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Sequential Changes in Red Cell Glycolytic Enzymes and Intermediates and Possible Control Mechanisms in the First Two Months of Postnatal Life in Lambs

S. F. TRAVIS, L. C. WAGERLE, C. M. DE ALVARADO, G. ROSE, AND M. DELIVORIA-PAPADOPOULOS

Department of Pediatrics and Cardeza Foundation for Hematologic Research, Jefferson Medical College, Thomas Jefferson University and Departments of Pediatrics and Physiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

ABSTRACT. The sequential changes in several glycolytic enzymes, glucose-6-phosphate dehydrogenase, glycolytic intermediates, and adenosine triphosphate, as well as intracellular pH and plasma inorganic phosphorus were followed simultaneously in eight lambs from birth to 2 months of age. The activities of all glycolytic enzymes and glucose-6-phosphate dehydrogenase were elevated at birth. The 2,3-diphosphoglycerate concentration increased markedly postnatally and was associated with a simultaneous increase in the concentrations of red cell glucose-6-phosphate and total triose phosphate and a decrease in intracellular pH. Inorganic phosphorus also increased and correlated with the 2,3-diphosphoglycerate concentration in the first 10 days of postnatal life. The content of red cell 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate, and ATP increased slightly. These results suggested increased glycolytic flux through the diphosphoglycerate mutase reaction which resulted in net synthesis of 2,3-diphosphoglycerate. The red cell total triose phosphate peaked and fell initially, followed by glucose-6-phosphate and 2,3-diphosphoglycerate suggesting inhibition of phosphofructokinase activity and a decrease in glycolysis secondary to decreased red cell intracellular pH. After 10 days of postnatal life all glycolytic intermediates fell simultaneously, which correlated with a decrease in activity of the glycolytic enzymes. (*Pediatr Res* 19: 272–277, 1985)

Abbreviations

2,3-DP, 2,3-diphosphoglycerate
G-6-P, glucose-6-phosphate
F-6-P, fructose-6-phosphate
TTP, total triose phosphate
3-PG, 3-phosphoglycerate
2-PG, 2-phosphoglycerate
PEP, phosphoenolpyruvate
FDP, fructose diphosphate
DHAP, dihydroxyacetone phosphate
G-3-P, glyceraldehyde-3-phosphate
Pi, inorganic phosphorus
ALD, aldolase
HK, hexokinase
PK, pyruvate kinase
PFK, phosphofructokinase
DPGM, diphosphoglyceratemutase
PGK, phosphoglyceratekinase
ENO, enolase
G-6-PD, glucose-6-phosphate dehydrogenase
RBC, red blood cells
pHvc, intracellular pH

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Send reprint requests to Dr. Susan F. Travis, Jefferson Medical College, Thomas Jefferson University, Department of Pediatrics, 1025 Walnut Street, Philadelphia, PA 19107.
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At birth, lambs are similar to human newborns since they possess a fetal Hb that has a high oxygen affinity (1, 3–6). Unlike the human, however, the lamb is capable of compensating for the presence of a high oxygen affinity Hb in the extrauterine environment by dramatically increasing the 2,3-DPG concentration (1, 3–6, 13). Similar to humans, 2,3-DPG has a low affinity for fetal deoxyhemoglobin in sheep, so that the marked increase in 2,3-DPG does not result in improved oxygen delivery by binding to deoxy-fetal Hb, but results in a decrease in intracellular pH which decreases Hb oxygen affinity via the Bohr effect (3). Unlike humans, both fetal and adult deoxyhemoglobins in sheep have a low affinity for 2,3-DPG but adult sheep Hb has an intrinsically low oxygen affinity which facilitates unloading of oxygen. It has been demonstrated that the dramatic rise in 2,3-DPG that occurs perinatally in lambs can be prevented by performing an exchange transfusion on the lamb with adult blood in the first few hours after birth (1). Thus, the rapid rise in 2,3-DPG in lambs in the first 2 days of life serves as a mechanism of lowering Hb oxygen affinity via the Bohr effect until an adequate amount of adult Hb is present. After the first 10 days of life, the 2,3-DPG concentration begins to decrease and usually falls to the minimal levels characteristic of adult sheep by 2 months of age (1, 3–5). The fall in 2,3-DPG postnatally correlates inversely with the rise in adult sheep Hb (1).

