 48 h ($p < 0.001$). [NTP]/[EPP] also decreased, but to a lesser degree. Intracellular pH remained unchanged. These findings appeared identical with those seen in birth-asphyxiated human infants. No changes in cerebral metabolite concentrations took place in six control piglets. The severity of secondary energy failure, as judged by the lowest [PCr]/[Pi] recorded at 24-48 h, was directly related to the extent of acute energy depletion, obtained as the time integral of reduction in [NTP]/[EPP] ($p < 0.0001$). This animal model of secondary energy failure may prove useful for testing cerebroprotective strategies. (*Pediatr Res* **36**: 699–706, 1994)

Abbreviations

[EPP], exchangeable phosphate pool concentration
FID, free induction decay
FiO$_2$, inspired oxygen fraction
MABP, mean arterial blood pressure
MRS, magnetic resonance spectroscopy
NTP, nucleotide triphosphate
[NTP], nucleotide triphosphate concentration
^{31}P, phosphorus
Paco$_2$, arterial partial pressure of carbon dioxide
Pao$_2$, arterial partial pressure of oxygen
PCr, phosphocreatine
[PCr], phosphocreatine concentration
PEt, phosphoethanolamine
pH$_a$, arterial pH
pH$_i$, intracellular pH
Pi, inorganic orthophosphate
[Pi], inorganic orthophosphate concentration
PME, phosphomonoester
[Ptot], total detectable phosphorus concentration
T$_R$, repetition time

^{31}P magnetic resonance spectra from the brains of babies with evidence of critically impaired intrapartum

gas exchange ("birth asphyxia") were commonly normal on the first day of life (1, 2). Subsequently, impairment of cerebral energy metabolism developed in some of the babies, as shown by decreasing [PCr]/[Pi] and eventually, in very severely affected ones, by a reduction in [NTP] [which is composed largely of ATP (3, 4)]. These changes in cerebral energetics took place without a decrease in pH$_i$ and in the absence of any evidence of

Table 2. *Physiologic variables in the experimental group (n = 12)**

	Time (h)				
	Baseline	0	2	24	48
Pao$_2$ (kPa)	11.4 (3.6)	3.1† (0.6)‡	11.6 (3.5)	13.7 (3.4)	13.4 (2.7)
Paco$_2$ (kPa)	5.8 (1.4)	6.6 (1.4)§	6.3 (1.9)	5.7 (1.5)	5.3 (1.4)
BE$_a$ (mmol · L^{-1})	3.8 (2.9)	−5.7† (5.1)‡	1.0‖ (2.6)	2.2 (2.7)	1.0 (3.0)
pH$_a$	7.45 (0.06)	7.26† (0.12)‡	7.36 (0.10)	7.40 (0.03)	7.42 (0.08)
Blood glucose (mmol · L^{-1})	8.3 (1.0)	9.8 (2.0)	8.4 (0.3)	7.6 (1.0)	8.1 (1.9)
MABP (kPa)	7.72 (0.82)	5.41† (0.78)‡	7.36 (1.18)	7.71 (1.30)	7.92 (1.41)
Heart rate (min^{-1})	172 (20)	223‖ (23)‡	205 (34)	185 (26)	174 (26)
Rectal temperature (°C)	38.5 (0.5)	38.4 (0.4)	38.7 (0.4)	38.6 (0.5)	38.6 (0.4)

* Times are from the start of resuscitation (at 0 h) after the acute insult. Values are mean (SD). BE$_a$, arterial base excess. At time 0, n = 11 for Pao$_2$, Paco$_2$, BE$_a$, pH$_a$, and blood glucose because of temporary catheter block in one animal. At 24 h n = 12 and at 48 h n = 10 because two animals died at 40 and 42 h. Analysis of variance on both groups of animals (excluding insult time 0) showed that changes in Pao$_2$ (p < 0.05), BE$_a$ (p < 0.01), and HR (p < 0.01) were all related to time. Heart rate (p < 0.05) was also related to hypoxia-ischemia.

