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Determination of concentrations of adenosine and other purines in human term placenta by reversed-phase high-performance liquid chromatography with photodiode-array detection: evidence for pathways of purine metabolism in the placenta

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ABSTRACT

A robust analytical method, using reversed-phase high-performance liquid chromatography with gradient elution and photodiode-array detection, was used to measure six purines and β-NAD^+ in acid-soluble extracts of samples taken from six different regions of human term placenta. Resolution of the analyte peaks in chromatographic profiles of the extracts, and the use of optimized integration, allowed simultaneous quantitation of all seven analytes from a single chromatogram. Peak purity was confirmed via on-line analysis of peak spectra, utilizing the purity parameter treatment of spectral data. Major placental purines were adenosine, inosine, hypoxanthine and adenine. Except for adenine, concentrations of the purines varied by two-fold or more between different regions of each placenta, but concentration ratios, *i.e.*, adenosine/inosine and inosine/hypoxanthine, were similar. The findings indicate that the pathway of ATP breakdown to hypoxanthine in ischemic human term placenta is via adenosine, and that regional differences in placental concentrations of adenosine and its metabolites may result from regional differences in degree of ischemia.

INTRODUCTION

The adenine nucleotide metabolite adenosine (Ado) has been implicated in a wide variety of regulatory processes related to normal functioning of organs and tissue systems [1,2]. The role of Ado as a mediator of local regulation of blood flow, for example, is well supported by experimental evidence [3]. Ado is vasoactive, generally eliciting an immediate relaxation of vascular smooth muscle. Ado is formed in tissues by breakdown of ATP as a result of oxygen deficiency. Ischemia in heart and brain causes enhanced Ado production accompanied by vasodilatation and, *in vivo*, by increased blood flow [3]. In some vascular beds, *e.g.* the dual-perfused human placental cotyledon, Ado causes vasoconstriction [4]. Ischemic challenge of the *in vitro* perfused placental cotyledon increases Ado release concomitant with an increase in perfusion pressure [5,6]. On delivery, the mean Ado content of seven human term placentas from uncompli-

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cated pregnancies, determined by reversed-phase high-performance liquid chromatography (HPLC), was 30.6 ± 11.5 nmol/g (mean ± S.D.) [7]. Further studies showed that Ado levels in different lobules of the one placenta varied by as much as two- to three-fold, and that levels of the Ado metabolites, inosine (Ino) and hypoxanthine (Hyp), also varied between lobules [8].

In the present study the regional variation in Ado concentration in human term placenta has been further investigated. Specifically, the relationship between the Ado level and levels of its metabolites Ino and Hyp in single placental samples was determined, using two samples taken from three lobules of each placenta, *i.e.* a total of six samples per placenta. Levels of guanosine (Guo), xanthine (Xan) and adenine (Ade) were also established for correlation with those of Ado and its metabolites. Concentrations of β-NAD^+, which is a prominent peak in chromatograms of placental extracts [8], were also determined. Measurements were performed using a reversed-phase HPLC method with photodiode-array detection, recently described for automated analysis of purines in placental extracts [9]. The resolution achieved by this method, the confirmation of peak purity via on-line analysis of peak spectra and the optimization of peak integration allowed all seven analytes in a placental extract to be quantitated simultaneously from one chromatogram.

EXPERIMENTAL

Materials

Nucleosides and nucleobases were obtained from Pharmacia P.L. Biochemicals (Piscataway, NJ, USA). Purine nucleotides were supplied by Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile, HPLC-grade 85% phosphoric acid and reagent-grade 1,1,2-trichloro-1,2,2-trifluoroethane (Freon) were obtained from Fisher Scientific (Springfield, NJ, USA). Baker-analyzed HPLC-grade ammonium dihydrogenphosphate was obtained from J. T. Baker (Phillipsburg, NJ, USA). Tri-*n*-octylamine was from Aldrich (Milwaukee, WI, USA). Nylon Acrodisc 0.45-μm disposable membrane filters were obtained from Gelman (Ann Arbor, MI, USA). Distilled water was passed through an IWT charcoal cartridge and redistilled in an all-glass system.