Our laboratory has investigated the sequential changes in several red cell glycolytic enzymes, G-6-PD, glycolytic intermediates and ATP from birth until approximately 2 months of life (56–58 days), as well as intracellular pH and plasma P_i in eight lambs simultaneously in order to determine the normal developmental pattern and to define the factors that influence the dramatic rise in 2,3-DPG that occurs in lambs during the first few days of postnatal life.

MATERIALS AND METHODS

Cross-bred sheep were used. The lambs were born and housed at the University of Pennsylvania. Most births were nocturnal and unattended. The lambs were kept in small pens with their dams. Feeding consisted of dam's milk and some grain. Adult sheep were fed grain and hay. Approximately 6 ml blood were obtained from each of eight lambs by jugular puncture on days 1, 3, 8, 10, 14–15, 21, 22, 29–30, 35–36, 42–43, 49–50, 56–58 of life. Two milliliters of whole blood were immediately pipetted into 4 ml of chilled 2N perchloric acid, reextracted, and neutralized by methods previously described (18). The concentrations of 2,3-DPG, ATP, G-6-P, F-6-P, TTP, 3-PG, 2-PG, and PEP were determined by methods previously described (18). TTP represents the simultaneous measurement of FDP, DHAP, and G-3-P as described by Keitt (8).

Aliquots for determination of intracellular and extracellular pH were removed when an adequate sample was obtained, and rapidly processed (2). The remaining blood was centrifuged, the plasma was removed, recentrifuged, and frozen for later determination of Pi (7). The packed cells were washed, filtered, and assayed for red cell glycolytic enzymes and G-6-PD, as previously described (19). Enzyme units were expressed as μmol of substrate converted/gHb/min at 37° C. Data were analyzed statistically by linear regression (correlation coefficient).

RESULTS

Glycolytic enzymes and G-6-PD (Table 1). All glycolytic enzymes studied except ALD were elevated at birth and declined progressively throughout the first 2 months of life. The age-dependent enzymes, HK, ALD, and PK transiently increased at 21–22 days of age and began to decrease again at 35–36 days of life. The mean decrease in HK activity from birth to 2 months of age was only 4% and HK activity was still 54% higher at 56–58 days of age than in adult sheep whereas mean PK activity decreased 24% from birth to 2 months of life and was only 13% higher than adult sheep at 56–58 days. The most marked decrease in enzyme activity in the first 2 months of life was seen in PFK (64%), DPGM (76%), PGK (45%), ENO (69%), and G-6-PD (46%). Although mean PFK activity was similar to adult sheep by 56–58 days and mean PGK and ENO activities were only 14–15% higher than in adult sheep, DPGM and G-6-PD activities were still 57 and 34% higher, respectively, at 56–58 days of life, than in adult sheep.

Glycolytic intermediates and ATP. There were marked increases in the concentrations of G-6-P, F-6-P, TTP, and 2,3-DPG with much less impressive increases in the concentrations of 3-PG, 2-PG, PEP, and ATP in the 1st wk of life when compared to adult sheep. The variation in the concentrations of these intermediates was so marked during this time period that the mean and SD for each day of sampling was not informative. For example, on day 1 the 2,3-DPG concentration varied from 475 to 6379.3 nmol/ml RBC; G-6-P varied from 50.5 to 624 nmol/ml RBC, and TTP varied from 16.9 to 403 nmol/ml RBC. Thus, the data were presented individually for each lamb as the percentage of normal adult sheep values (Fig. 1–3). The adult sheep values used as controls are in Table 2. Sequential changes in the concentrations of G-6-P, TTP, and 2,3-DPG were the most marked and are presented for each lamb. Changes in the concentration of F-6-P are not included since they followed the same pattern as the G-6-P. Since the changes in 3-PG, 2-PG, PEP, and ATP were of a lesser magnitude and tended to parallel each other, only 3-PG is presented. The numbers in parentheses represent intracellular pH. Three patterns were observed (Figs. 1–3).

A. In lambs 1–4, the concentrations of G-6-P, TTP, and 2,3-DPG increased simultaneously on day 1, peaked between days 3–8, and tended to decrease simultaneously also, except lamb 3, whose red cell TTP concentration decreased significantly between days 3 and 8, whereas 2,3-DPG continued to increase and the G-6-P concentration was stable (Fig. 1).

B. In lambs 5 and 6, the TTP concentration had already peaked on day 1 and fell significantly between days 1 and 3 as the concentrations of G-6-P and 2,3-DPG continued to increase (Fig. 2).