† p < 0.001 for difference from baseline (paired t test).

‡ p < 0.001 for difference from controls at equivalent time (unpaired t test). Baseline and time 0 were compared with baseline time 0 in controls.

§ p < 0.01 for difference from controls at equivalent time (unpaired t test).

‖ p < 0.01 for difference from baseline (paired t test).

ischemic insult; heart rate and Paco$_2$ increased. These variables subsequently returned to normal.

^{31}P MRS. A typical baseline ^{31}P spectrum is illustrated in Figure 1. Eleven peaks could be identified from their characteristic resonance frequencies (15). The peaks were assigned, from left to right, to two PME (PEt and phosphocholine); a bifid peak due to Pi; two phosphodiesters (phosphatidylethanolamine and phosphatidylcholine); PCr; the γ, α, and β peaks of NTP; and, between the α and β peaks, NAD plus NADH and uridine diphosphate sugars.

Figure 2 shows the changes in the spectra that took place in an animal subjected to an acute cerebral hypoxic-ischemic insult lasting 43 min that caused severe intracellular acidosis and depletion of high-energy metabolites, which was followed by recovery and later by depletion of [PCr]/[Pi] and [NTP]/[EPP] without intracellular acidosis.

Mean values for metabolite ratios and pH$_i$ in the control and experimental groups are given in Tables 3 and 4, respectively. No changes took place over 48 h in the
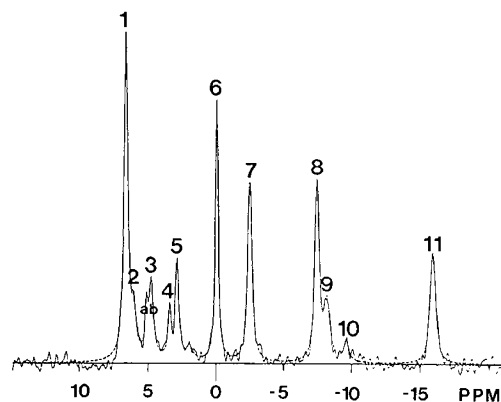
Figure 1. ^{31}P spectrum from a control animal. Resonances are assigned as follows: *1*, PEt; *2*, phosphocholine; *3a* and *b*, Pi components; *4*, phosphatidylethanolamine; *5*, phosphatidylcholine; *6*, PCr; *7*, γ-NTP; *8*, α-NTP; *9*, NAD and NADH; *10*, uridine diphosphate sugars; and *11*, β-NTP.

control group. In the experimental group, [PCr]/[Pi] decreased during the insult from 1.40 (SD 0.29) to 0.01 (SD 0.02); this was due to a reduction in [PCr] together with a reciprocal and then larger increase in [Pi]. The Pi peak broadened and appeared to include several components indicating a range of compartments with different pH$_i$. Eventually, the Pi peak narrowed at a chemical shift indicating a pH$_i$ of 5.84 (SD 0.30) compared with a baseline value of 7.06 (SD 0.05). Many other resonances (*e.g.* PEt, phosphocholine, and γ- and β-NTP) also showed acid chemical shifts (data not given), and the broadening of the γ-NTP peak was also attributable to very low pH$_i$, as previously found *in vitro* (14).

