Optimized separation of purine bases and nucleosides in human cord plasma by capillary zone electrophoresis

Tilman Grune*

Institute of Biochemistry, Medical Faculty (Charité), Humboldt University, Hessische Strasse 3–4, O-1040 Berlin (Germany)

Gordon A. Ross

Department of Medicine, St. Bartholomew's Hospital Medical College, London EC1A 7BE (UK)

Heike Schmidt and Werner Siems

Institute of Biochemistry, Medical Faculty (Charité), Humboldt University, Hessische Strasse 3–4, O-1040 Berlin (Germany)

David Perrett

Department of Medicine, St. Bartholomew's Hospital Medical College, London EC1AS 7BE (UK)

ABSTRACT

An optimized separation of the main purine compounds of human serum by capillary zone electrophoresis is presented. Separations were performed in an uncoated silica capillary (44 cm × 75 μm I.D., 37 cm to window) on a SpectraPhoresis 1000 system with UV detection. The separation of adenine (Ade), adenosine (Ado), guanine (Gua), guanosine (Guo), hypoxanthine (Hyp), inosine (Ino), xanthine (Xan) and uric acid (UA) was optimized with respect to pH, temperature, applied potential and hydrodynamic injection time. Optimum conditions were 20 mM borate buffer (pH 9.4), 37°C, 20 kV and 9 s load and detection at 260 nm. Linearity extended from 1 to 125 μM. The sensitivity of the method was 0.5 μM, which is adequate for measuring Ade, Gua, Hyp and UA in plasma samples. Plasma samples from newborns were precipitated with an equal volume of perchloric acid (7%, v/v), the supernatant was adjusted to neutral pH with potassium carbonate and, before injection, the sample was alkalized with sodium hydroxide. The method presented here allows the determination of Ade, Guo, Hyp and UA. The levels of the determined purines were compared in samples from control newborns, preterm babies and newborns with asphyxia or acidic serum pH values.

INTRODUCTION

The history of the separation of purine nucleosides and bases includes various separation principles, starting with the first column chromatography in 1949 [1]. After this first application of ion-exchange chromatography, thin-layer chromatography [2], gas chromatography [3] and also electrophoretic techniques [4] were used. With the devel-

opment of HPLC instrumentation, a number of different separation techniques, such as ion-exchange [5], reversed-phase [6,7] and ion-pair reversed-phase [8,9] chromatography, have been applied and HPLC is currently the favoured technique.

Today capillary zone electrophoresis is one of the most efficient separation techniques. It has been rapidly developed since Jorgenson and Lucacs [10] used small-diameter capillaires for separation of ions in an electrical field. One of the greatest advantages of capillary electrophoresis is the use of

---

* Corresponding author.

small sample amounts, from a few nanolitoes of sample to the cytosolic fluid of single cells [11]. These small sample volumes make it possible to solve a number of biological and clinical problems where the amount of biological material is limited. One such problem is the analysis of serum samples from newborns and preterm babies. A common clinical problem is the evaluation of the anoxic period during the birth period and of the oxygen supply to organs of newborns and preterm babies during the first week of life.

The aim of this study was to optimize capillary zone electrophoresis for the separation of common naturally occurring purine nucleosides and bases and to use the developed technique for the analysis of plasma from newborn babies.

EXPERIMENTAL

Materials

Purine reference standards for HPLC were purchased from Sigma (Poole, UK). Sodium borate was obtained from Fisher Scientific (Fairlawn, NJ, USA).

Instrumentation

For all electrophoretic separations a SpectraPhoresis 1000 instrument (Spectra-Physics, San Jose, CA, USA) was used. Analyses were performed in an uncoated silica capillary of 44 cm × 75 μm I.D. with the window located at a distance of 37 cm. UV detection over the range 200–300 nm was performed. Hydrodynamic injection was used.

Separation conditions

For separation, a 20 mM borate buffer was employed. The separation was optimized with respect to pH, temperature, applied potential and injection time. The final optimized conditions, used for serum analysis, were pH 9.4, 37°C, 20 kV and a 9 s load.

Patient groups

Twenty-seven neonates, hospitalized at the Department of Pediatrics and the Department of Gynecology and Obsterics of the Medical Faculty (Charité) of Humboldt University, Berlin, were studied. Clinical data for the patients are shown in Table I. The newborns were arranged in one of the following groups.