C. In lambs 7 and 8, the concentrations of G-6-P and TTP had already peaked on day 1 and both decreased in concentration between days 1 and 3. The concentration of 2,3-DPG was also

Table 1. *Red cell enzyme activity* (U/g Hb) in neonatal lambs and adult sheep*

	Neonatal lambs (n = 8) (age in days)											Adult sheep (n = 13)
	1	3	8	10	14–15	21–22	29–30	35–36	42–43	49–50	56–58	
HK	0.712 ±0.256	0.748 ±0.243	0.701 ±0.122	0.776 ±0.123	0.835 ±0.167	0.927 ±0.306	0.901 ±0.306	0.864 ±0.240	0.844 ±0.212	0.713 ±0.257	0.682 ±0.158	0.313 ±0.102
PFK	5.22 ±1.19	5.01 ±1.24	4.61 ±0.91	4.12 ±0.84	3.89 ±1.18	3.38 ±1.08	3.02 ±1.16	2.49 ±0.91	2.12 ±0.85	2.06 ±0.82	1.90 ±0.43	1.96 ±0.21
ALD	0.674 ±0.109	0.612 ±0.111	0.688 ±0.167	0.890 ±0.277	0.999 ±0.171	1.24 ±0.217	1.34 ±0.29	1.30 ±0.28	1.14 ±0.33	1.07 ±0.22	0.806 ±0.104	0.692 ±0.144
PGK	103.2 ±10.5	104.7 ±8.16	102.1 ±4.72	97.0 ±10.1	92.9 ±12.8	89.0 ±13.9	85.2 ±17.2	72.8 ±18.2	71.0 ±18.9	61.9 ±15.7	57.1 ±7.78	48.6 ±8.57
DPGM	1.96 ±0.63	1.84 ±0.49	1.72 ±0.46	1.59 ±0.31	1.41 ±0.40	1.24 ±0.34	0.874 ±0.263	0.800 ±0.289	0.705 ±0.172	0.613 ±0.145	0.481 ±0.138	0.205 ±0.092
ENO	28.9 ±8.02	27.3 ±6.88	22.9 ±6.89	21.3 ±6.45	20.0 ±6.34	17.1 ±6.48	16.9 ±3.96	14.1 ±6.82	12.3 ±6.07	10.8 ±4.89	9.00 ±2.99	7.78 ±2.95
PK	37.3 ±13.5	37.9 ±10.8	35.8 ±10.5	32.7 ±7.63	34.7 ±9.55	35.2 ±9.48	34.1 ±9.09	33.0 ±7.21	31.8 ±5.65	28.4 ±5.46	28.2 ±4.22	24.6 ±3.08
G6PD	4.48 ±1.31	4.49 ±0.93	4.17 ±0.70	4.07 ±0.61	4.07 ±0.49	4.37 ±0.85	3.98 ±0.88	3.87 ±0.86	3.25 ±0.59	2.95 ±0.56	2.42 ±0.28	1.59 ±0.31

* Mean ± SD.