Instrumentation

HPLC was performed on a Varian 5560 chromatograph with column heater, 8085 autosampler, Rheodyne 7126 valve injector and Varian 9060 diode-array detector. A Spectra-Physics 4270 data integrator with memory module was used to record chromatographic profiles, integrate peak areas and store chromatograms for re-processing. Chromatographic data were also stored in a computer for subsequent replaying of chromatograms. The monitor of the 9060 detector displayed real-time peak spectra and chromatograms; a Hewlett Packard Thinkjet printer recorded peak spectra and peak purity parameters from a single pre-set wavelength range. Alternatively, spectral data were stored on floppy disks, and peak spectra and purity parameters were obtained subsequently via Varian Polysoc 3.0 software.

Purity parameters

The purity parameter treatment of spectral data of a peak employs a weighting factor, the square of the absorbance, to reduce the data to a single value, the purity parameter, which represents the characteristic average wavelength of the peak spectrum [9,10]. Comparison of purity parameters of sample peaks with those of peaks of standards under the same chromatographic conditions can be used to confirm sample peak identity and homogeneity. Upslope, apex and downslope purity parameters of homogeneous peaks are identical. With Polysoc 3.0 software, selection of the wavelength range for determination of peak purity parameters can be optimized, and upslope, apex and downslope spectra can be normalized and superimposed to confirm identity.

Chromatographic conditions

Development of the chromatographic method has been reported [9]. The column was a 150 mm × 4.6 mm I.D. Shandon C_{18} Hypersil, particle

size 3 μm (Keystone Scientific, State College, PA, USA). Brownlee guard cartridges, 15 mm × 3.2 mm I.D., packed with 7 μm, wide-pore, spherical C_{18} silica, were used routinely. Placental extracts were analyzed by gradient elution using two different mobile phases in which ammonium dihydrogenphosphate was the low-strength eluent and acetonitrile was the organic modifier. Ammonium dihydrogenphosphate was either 0.0075 *M* pH 6.0 or 0.05 *M* pH 3.3; pH was adjusted by addition of ammonium hydroxide or orthophosphoric acid, and solutions were filtered through a 0.45-μm Millipore membrane. The gradient program for both mobile phases was: 0–6 min, 0–4% acetonitrile; 6–10 min, 4–20% acetonitrile; 10–12 min, 20% acetonitrile; 12–13 min, 20–0% acetonitrile; re-equilibration time at 0% acetonitrile was 9 min. Flow-rate was 1.0 ml min^{-1}. System dwell time was 1.9 min. Column temperature was 40°C. Detection wavelength was 254 nm.

Preparation of placental extracts

Full term placentas from normal pregnancies were obtained as soon as possible after spontaneous vaginal delivery or caesarean section. Excess maternal blood was rapidly washed off with ice-cold normal saline. Tissue sampling was performed from incisions made beneath the maternal surface, after rapid blotting of blood with gauze pads, using bone rongeurs cooled in liquid nitrogen. Two samples were taken from three different lobules of each placenta; sampling took 4–6 min. Samples were stored at −80°C. Each pellet was powdered under liquid nitrogen and the powder (100–200 mg) was vortex-mixed with 2 ml of 12% ice-cold trichloroacetic acid for 30 s. Acid-insoluble material was sedimented by centrifugation at 4°C, and trichloroacetic acid was removed from the supernatant by vortex-mixing with 4 ml of 0.5 *M* tri-*n*-octylamine in Freon for 30 s [7]. Acid-soluble extracts were filtered through 0.45-μm Acrodisc membrane filters and stored at −20°C until analysis.

Chromatographic analysis

Placental extracts were analyzed in duplicate or triplicate, using automatic or manual injection and 10- or 20-μl sample loops. Peak apex purity parameters were routinely determined over 220 to 311 nm to evaluate peak purity. Agreement to ±1 nm with values established for standards indicated purity of peaks, except for Hyp which required further confirmation by spectral analysis. Ado, Ino, Guo, Ade, Hyp, Xan and β-NAD^+ concentrations in the extracts were quantitated simultaneously from chromatograms developed under pH 6.0 mobile phase conditions, using calibration curves prepared with standards in concentration ranges comparable to those in the extracts. Hyp was also analyzed from chromatograms developed under the pH 3.3 mobile phase conditions. Weight of the extracted tissue powder was used to convert analyte concentrations in the extract to nmol analyte per g placental tissue.