Group A, serving as control, consisted of eight healthy full-term neonates delivered after normal pregnancy and without signs of perinatal asphyxia.

Group B included five preterm newborns delivered after complicated pregnancy but without clinical and biochemical indicators of asphyxia. The complications in pregnancy were foetal retardation 1, hypertension 1 and cervical insufficiency and tocolysis 5.

Group C consisted of seven full-term neonates with foetal acidosis but without clinical signs of asphyxia. These newborn infants were delivered after normal pregnancy.

Group D consisted of four full-term and three preterm newborn infants with perinatal asphyxia. Four of these seven infants were delivered after normal pregnancy. The pregnancies of the other three

TABLE I

CLINICAL DATA OF THE NEWBORNS STUDIED (MEANS ± S.D.)

Group A, full-term newborns without asphyxia; Group B, preterm infants without asphyxia; Group C, full-term neonates with acidosis; Group D, full-term and preterm newborns with asphyxia. The Apgar score was determined according to ref. 12.

Parameter	Group			
	A	B	C	D
Gestational age (weeks)	39.2 ± 1.0	35.8 ± 0.4	39.6 ± 1.6	38.0 ± 2.1
Birth mass (g)	3429 ± 367	2730 ± 444	3534 ± 315	3126 ± 450
Umbilical artery pH	7.29 ± 0.05	7.32 ± 0.04	7.13 ± 0.06	7.16 ± 0.10
Apgar score:				
1 min	8.9 ± 1.0	8.6 ± 1.1	7.0 ± 2.2	4.8 ± 1.3
5 min	9.4 ± 0.5	9.0 ± 1.0	8.2 ± 1.4	6.6 ± 0.5
10 min	9.9 ± 0.4	9.2 ± 0.8	8.4 ± 1.1	7.3 ± 0.5

infants were complicated by servical insufficiency and tocolysis.

Sample pretreatment

A 20-µl volume of serum was extracted with an equal amount of perchloric acid (7%, v/v). The extracts were centrifuged for 10 min at 1200 g. The supernatant was neutralized with 1.3 M potassium carbonate (ca. 5 µl), cooled for 1 h and centrifuged. The final supernatant was stored at −20°C until analysis. Immediately before injection the sample was alkalized with 0.1 M sodium hydroxide solution.

RESULTS AND DISCUSSION

The purine bases and nucleosides employed were selected on the basis of their known occurrence in plasma. The purines adenine (Ade), adenosine (Ado), guanine (Gua), guanosine (Guo), hypoxanthine (Hyp), inosine (Ino), xanthine (Xan) and uric acid (UA) were used.

Influence of pH

The influence of pH on the separation characteristics was investigated in the pH range 7.5–11.5. With increasing pH the migration time of purine nucleosides and bases increased with slight differences in the migration sequence (Fig. 1A). The number of theoretical plates (N) achieved a plateau in the pH range 9–9.5 (Fig. 1B). The number of theoretical plates for Ade, Gua, Hyp, Guo, Xan and Ino was in the ragne 170 000–210 000 and for Ado and Ino was ca. 125 000. The optimum analysis, with regard to short migration times and good efficiency of separation, of the selected purine compounds was achieved at pH 9.4.

Temperature effects

Temperature effects on the separation quality at pH 9.4 were investigated in the range 25–50°C. High temperatures caused faster migration times (Fig. 2A), but the migration sequence of purine compounds was not influenced by temperature. There were drastic changes in the separation efficiency with dependence on temperature (Fig. 2B). Whereas the separation efficiencies for Guo, Hyp, Xan, Ino and uric acid were maximum at 35°C, for Ade, Ado and Gua the were minimum at this temperature. The optimum temperature for the analysis of the complex purine mixture was 37°C, giving low migration times and a good separation quality.