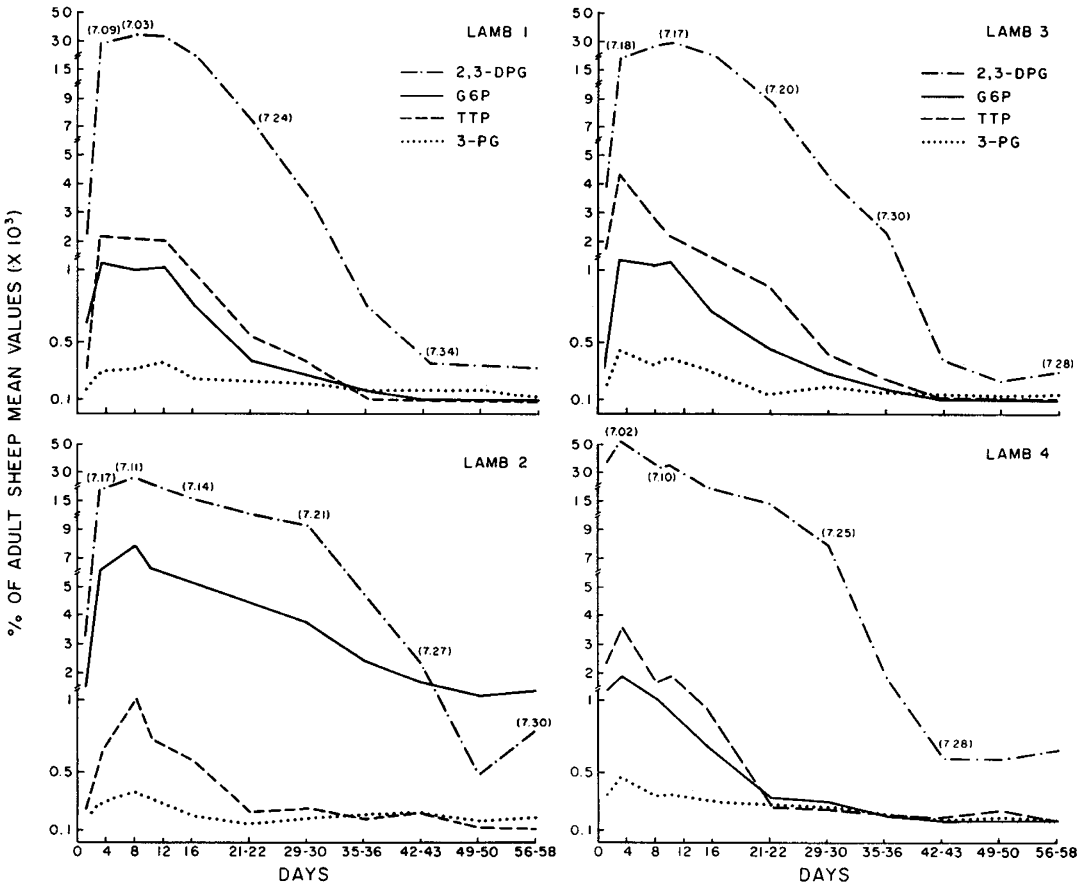

Fig. 1. Red cell glycolytic intermediates in postnatal lambs—pattern A.

markedly increased on day 1 and either remained relatively stable (lamb 8) or continued to increase further (lamb 7) between days 1 and 3 (Fig. 3).

After approximately 2 wk of postnatal life all red cell glycolytic intermediates tend to decrease simultaneously toward the low levels observed in adult sheep.

Red cell pHvc and plasma Pi (Table 3). The ΔpH was significantly increased from days 3–10 which coincided with peak 2,3-DPG levels and decreased pHvc. Plasma Pi was slightly higher than in adult sheep on days 1 and 3, progressively increased and peaked on day 10 and remained elevated the entire 2 months of postnatal life.

Possible control mechanisms. The concentration of red cell 2,3-DPG correlated inversely with pHvc from day 1 to 56–58 of life ($r = -0.82$; $p < 0.001$, Fig. 4). During the first 10 postnatal days, when 2,3-DPG was most elevated there was a positive correlation between Pi and 2,3-DPG ($r = 0.66$; $p < 0.001$; Fig. 5). After 10–14 days of life when all intermediates fell simultaneously, the decrease in 2,3-DPG from days 10 through 56–58 of postnatal life correlated positively with the decrease in DPGM activity ($r = 0.81$; $p < 0.001$, Fig. 6).

DISCUSSION

Red cell enzyme activity, the concentration of red cell glycolytic intermediates, red cell intracellular pH, and plasma Pi have been measured simultaneously in lambs from birth to 2 months of postnatal life in order to document the sequential changes in glycolytic enzymes and intermediates toward normal adult sheep values, and to more clearly define the possible control mechanisms responsible for the marked postnatal rise and fall in the red cell 2,3-DPG concentration. Prior studies have not evaluated glycolytic enzymes and intermediates simultaneously, so that direct comparisons between enzyme activity and levels of glycolytic intermediates could not be made.

At birth the activities of HK, PFK, PGK, DPGM, ENO, PK, and G-6-PD were elevated when compared to adult sheep, and decreased in activity throughout the first 2 months of life. Red cell PFK, DPGM, PGK, ENO, and G-6-PD demonstrated the most marked decrease in activity from birth to 2 months of age (46–76%). Red cell PFK, PGK, ENO, and PK activities approached adult values by 2 months of age but DPGM activity remained 57% higher than in adult sheep, despite a 75% decrease in enzyme activity between days 1 and 58 of life. These results are similar to those reported by Noble *et al.* (12) in sheep during the first 17 days of postnatal life, with the exception of PK activity which was similar in newborn and adult sheep in their study.