When [PCr]/[Pi] and [PCr]/[Ptot] had started to decrease, [NTP]/[EPP] and [NTP]/[Ptot] began to decline but at a slower rate; immediately before resuscitation [NTP]/[EPP] had fallen from 0.19 (SD 0.02) to 0.06 (SD 0.04). During hypoxia-ischemia, the PME region of the spectrum became difficult to analyze in terms of individual constituents. PME concentration/[Ptot] increased, possibly in part due to the generation of sugar phosphate resonances in the PME region. Two h after resuscitation began, all the abnormal values had on average reverted to or close to baseline, although [NTP]/[EPP] was slightly lower than in controls. Recovery of [PCr]/[Pi] and [NTP]/[EPP] was incomplete in two animals, although both indices returned to within 3 SD of mean baseline values within 5 h. Table 4 also shows that by 24 h after the insult mean [PCr]/[Pi] had again fallen below baseline and control values and decreased further to 0.49 (0.37) by 48 h. [NTP]/[EPP] was also decreasing by 24 h. No change took place in pH$_i$. Data for [PCr]/[Pi] for the control and experimental groups are summarized in Figure 3. Inspection of data from individual piglets showed that progressive deterioration in [NTP]/[EPP] took place in eight animals, whereas in three animals recovery was under way by 32–42 h. There was no change in [NTP]/[EPP] in one animal, although [PCr]/[Pi] declined slightly.
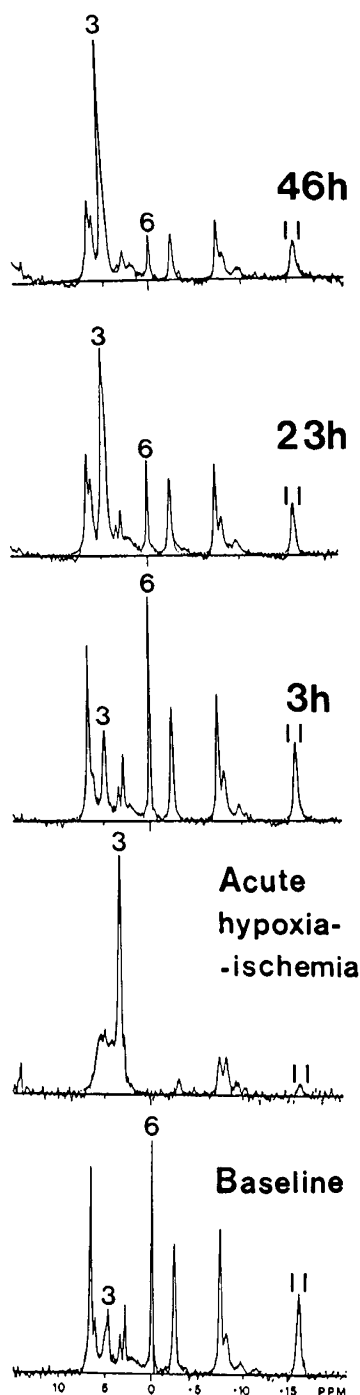
Figure 2. Series of spectra from an animal subjected to 43 min of acute cerebral hypoxia-ischemia. Pi (*peak 3*), PCr (*peak 6*), and β-NTP (*peak 11*) are assigned as in Figure 1. The spectrum during the hypoxic-ischemic insult was obtained immediately before resuscitation (time 0). Subsequent spectra have been timed from the start of resuscitation. During hypoxia-ischemia, [PCr]/[Pi] and [NTP]/[EPP] decreased from 1.20 to 0.00 and 0.18 to 0.04, respectively, and pH_i decreased from 7.10 to 6.34. [PCr]/[Pi] and [NTP]/[EPP] recovered to 1.17 and 0.18 at 3 h before decreasing again over the next 2 d to 0.12 and 0.12 at 46 h. pH_i at 3 h was 7.11, at 23 h 6.94, and at 46 h 7.19.

Figure 4 shows that a positive linear relationship was found between the severity of the acute cerebral hypoxic-ischemic insult, as determined by the time integral of depletion of [NTP]/[EPP], and the minimum value for

Table 3. *Cerebral ^{31}P metabolite ratios and pH_i in the control group (n = 6)**