Voltage dependence

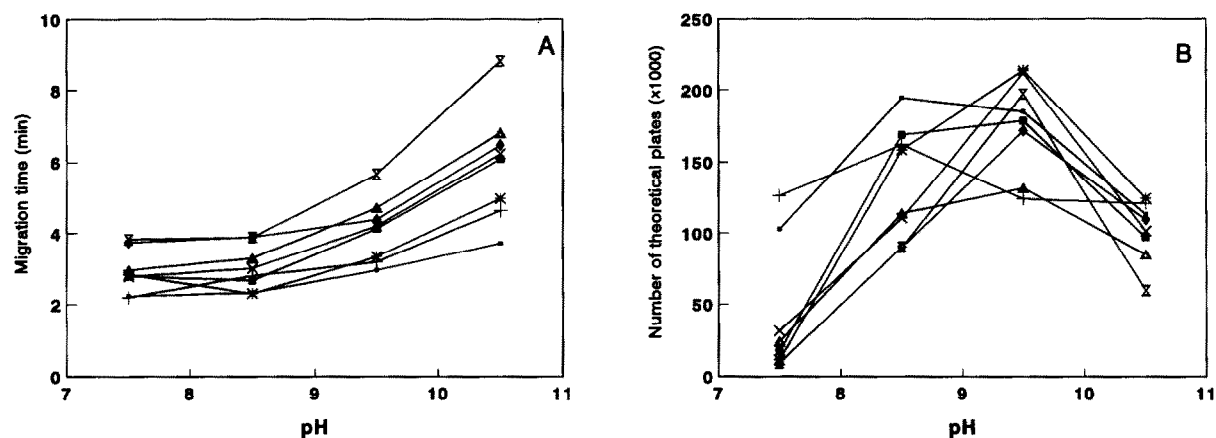The dependence of voltage on separation quality is shown in Fig. 3. The drastic decrease in migration

Fig. 1. Influence of pH on (A) migration times and (B) separation efficiency of purine compounds in 20 mM borate buffer at 25°C and 20 kV (capillary: uncoated silica, 75 µm I.D. × 44 cm, 37 cm to window). ■ = Ade; + = Ado; ✳ = Gua; × = Guo; □ = Hyp; △ = Ino; ◇ = Xan; X = uric acid.
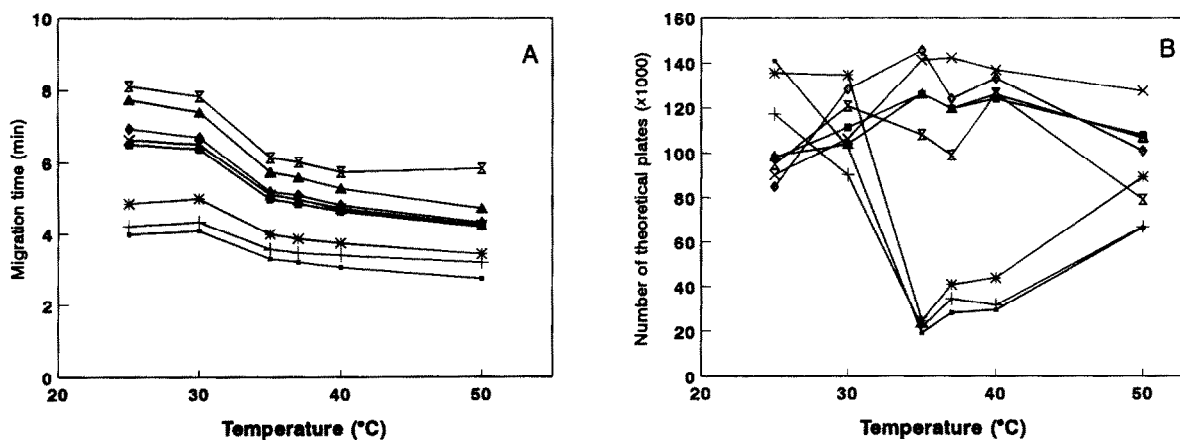
Fig. 2. Effect of temperature on the separation of purine compounds in 20 mM borate buffer (pH 9.4) with 20 kV (capillary: uncoated silica, 75 μm I.D. × 44 cm, 37 cm of window). (A) Migration time; (B) theoretical plates. Symbols as in Fig. 1.

times with increase in voltage was in agreement with the increasing number of theoretical plates. Hence it seems that high voltages lead to optimization of separation. Further increase in voltage is limited by the increasing current levels and the associated Joule heating effects.