Thus, newborn lambs have markedly elevated levels of enzymes involved in the synthesis of 2,3-DPG and other glycolytic intermediates. In the first 3 days of life, the concentration of red cell 2,3-DPG increases markedly and peaks at levels 250–460 times greater than that observed in adult sheep (adult mean 21.2 nmol/ml RBC; peak in lambs ranged from 4821–9800 nmol/ml RBC), which is similar to results obtained by others (1, 3–5, 13). This rapid rise in 2,3-DPG was associated with a significant increase in ΔpH secondary to a decrease in intracellular pH from days 3 to 10 which coincided with peak 2,3-DPG levels, similar to prior studies (3). From 10 days to 2 months of life, as the levels of 2,3-DPG decreased significantly toward the low levels observed in adults, there was a corresponding decrease in DPGM activity. There was also a positive, but less impressive, correlation between the decrease in PFK activity and the TTP concentration from 10 to 58 days of postnatal life ($r = 0.45$; $p < 0.001$). The decrease in the 2,3-DPG concentration after 10 days of life was associated with an increase in intracellular pH. Thus, there was

a significant inverse correlation ($p < 0.001$) between intracellular pH and the red cell 2,3-DPG content throughout the first 2 months of postnatal life.

Plasma Pi was also evaluated sequentially and appeared to influence the concentration of red cell 2,3-DPG since there was a significant positive correlation between plasma Pi and red cell 2,3-DPG during the first 10 postnatal days when the 2,3-DPG concentration was most elevated. These results are consistent with the known effects of Pi on red cell metabolism and have been described previously (9–11, 14, 15, 16, 20).

The marked increase in red cell 2,3-DPG content in early postnatal life was also associated with a marked increase in G-6-P, F-6-P and TTP (simultaneous measurement of FDP, DHAP, and G-3-P), whereas the concentrations of 3-PG, 2-PG, PEP, and ATP increased slightly. This pattern of glycolytic intermediates was suggestive of an increased glycolytic rate with preferential flow through the DPGM step, rather than the PGK reaction, resulting in net synthesis of 2,3-DPG. The increase in the 2,3-DPG concentration was associated with a decrease in red cell pHvc and it is proposed that this decrease in pH then led to inhibition of PFK activity and a resultant decrease in the concentration of TTP and a decrease in the glycolytic rate. A crossover at the PFK step was demonstrated by the simultaneous decrease in the red cell TTP concentration and increase in the G-6-P (and F-6-P) concentrations in lambs 5 and 6, and in lamb

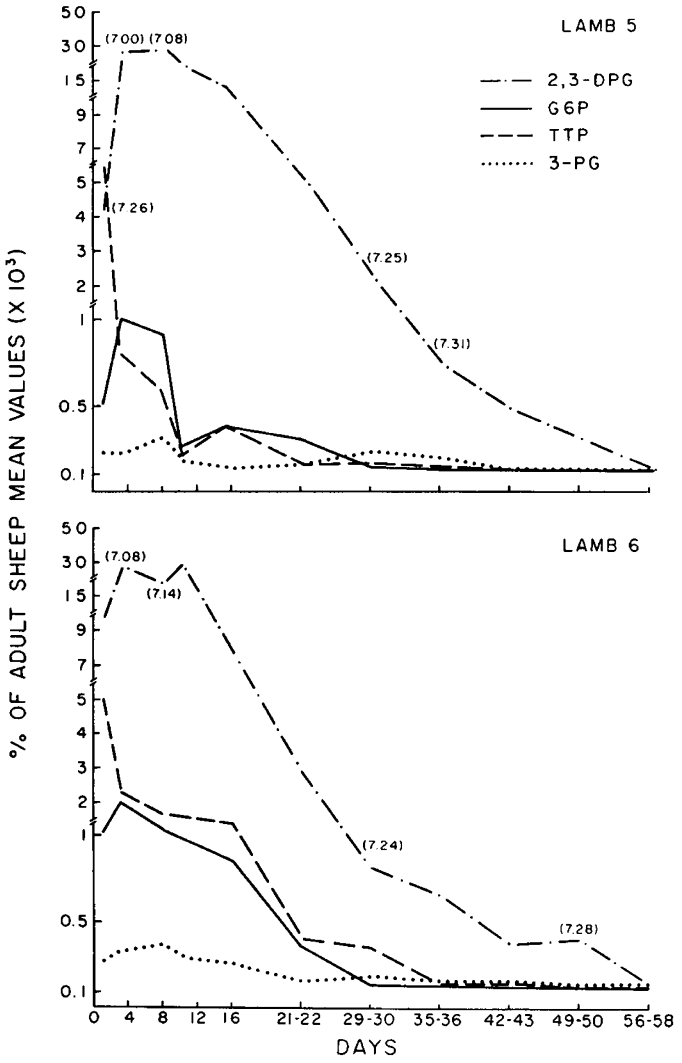

Fig. 2. Red cell glycolytic intermediates in postnatal lambs—pattern B.