	Time (h)			
	0	2	24	48
[PCr]/[Pi]	1.45 (0.31)	1.27 (0.19)	1.50 (0.24)	1.49 (0.40)
[PCr]/[Ptot]	0.13 (0.01)	0.12 (0.01)	0.14 (0.01)	0.15 (0.01)
[Pi]/[Ptot]	0.09 (0.01)	0.10 (0.01)	0.10 (0.01)	0.10 (0.02)
[NTP]/[EPP]	0.21 (0.01)	0.20 (0.00)	0.20 (0.01)	0.19 (0.01)
[NTP]/[Ptot]	0.12 (0.01)	0.12 (0.00)	0.12 (0.01)	0.12 (0.01)
[PME]/[Ptot]	0.21 (0.01)	0.21 (0.01)	0.20 (0.01)	0.19 (0.02)
[PDE]/[Ptot]	0.10 (0.02)	0.11 (0.02)	0.10 (0.01)	0.11 (0.02)
[NAD+NADH]/[Ptot]	0.08 (0.01)	0.07 (0.01)	0.07 (0.01)	0.06 (0.01)
[UDP]/[Ptot]	0.02 (0.01)	0.02 (0.01)	0.03 (0.01)	0.02 (0.01)
pH_i	7.04 (0.03)	7.04 (0.02)	7.08 (0.03)	7.07 (0.02)

* Times are from the first observations made (at 0 h) and 2, 24, and 48 h afterward. Values are mean (SD). [PDE], phosphodiester concentration; [UDP], uridine diphosphate sugar concentration.

[PCr]/[Pi] at 24–48 h ($r = 0.91, p < 0.0001$). A similar relation was found between the acute decrement of [NTP]/[Ptot] and minimum [PCr]/[Pi] at 24–48 h ($r = 0.87, p < 0.0003$).

Neuropathology. Histologic examination of the brains of the experimental group of animals (Squier MV, Gould S, personal communication) showed damage involving the cerebral and cerebellar cortex, basal ganglia, cerebral white matter, and brain stem in descending order of severity. Two separate patterns of neuronal changes were seen. Neuronal cytoplasmic eosinophilia with dissolution of the nuclear membrane and loss of nuclear staining were found in some of these areas, and in others intense nuclear basophilia with shrinkage or fragmentation of nuclear material into densely staining round granules, suggestive of apoptotic cell death. In the more severely damaged brains, there was edema, microglial reaction, and astrocytic proliferation in the white matter. A full account of the histopathologic findings will be published separately.

DISCUSSION

The major purpose of this study was to test the hypothesis that the sequence of cerebral metabolic changes that follows severe birth asphyxia in human infants, and that we have described as secondary energy failure (1, 2, 5, 6), could be reproduced in the newborn piglet. This animal was chosen because the brain is comparable to, although in some ways slightly more mature than, the brain of a human infant (16), because it has been well characterized by previous investigators (9, 17), and because it is large enough to allow simultaneous studies by MRS and other techniques. The animal is similar in size to a preterm infant, so methods for monitoring physiologic variables and maintaining it in good condition for more than 48 h were readily available. The anesthetic agents were chosen to provide satisfactory anesthesia without causing effects that would interfere with the interpretation of the results. Isoflurane, which was used as the main anesthetic, reduces cerebral metabolic rate (18), and nitrous oxide, which was also used, are known to increase cere-

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Table 4. *Cerebral ^{31}P metabolite ratios and pH$_i$ in the experimental group (n = 12)**