Calibration and reproducibility

The linearity of the calibration graph for standard solutions with concentrations between 1.25

and 125 μM and the influence of the hydrophobic injection time between 1 and 9 s was investigated and correlation coefficients from 0.96 to 0.99 were achieved, which allow the determination of the purines studied. The optimum injection time for the low-concentration solution of purine nucleosides and bases with respect to sensitivity is 9 s, which leads to a detection limit of 0.5 μM. (Since this work was performed, updated instrument software has increased the maximum possible hydrodynamic
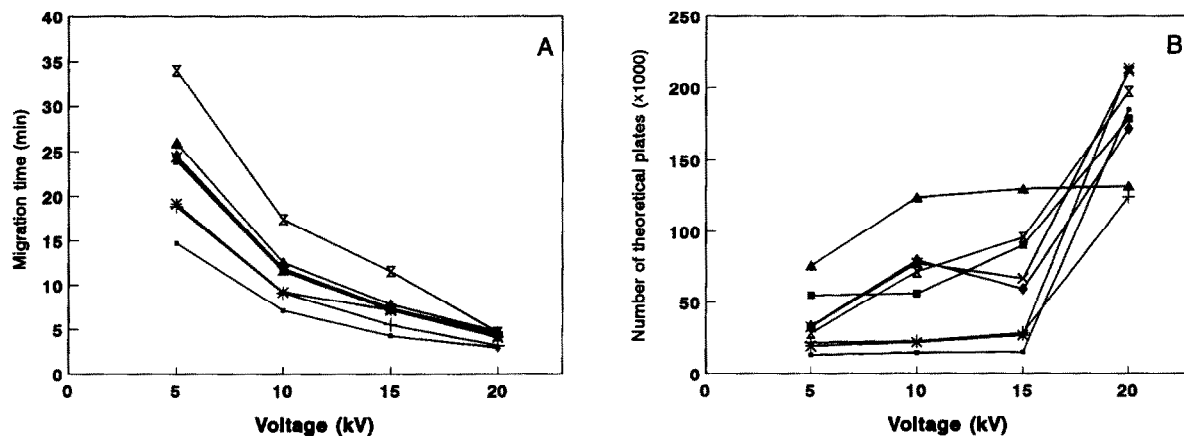