Statistical analysis

The form of distribution of analyte concentrations was examined via the SAS PROC UNIVARIATE statistical program (SAS Institute, Cary, NC, USA) after normalizing the data. Differences in analyte concentrations between placentas and between central and peripheral lobules and the effect of time elapsed from delivery to sampling on analyte concentrations within a placenta were examined via analysis of covariance with time as the covariate using the PROC GLM statistical program.

RESULTS

Analysis of purines and β-NAD^+ in placental extracts

Fig. 1 shows a chromatogram of a mixture of standards of thirteen nucleotide metabolites which are present in human term placenta, obtained using pH 6.0 mobile phase conditions. Table I lists retention times and purity parameters. Baseline resolution of Ado, Ino, Guo, Ade, Hyp, Xan and β-NAD^+ was achieved and with the exception of Ade, peaks were sharp and symmetrical. At pH 6.0 the Ade peak is characteristically rather broad, but is quantifiable. Chromatograms of extracts from two placentas are shown in Fig. 2 and demonstrate that analyte peaks

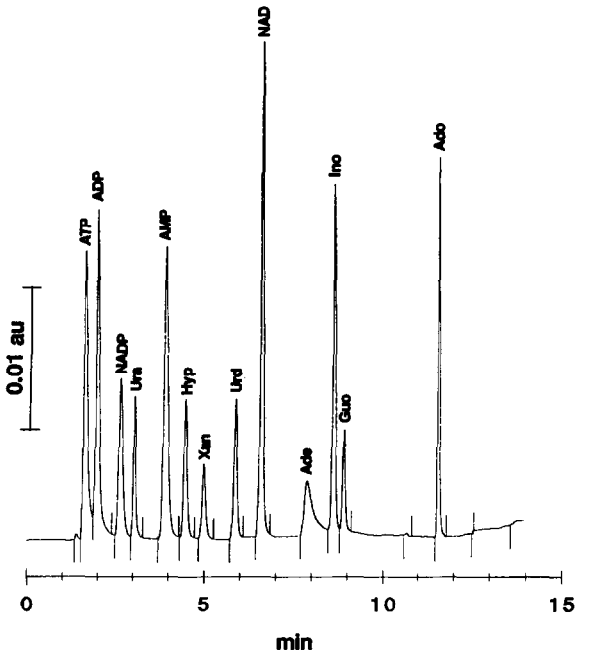

Fig. 1. Chromatogram of standards of nucleotide metabolites present in human term placenta. Column, 150 mm × 4.6 mm I.D. Shandon C_{18} Hypersil, 3 μm average particle size. Mobile phase, programmed gradient of acetonitrile in 0.0075 *M* ammonium dihydrogenphosphate pH 6.0. Details are given in the text. Flow-rate, 1.0 ml/min. Column temperature, 40°C. Detection wavelength, 254 nm. Chart speed, 1 cm/min. Injection volume, 20 μl containing 200 pmol of ATP, ADP, AMP, β-NAD^+ and Ino, 100 pmol of $NADP^+$, Hyp, Xan, Urd, Ura, Ade and Ado, and 50 pmol of Guo.

were well resolved from other components, with the exception of Ade and Ino. An unknown peak eluted between the Ade and Ino peaks, but was usually smaller and did not interfere with measurement of Ade or Ino. Spectra of a typical sample Ade peak are shown in Fig. 3. Analyte peaks were pure as indicated by their peak apex purity parameters. However, the peak apex purity parameter of Hyp was not a sensitive indicator of the presence of Gua [9], which if present co-eluted with Hyp at pH 6.0. Unless Hyp peak purity was confirmed by post-run spectral analysis, Hyp was also measured in a second chromatographic run using pH 3.3 mobile phase conditions which moved Gua to a shorter retention time and yielded a pure Hyp peak [9].