	Time (h)				
	Baseline	0	2	24	48
[PCr]/[Pi]	1.40 (0.29)	0.01† (0.02)	1.32 (0.46)	0.62‡ (0.61)‖	0.49† (0.37)§
[PCr]/[Ptot]	0.13 (0.01)	0.00† (0.01)§	0.14 (0.02)	0.09 (0.05)‖	0.09‡ (0.04)§
[Pi]/[Ptot]	0.10 (0.02)	0.42† (0.08)§	0.12 (0.04)	0.31‡ (0.24)‖	0.25† (0.10)§
[NTP]/[EPP]	0.19 (0.02)	0.06† (0.04)§	0.18 (0.00)§	0.13‡ (0.07)‖	0.15† (0.03)‖
[NTP]/[Ptot]	0.11 (0.01)	0.03† (0.02)§	0.10 (0.01)‖	0.08 (0.05)‖	0.10† (0.02)‖
[PME]/[Ptot]	0.21 (0.02)	0.32† (0.06)§	0.21 (0.02)	0.16 (0.05)	0.19 (0.03)
[PDE]/[Ptot]	0.11 (0.02)	0.03† (0.02)§	0.11 (0.02)	0.10 (0.05)	0.11 (0.06)
[NAD+NADH]/[Ptot]	0.07 (0.02)	0.07 (0.01)	0.07 (0.01)	0.05‡ (0.01)‖	0.05‡ (0.02)‖
[UDP]/[Ptot]	0.03 (0.01)	0.02 (0.02)	0.02 (0.01)	0.02 (0.01)	0.02 (0.01)
pH$_i$	7.06 (0.05)	5.84† (0.30)§	7.02 (0.10)	6.89 (0.36)	7.09 (0.08)

* Times are taken from the start of resuscitation after the acute insult. At 24 h n = 11 because of temporary spectrometer failure, and at 48 h n = 10 because two animals died with severe brain injury. Values are mean (SD). [PDE], phosphodiester concentration; [UDP] uridine diphosphate sugar concentration. Analysis of variance comparing the experimental and control groups (omitting values at insult time 0) showed differences in [PCr]/[Pi] ($p < 0.001$), [PCr]/[Ptot] ($p < 0.05$), [Pi]/[Ptot] ($p < 0.001$), [NTP]/[EPP] ($p < 0.001$), [NTP]/[Ptot] ($p < 0.01$), [PME]/[Ptot] (time dependent $p < 0.05$), and [NAD]/[Ptot] ($p < 0.001$).

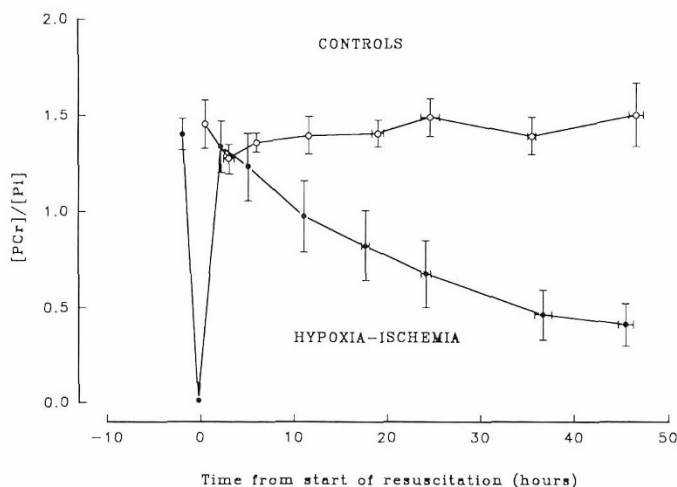 † $p < 0.001$ for differences from baseline (paired t test).

  ‡ $p < 0.01$ for differences from baseline (paired t test).

  § $p < 0.001$ for difference from controls at equivalent time (unpaired t test).

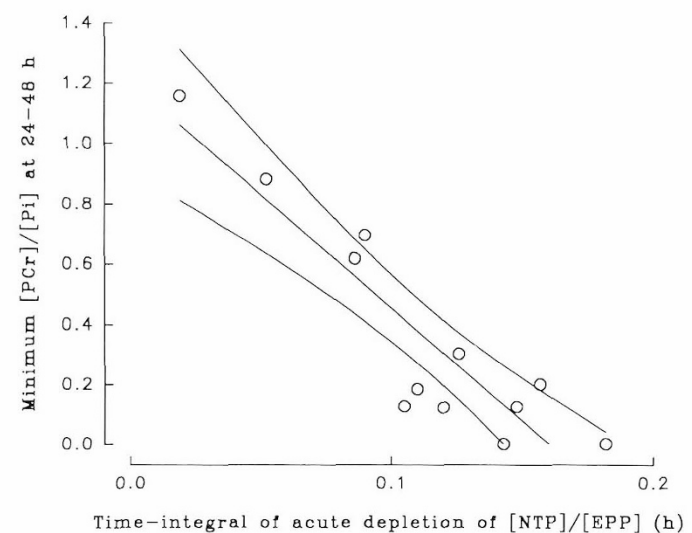 ‖ $p < 0.01$ for difference from controls at equivalent time (unpaired t test).