Fig. 3. Effect of voltage on the separation of purine compounds in 20 mM borate buffer (pH 9.4) at 37°C (capillary: uncoated silica, 75 μm I.D. × 44 cm, 37 cm to window). (A) Migration time; (B) theoretical plates. Symbols as in Fig. 1.
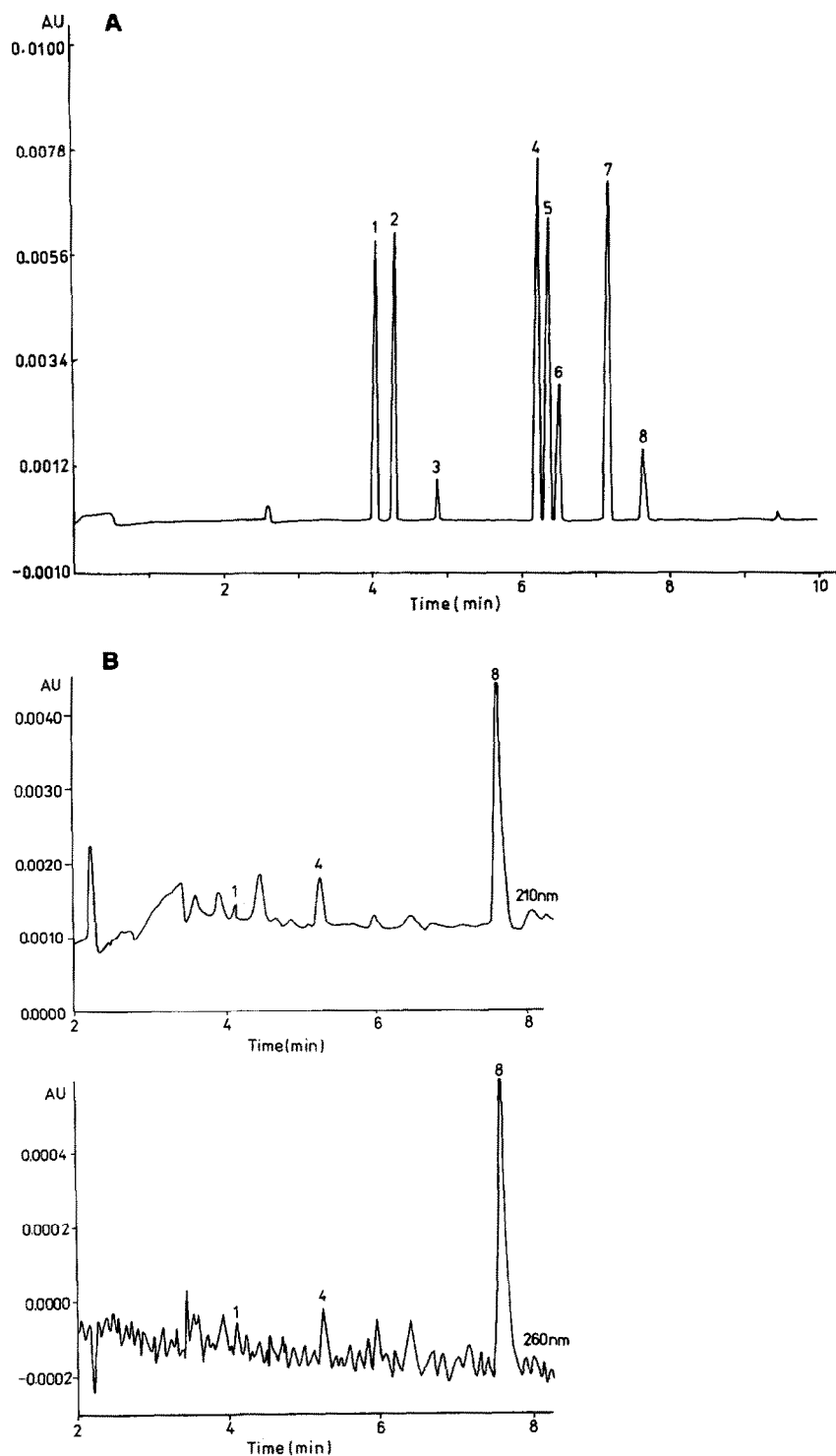
Fig. 4. Electropherograms of (A) a standard solution of purines and (B) a serum extract. Separation conditions as in Table II. The detection wavelength was 260 nm and for comparison of the increased sensitivity in (B) (upper panel) a chromatogram at 210 nm is shown. Peaks: 1 = Ade; 2 = Ado; 3 = Gua; 4 = Hyp; 5 = Guo; 6 = Xan; 7 = Ino; 8 = UA.

TABLE II

RETENTION TIMES AND REPRODUCIBILITY OF RETENTION TIMES AND PEAK AREA OF PURINE NUCLEOSIDES AND BASES

Separation conditions: uncoated silica capillary (75 μm I.D. × 44 cm, 37 cm to window), 20 mM borate buffer (pH 9.4), 37°C, 20 kV, 9 s hydrodynamic load, detection wavelength 254 nm.

Purine compound	Retention time (min)	Reproducibility (n = 10) (%)	
		Retention time	Peak area
Ade	3.16	2.09	6.31
Ado	4.08	2.13	3.51
Gua	4.27	1.86	4.28
Hyp	5.14	2.39	2.86
Guo	5.27	2.54	3.14
Xan	5.44	3.64	4.29
Ino	5.77	3.21	3.29
UA	7.67	4.11	4.07

load to 30 s with a corresponding increase in sensitivity.)

Under the optimized conditions of 20 mM borate buffer (pH 9.4), 37°C, 20 kV, 9 s load and detection at 254 nm, the reproducibility of the retention times and peak areas was investigated for a standard mixture. The results are presented in Table II. The reproducibility of migration times was in the range 2–4% for the selected purines. The peak-area reproducibility was in the range 3–6%. This allowed for the satisfactory determination of the purines in biological extracts.

Detection

Detection was performed in the wavelength range 200–300 nm. Detection at 210 nm was significantly more sensitive than at 260 nm (Fig. 4).