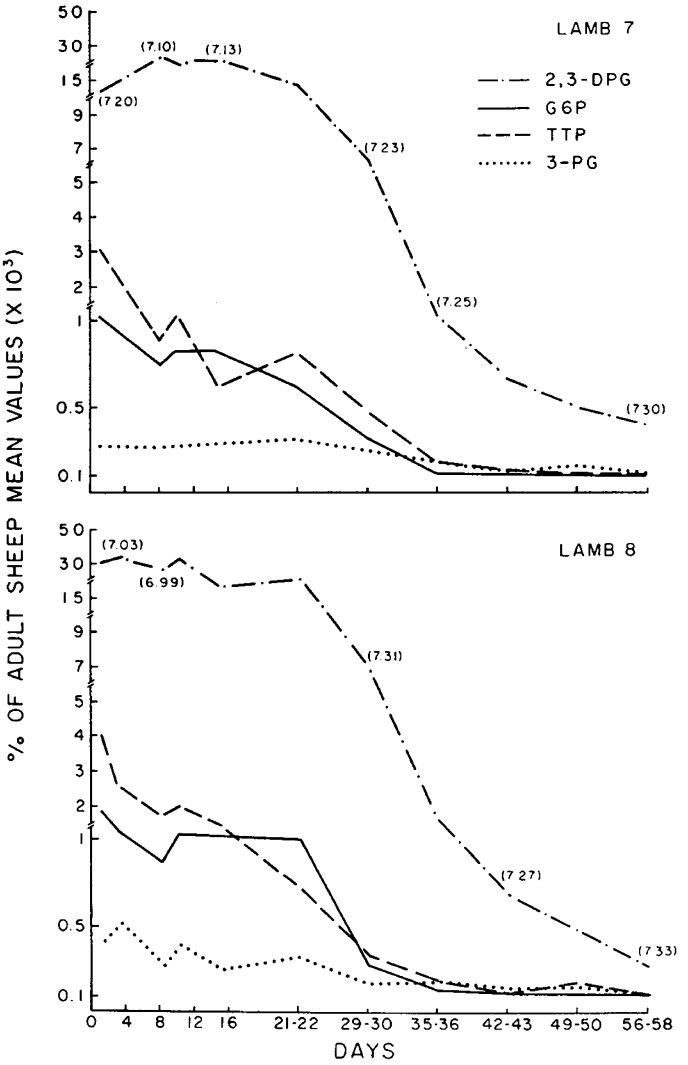

Fig. 3. Red cell glycolytic intermediates in postnatal lambs—pattern C.

Table 2. *Red cell glycolytic intermediates* and ATP (nmol/ml RBC) in adult sheep (n = 13)*

G-6-P	34.2 ± 8.47
F-6-P	9.50 ± 2.62
TTP	7.21 ± 2.19
2,3-DPG	21.2 ± 16.2
3-PG	20.4 ± 5.16
2-PG	4.44 ± 2.70
PEP	9.16 ± 4.60
ATP	532 ± 126

* Mean ± SD.

3, the TTP concentration also decreased prior to the fall in the G-6-P concentration. It is likely that the three different patterns of glycolytic intermediates that were observed (A, B, and C) represented differences in the time the samples were drawn in relation to the birth of the lambs, and to the stimulus to glycolysis, since the birth of the lambs was nocturnal and unattended in most instances. For example, in pattern C the greatest time interval had probably elapsed between the stimulus to glycolysis and day 1 blood sampling, since the concentrations of G-6-P, TTP, and 2,3-DPG had already peaked and were decreasing. In pattern B, only the TTP was falling which was consistent with inhibition of PFK activity secondary to decreased intracellular

Table 3. *Serum P_i, whole blood pH(pHv), pHvc, and Δ pH in neonatal lambs and adult sheep*