bral blood flow (17). The data given in Table 1 demonstrate that a range of physiologic variables in both the experimental and control groups of animals remained at satisfactory levels throughout the studies.

MRS was carried out using surface coil localization, and because of the high field strength it was possible to obtain spectra of good quality. Corrections for saturation were not required (because of the long T_R), so accurate estimates of the relative concentrations of the ^{31}P metabolites could be obtained irrespective of whether spin-lattice relaxation rates altered. The volume of brain interrogated, approximately 4 mL, included the occipito-parietal regions, and the spectra appeared uncontaminated from extracerebral tissue, inasmuch as [PCr]/[Pi] decreased to zero in the animals that were worst affected by secondary energy failure, although tissues other than the brain remained well oxygenated and perfused.

The control spectrum illustrated in Figure 1 shows the expected prominent peaks due to PCr (peak 6) and NTP (peaks 7, 8, and 11). The β-NTP peak (peak 11) is mainly due to ATP. Other peaks are due to mobile components of cells, including PEt (peak 1) and phosphodiester (peaks 4 and 5). Two peaks (labeled 3a and b) were found to resonate around the Pi frequency at about 5 ppm. The left-hand peak is thought to be due to extracellular and possibly mitochondrial Pi, and the right-hand one to cytosolic Pi (19–21). An averaging method was used to derive the reported pH$_i$. The data given in Table 3 show that the ^{31}P metabolite concentration ratios in the control piglets remained close to baseline values for the 48 h during which observations continued.

Figure 3. [PCr]/[Pi] in the control group (n = 6) and the experimental group of piglets whose brains were subjected to acute hypoxia-ischemia (n = 12). Values are means and SEM.

Figure 4. Relation between the extent of acute cerebral energy depletion, indicated by the time integral of depletion of [NTP]/[EPP], and the severity of subsequent secondary energy failure as shown by the minimum recorded values for [PCr]/[Pi] at 24–48 h. The regression line (r = 0.91, $p < 0.0001$) and its 95% confidence limits are shown.

The cerebral circulation of the piglet is supplied by an intact circle of Willis (22) and includes vertebral as well as carotid components, so occluding the common carotid arteries leads to variably but substantially reduced cerebral blood flow. The reduction in FiO_2 that caused Pao_2 to decrease to 3.1 ± 0.6 kPa (Table 2) then critically curtailed the oxygen supply to the brain. This situation is likely to have been similar to that during severe birth asphyxia in human infants.