Analysis of serum from newborns

The electropherogram of a standard solution and a perchloric acid extract of newborn serum is shown in Fig. 4. The method allows the separation of the major naturally occurring purine nucleosides and bases. The results of serum analysis are given in Table III. The method permits the resolution of four purine compounds, namely adenine, hypoxanthine, guanosine and uric acid, in serum samples. The determinaton of the other purine compounds of serum is not yet possible, owing to the detection limit of about 0.5 μM and the sample pretreatment procedure. The separation quality is comparable to those of other capillary electrophoresis systems [13,14]. Atamna et al. [13] used a micellar electrokinetic capillary electrophoresis system for the separation of xanthine and uric acid derivatives. Uric acid and hypoxanthine in human serum were determined by Schoots et al. [14]. There have been no reports on the determination of other purine compounds in human serum, such as adenine and guanosine.

The determined purine concentrations agree with those of other workers [15–17] using HPLC. All investigated groups of newborns showed about the same concentration of uric acid. The concentrations of adenine, guanosine and hypoxanthine in serum of babies with asphyxia and acidic pH were

TABLE III

CONCENTRATIONS OF PURINE COMPOUNDS IN SERUM FROM NEWBORNS IMMEDIATELY AFTER BIRTH

Concentrations are given in μmol/l (mean ± S.D.).

Purine	Group			
	A (8)[a]	B (5)[a]	C (7)[a]	D (7)[a]
Adenine	3.13 ± 0.85	2.98 ± 1.33	5.16 ± 3.67	4.62 ± 1.89[b]
Guanosine	2.15 ± 0.71	2.70 ± 1.63	5.20 ± 2.01[b]	5.33 ± 2.07[b]
Hypoxanthine	6.87 ± 1.37	6.49 ± 2.56	10.13 ± 3.18[b]	8.75 ± 2.08[b]
Uric acid	358.19 ± 117.75	321.75 ± 135.46	371.43 ± 218.76	376.71 ± 215.02

[a] Number of patients.
[b] $P < 0.05$ in comparison with control group.

significantly increased in comparison with those of control babies. This may be the consequence of increased purine degradation during the birth and a asphyxia and/or acidosis.

In conclusion, the method presented allows the separation of some purine compounds in human serum using small-volume samples, including hypoxanthine and uric acid, two of the principal markers of asphyxia and/or ischaemia in human biofluids.

ACKNOWLEDGEMENTS

REFERENCES

1 W. E. Cohn, *Science*, 109 (1949) 377.

2 A. H. van Gennip, D. Y. van Noordenbury-Huistra, P. K. de Bree and S. K. Wadman, *Clin. Chim. Acta*, 86 (1978) 7.

3 W. G. Stillwell, H. X. Xu, J. A. Adkins, J. S. Wishnok and S. R. Tannenbaum, *Chem. Res. Toxicol.*, 2 (1989) 94.

4 J.-E. Edström, *Biochim. Biophys. Acta*, 22 (1956) 378.

5 D. Perrett, L. Bhusate, J. Patel and K. Herbert, *Biomed. Chromatogr.*, 5 (1991) 207.

6 R. A. Harkness, *J. Chromatogr.*, 429 (1988) 255.

7 T. Grune, W. Siems, G. Gerber, Y. V. Tikhonov, A. M. Pimenov and R. T. Toguzov, *J. Chromatogr.*, 563 (1991) 53.

8 T. Grune, W. G. Siems, G. Gerber and R. Uhlig, *J. Chromatogr.*, 553 (1991) 193.

9 V. Stocchi, L. Cucchiarini, F. Canestrari, M. P. Piacentini and G. Fornaini, *Anal. Biochem.*, 167 (1987) 181.

10 J. W. Jorgenson and K. D. Lucacs, *J. Chromatogr.*, 218 (1981) 209.

11 T. M. Olefirowicz and A. G. Ewing, *Anal. Chem.*, 62 (1990) 1872.

12 V. Apgar, *Curr. Res. Anesth.*, 32 (1953) 4.

13 I. Z. Atamna, G. M. Janini, G. M. Muschik and H. J. Issaq, *J. Liq. Chromatogr.*, 14 (1991) 427.

14 A. C. Schoots, T. P. E. M. Verheggen, P. M. J. M. de Vries and F. M. Everaets, *Clin. Chem.*, 36 (1990) 435.

15 M. C. O'Conner, R. A. Harkness and R. J. Simmonds, *Br. J. Obstetr. Gynaecol.*, 88 (1981) 381.

16 K. Thiringer, *Acta Paediatr. Scand.*, 72 (1983) 231.

17 O. D. Saugstad and L. Gluck, *J. Perinat. Med.*, 10 (1982) 266.