Age in days	P_i(mg/dl)*	pHv*	pHvc*	ΔpH*
Lambs				
1	7.55 ± 1.14	7.419 ± 0.080	7.235 ± 0.049	0.184 ± 0.040
3	7.55 ± 3.16	7.420 ± 0.061	7.088 ± 0.069	0.332 ± 0.070
8	8.99 ± 1.29	7.388 ± 0.083	7.088 ± 0.058	0.300 ± 0.040
10	9.51 ± 0.50	7.404 ± 0.079	7.126 ± 0.043	0.278 ± 0.060
15	9.00 ± 1.31	7.419 ± 0.090	7.172 ± 0.076	0.247 ± 0.045
21–22	9.41 ± 1.04	7.409 ± 0.068	7.210 ± 0.045	0.199 ± 0.036
29–30	9.53 ± 0.92	7.431 ± 0.043	7.246 ± 0.030	0.185 ± 0.036
35–36	9.96 ± 1.15	7.409 ± 0.061	7.251 ± 0.068	0.158 ± 0.023
42–43	8.51 ± 3.28	7.437 ± 0.061	7.266 ± 0.059	0.171 ± 0.020
49–50	8.74 ± 1.18	7.415 ± 0.048	7.263 ± 0.049	0.152 ± 0.013
56–58	8.81 ± 1.70	7.426 ± 0.075	7.260 ± 0.070	0.166 ± 0.020
Adult sheep	7.31 ± 1.30	7.415 ± 0.038	7.259 ± 0.029	0.156 ± 0.026

* Mean ± SD.

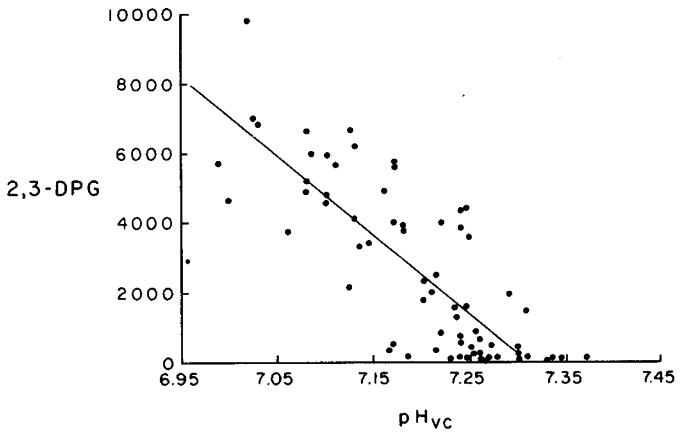

Fig. 4. Relationship between red cell 2,3-DPG concentration (nmol/ml RBC) and red cell intracellular pH(pHvc) from day 1 to 56–58 of postnatal life ($n = 74$; $r = -0.82$; $p < 0.001$).

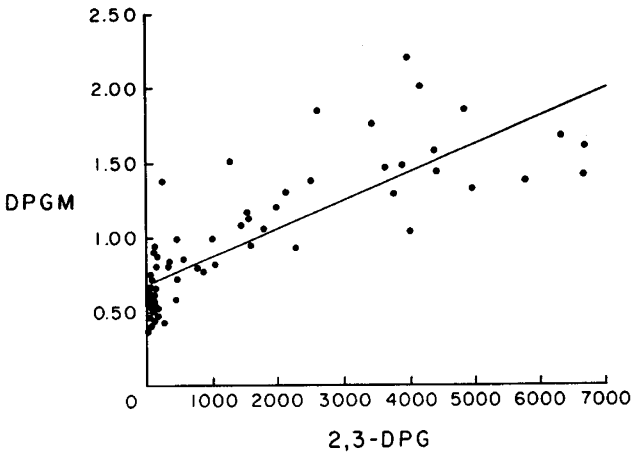

Fig. 6. Relationship between red cell 2,3-DPG concentration (nmol/ml RBC) and DPGM activity (U/g Hb) from days 10 to 56–58 of postnatal life. ($n = 64$; $r = 0.81$; $p < 0.001$).

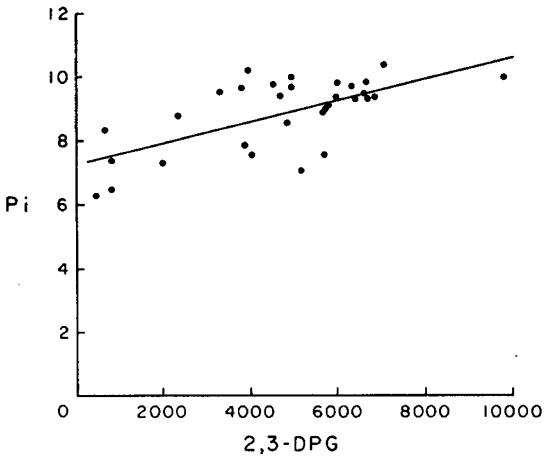

Fig. 5. Relationship between red cell 2,3-DPG concentration (nmol/ml RBC) and plasma inorganic phosphorus (Pi in mg/dl) in the first 10 days of postnatal life. ($n = 31$; $r = 0.66$; $p < 0.001$).

pH and the stimulus to glycolysis presumably occurred closer to the time of blood sampling than in C. Pattern A probably represented the most recent events with a simultaneous increase in all three intermediates.