The changes in ^{31}P metabolites and pH_i during the acute cerebral insult (Fig. 2, Table 4) resembled those reported in previous studies, including those of newborn animals (8, 9, 23–25). The profound decrease in pH_i was attributable in part to production of lactic acid. The objective of the acute insult, to reduce [NTP] as well as [PCr]/[Pi], was achieved, thus indicating severe cerebral energy depletion. On reoxygenating and reperfusing the brain, recovery of ^{31}P metabolites and pH_i was rapid, starting within 15 min and on average returning to baseline by 2 h. This recovery was more complete than that in newborn rats subjected to permanent unilateral carotid occlusion and hypoxemia (25), which provide a more severe ongoing cerebral insult than that in the present experiments. Subsequently and in spite of normal values for arterial blood gases, blood glucose, and MABP, [PCr]/[Pi] gradually declined, followed by [NTP]/[EPP], in all but one experimental animal. These changes appeared identical with those previously described as secondary energy failure in birth-asphyxiated human infants (2, 5, 6). Also as in the infants, pH_i did not decrease, and in some animals progressive deterioration in cerebral energetics was seen, whereas in others apparent recovery was beginning by 48 h. Prominent among mechanisms that may be implicated in the progression from primary to secondary energy failure are those involving excitotoxins such as glutamate, which initiates cellular damage mediated by nitric oxide and calcium; damage due to free radicals; immunocytotoxic reactions; impairment of protein synthesis; and lack of essential growth factors (24, 26–28). Ongoing ischemia is an unlikely explanation because cerebral blood flow appears increased in newborn infants with secondary energy failure (29), although some local microvascular failure may contribute. pH_i is expected to decrease when mitochondrial energetics are compromised, *e.g.* by lack of oxygen during the primary insult, which decreases the metabolism of pyruvate by the mitochondrial respiratory chain. There are several possible explanations for the absence of a similar decrease during secondary energy failure. The time course was very slow. Lactic acid produced by damaged cells may have been washed out of the brain. Also, it is likely that damage was very heterogeneous. Mitochondrial function may have been preserved in some cells, whereas in others local ischemia could have caused lack of substrate (and thus no decrease in pH_i). Furthermore, dead cells would not produce lactate, and membrane failure would lead to the equalization of pH_i and extracellular pH.

During the acute cerebral hypoxic-ischemic insult, reduction of [NTP], quantified as [NTP]/[EPP], was taken as the best index of energy depletion. These values were related to the subsequent decrease in [PCr]/[Pi], which has been found to be the most sensitive index of secondary energy failure in human infants and to be directly related to prognosis (2, 5). Figure 4 shows that the greater the depletion of [NTP]/[EPP] during the acute insult, the lower the [PCr]/[Pi] 24–48 h later. A similar relation was demonstrated between [NTP]/[Ptot] [which has previously been used as an index of energy depletion (1, 2)] and later minimum [PCr]/[Pi]. Thus, the worse the episode of acute energy depletion, the greater the extent of the subsequent secondary energy failure. These data strongly suggest that the reason for the variation in the severity of secondary energy failure observed in human infants has a similar basis; specifically, the ones who display the most severe changes have been subjected to the most severe cerebral energy depletion before or around the time of birth.

The results of neuropathologic examination of the brain indicated necrosis, apoptosis, and secondary damage associated with cerebral edema. These changes were closely similar in nature and regional distribution to those seen in birth-asphyxiated infants; they will be described in detail elsewhere. Because the severity of secondary energy failure in the human infant is directly related to the chances of death or disability (2, 5), it is important that means are found to prevent it. The best way would be to prevent, by appropriate obstetric surveillance and intervention, the episode of primary energy failure that sets in train the biochemical reactions that lead later to the secondary phase. If this cannot be achieved, then it is possible that means may be found to interrupt the reactions. Methods for blocking them are becoming available, and evidence is also accumulating that hypothermia may have a useful role (27, 28, 30).

Most studies of cerebroprotection have involved very small animals, such as the newborn rat, which are particularly useful for screening for suitable pharmacologic agents (23, 28). Before cerebroprotective strategies are applied to human infants who have suffered a severe acute hypoxic-ischemic insult to the brain, it will be important to prove their worth in a suitable animal model that is large enough for a wide range of systemic as well as cerebral observations to be made. The newborn piglet provides such a model, and we suggest that the prevention of secondary energy failure represented by minimum [PCr]/[Pi] within 48 h could be used as an initial end point.

We conclude that, in the newborn piglet, *1*) a severe acute episode of cerebral hypoxia-ischemia that is then reversed leads, after a delay of many hours, to the phenomenon that has previously been described as secondary energy failure in the birth-asphyxiated human infant, and *2*) the severity of secondary energy failure is directly related to that of the primary insult. This animal model may be suitable for exploring further the mechanisms

involved in the progression from primary to secondary energy failure and for testing strategies for preventing this progression.

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