TABLE I

RETENTION TIMES AND PURITY PARAMETERS OF PURINE NUCLEOTIDES, NUCLEOSIDES AND BASES PRESENT IN HUMAN TERM PLACENTA

Chromatographic conditions as described under Experimental, using a gradient of acetonitrile in 0.0075 *M* ammonium dihydrogenphosphate, pH 6.0.

Analyte	Retention time (mean ± S.D., n = 5) (min)	Purity parameter[a] (mean ± S.D., n = 5) (nm)
ATP	1.57 ± 0.00	255.91 ± 0.03
ADP	1.91 ± 0.02	255.95 ± 0.03
$NADP^+$	2.59 ± 0.03	251.19 ± 0.11
Uracil	2.98 ± 0.01	255.42 ± 0.07
AMP	3.85 ± 0.03	255.93 ± 0.01
Hyp	4.40 ± 0.02	248.84 ± 0.01
Xan	4.92 ± 0.02	260.35 ± 0.04
Uridine	5.81 ± 0.02	257.66 ± 0.02
β-NAD^+	6.55 ± 0.05	251.19 ± 0.02
Ade	7.85 ± 0.04	255.98 ± 0.08
Ino	8.57 ± 0.04	247.28 ± 0.06
Guo	8.85 ± 0.03	256.63 ± 0.11
Ado	11.54 ± 0.02	256.29 ± 0.01

[a] 220–311 nm range.

Analyte peaks varied greatly in size within a single chromatogram, reflecting different concentrations of the analytes in the particular extract, in addition to different absorptivity of analytes at 254 nm. Relative sizes of the peaks in chromatographic profiles of different extracts also varied, reflecting differences in relative concentrations of the analytes between extracts. Integration of peak areas of the seven analytes was performed using automatic peak width recognition with high sampling rate and provided good precision and reproducibility of peak measurement. The baseline was sometimes affected by changes in mobile phase composition due to the gradient, as evidenced by a shallow increase of approximately $2 \cdot 10^{-4}$ a.u.f. in baseline over the 12-min chromatographic run. Gradient changes also influenced the signal-to-noise ratio, which decreased during the run. The influence of these phenomena on peak integration was more important with later and smaller peaks, as *e.g.* Guo. If incorrect integration was indicated, *e.g.* by poor