Pattern A was suggestive that 2,3-DPG inhibition of red cell hexokinase activity, as previously described by Srivastava and Beutler (17), may also have contributed to the pattern of glycolytic intermediates observed, especially in lambs 1, 2, and 4 in whom the concentrations of G-6-P, TTP, and 2,3-DPG increased and fell simultaneously. However, an inverse correlation between the red cell 2,3-DPG concentration and hexokinase activity could not be demonstrated.

In recent studies, Noble *et al.* (13) postulated activation of PFK as the stimulus to glycolysis in newborn lambs. Our results differ from those of Noble *et al.* (13) who found the rise of TTP always preceded the increase in 2,3-DPG and they also postulated a partial block at the glyceraldehyde-3-phosphate dehydrogenase step which was relieved as the concentration of serum Pi increased. The simultaneous increase in G-6-P, TTP, and 2,3-DPG in our studies is more suggestive of activation of PFK and preferential flow through the DPGM step, as the mechanism responsible for the pattern of glycolytic intermediates observed. The differences in the results may be secondary to species variation in the sheep studied.

The stimulus to glycolysis at birth has been proposed to be a rise in venous pH shortly after birth, which leads to activation of PFK, and an increase in plasma glucose which supplies increased substrate for the fetal red cell (13). It is proposed on the basis of our studies that this increased glycolytic rate results in net synthesis of 2,3-DPG due to preferential flow through the DPGM step. The dramatic increase in the concentration of red cell 2,3-DPG is self-limited since it results in a decrease in intracellular pH which appears to inhibit PFK activity resulting in a decrease in the TTP concentration and glycolytic rate, which was followed by a decrease in all glycolytic intermediates. Inhi-

bition of HK activity by 2,3-DPG may also have contributed to the decrease in the concentration of G-6-P and other intermediates distal to this step. The magnitude of the increase in the 2,3-DPG concentration was probably further enhanced by the increasing plasma Pi concentration. After the first 10–14 days of life all glycolytic intermediates fell simultaneously. At this time other control mechanisms became operative since glycolytic enzyme activities were also decreasing, as demonstrated by the significant correlation between the decreasing activity of red cell DPGM and corresponding decrease in the red cell 2,3-DPG concentration.

These studies have demonstrated that red cell PFK plays a key regulatory role in postnatal life in lambs. Thus, the fetal lamb can serve as a model for the study PFK activation and inhibition *in vivo* and the regulation of 2,3-DPG synthesis.

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Epidermal Growth Factor Binding to Neonatal Mouse Skin Explants and Membrane Preparations—Effect of Triiodothyronine

S. B. HOATH,[1] J. LAKSHMANAN, AND D. A. FISHER

Department of Pediatrics, Harbor-UCLA Medical Center, UCLA School of Medicine, Torrance, California 90509

ABSTRACT. Daily treatment of newborn Swiss-Webster mice with triiodothyronine (T3, 500 ng/day) increased epidermal growth factor (EGF) content in whole skin (epidermis + dermis). Separation of the epidermis using 0.01 M dithiothreitol followed by processing for radioimmunoassay measurement reveals levels of EGF 2-to 3-fold higher in epidermis than in whole skin. *In vitro* flotation of circular skin sections from control and T3 treated neonatal mice in medium containing [I^{125}]EGF showed increased uptake of label following 5 days of *in vivo* T3 treatment. Mouse skin membrane preparations exhibit saturable, specific binding of [I^{125}]EGF. T3 treatment for 5 days *in vivo* significantly increased EGF binding capacity in skin membrane preparations but did not alter EGF receptor affinity (Kd 4.5 nM). Protein, RNA, and DNA concentrations were significantly increased in whole neonatal mouse skin following T3 administration. These results suggest one mechanism by which thyroid hormones increase skin EGF concentration is augmentation of skin EGF receptor binding. (*Pediatr Res* 19: 277–281, 1985)

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Reprint requests Dr. Steven B. Hoath, Department of Pediatrics, University of Cincinnati Medical Center, Mail Location 541, Room 6263, 231 Bethesda Avenue Cincinnati, OH 45267.

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[1] Current address: Department of Pediatrics, ML 541, University of Cincinnati College of Medicine, Cincinnati, OH 45267.