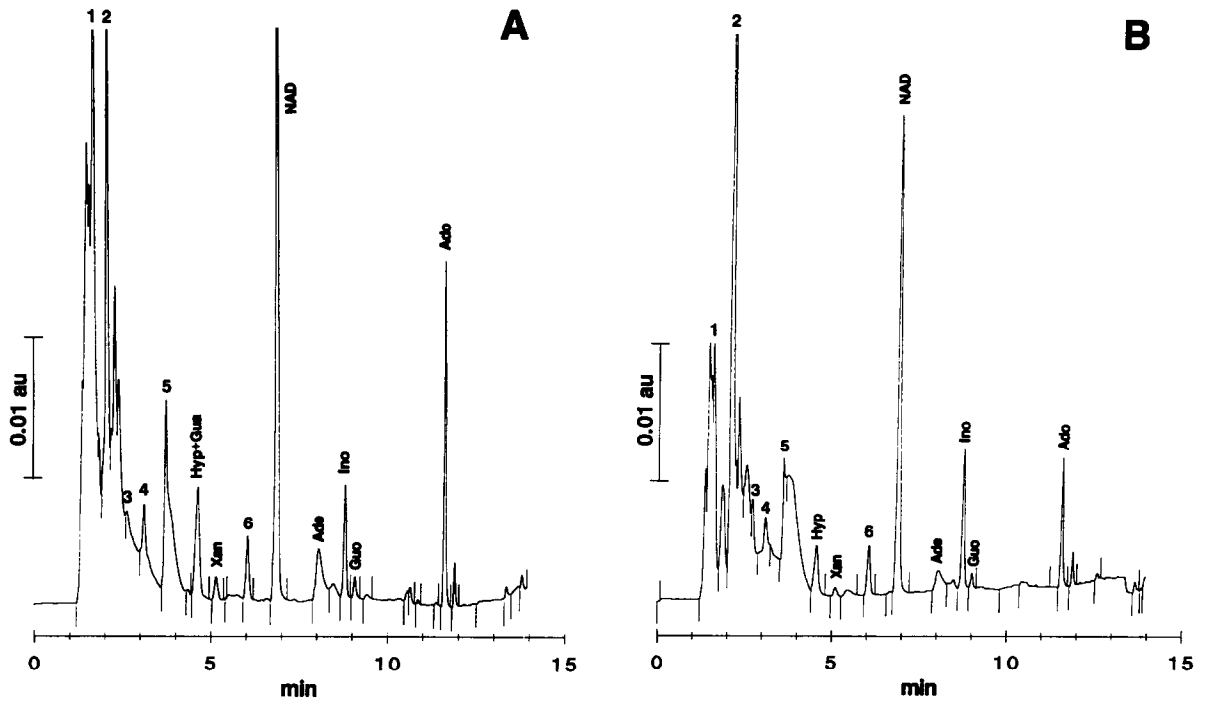

Fig. 2. Chromatograms of acid-soluble extracts of samples from different placentas. For column and chromatographic conditions, see legend to Fig. 1. Injection volume was 20 μl. Peaks 1, 2, 3, 4, 5 and 6 are ATP, ADP, $NADP^+$, Ura, AMP and Urd, respectively. The Hyp peak in A contained Gua, but in B was pure, as confirmed by spectral analysis. Peaks of Ado, Guo, Ino, Ade, β-NAD^+, Urd, Xan and Ura were pure as indicated by peak apex purity parameters.

duplicates or by an inappropriate position of peak event marks, the stored chromatogram was replayed using corrected integration settings, in particular a higher peak threshold. Means of duplicates of integrated peak areas differed from individual values by less than 6% except in the case of Guo, the smallest peak, where difference of the individual values from means was sometimes greater than 6% but less than 12%.

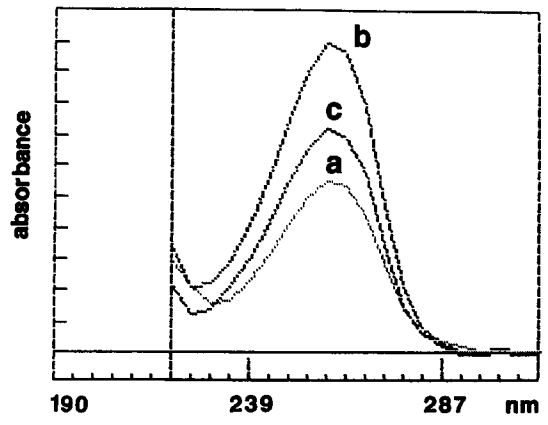

Fig. 3. Typical Ade peak spectra from a chromatogram of a placental extract. Purity parameters: (a) upslope 256.5 nm; (b) apex 257.0 nm; (c) downslope 257.1 nm. Wavelength range 220–311 nm.

Calibration curves for the seven analytes were linear over the ranges tested. Using pH 6.0 mobile phase conditions, linear regression analyses of the dependence of peak area (y) on concentrations of the standards (x) gave equations as follows: Hyp (4–20 μM), $y = 388.4x + 232$; Xan (1–15 μM), $y = 282.3x - 299$; β-NAD^+ (4–40 μM), $y = 634x - 1746$; Ade (1–15 μM), $y = 492.9x - 3.0$; Ino (1.5–20 μM), $y = 434.7x + 522$; Guo (0.5–6 μM), $y = 556.5x + 42$; Ado (1.5–20 μM), $y = 630.9x + 181$. Correlation coefficients were ≥ 0.999. The equation for Hyp at pH 3.3 was $y = 380.1x + 226$ (4–50 μM, $r > 0.999$).

Recovery of analytes from 12% trichloroacetic acid was determined by extracting two different

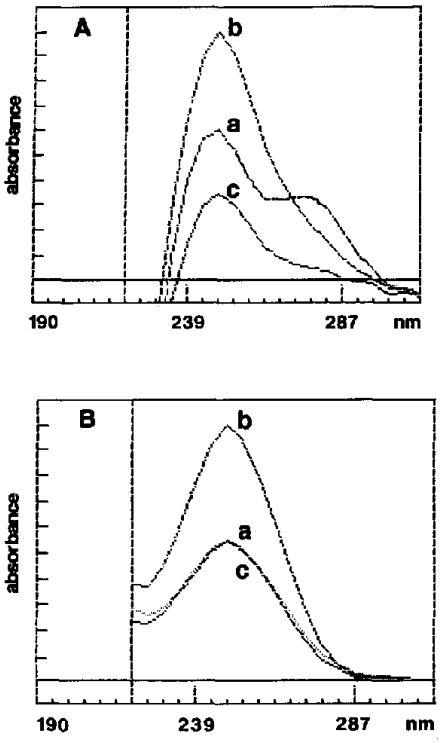

Fig. 4. Spectra of Hyp peaks from chromatograms of a placental extract. (A) Before treatment of the extract with Gua deaminase. Purity parameters: (a) upslope 259.2 nm; (b) apex 252.7 nm; (c) downslope 251.8 nm. (B) After treatment with Gua deaminase. Purity parameters: (a) upslope 247.3 nm; (b) apex 248.1 nm; (c) downslope 247.5 nm. Wavelength range 220–311 nm.

concentrations of each standard in 12% trichloroacetic acid, with tri-*n*-octylamine in Freon, as described under Experimental; standards recovered after extraction were compared with the identical concentrations in water. Concentrations used were similar to those found in placental extracts. Concentrations, recoveries (mean ± S.D.) and number of experiments were as follows: Ado, 7 and 14 μM, 104.5 ± 3.1% (n = 15); inosine, 3 and 6 μM, 103.1 ± 2.5% (n = 20); Guo, 0.5 and 1 μM, 102.6 ± 8.6% (n = 20); Ade, 5 and 10 μM, 101.9 ± 4.7% (n = 20); Hyp 4 and 8 μM, 104.7 ± 2.7% (n = 14); Xan, 4 and 8 μM, 106.4 ± 2.8% (n = 20); β-NAD^+, 15 and 30 μM, 96.5 ± 5.8% (n = 15).

Guanine in placental extracts

That Gua was present in a placental extract was inferred if the Hyp concentration determined using the pH 6.0 mobile phase was greater than the concentration determined using pH 3.3 mobile phase conditions. Direct evidence for the presence of Gua in the Hyp peak was obtained via comparison of spectra of upslope, apex and downslope of Hyp peaks at pH 6.0, as indicated in Fig. 4A, which shows spectra of the Hyp peak in a placental extract (sample 1/2 in Table II; Fig. 2A). Gua elutes 1–2 s before Hyp. The peak upslope spectrum, λ_{max} 244 and 277 nm, is that of

TABLE II

CONCENTRATIONS OF PURINE NUCLEOSIDES, BASES AND β-NAD^+ IN THREE LOBULES OF A TERM HUMAN PLACENTA

Lobule No./ Sample No.	Location[a]	Time[b] (min)	Concentration[c] (nmol/g)						
			Ado	Ino	Guo	Ade	Hyp	Xan	β-NAD^+
1/1	c	7.0	56.2	20.1	5.7	58.2	27.8[d]	8.2	274.9
1/2	c	8.5	71.9	48.2	7.3	55.3	57.3[d]	19.4	249.9
2/1	p	9.0	44.8	14.9	4.3	58.2	32.6[d]	9.5	260.1
2/2	p	10.5	50.1	39.3	5.9	51.4	111.6	16.7	289.2
3/1	p	12.0	78.0	60.9	10.1	52.8	120.1[d]	31.0	263.5
3/2	p	12.5	63.6	48.4	7.4	49.7	140.6	44.1	237.5

[a] Central (c) or peripheral (p) lobule.
[b] Time from delivery to sampling.
[c] Means from duplicate injections; difference of values from mean <6%.
[d] Gua eluted with Hyp at pH 6.0.

Gua. The apex and downslope spectra have the λ_{max}, 249 nm, of Hyp, but both show absorption around 277 nm indicating the presence of some Gua. After treatment of the sample with guanine deaminase, upslope, apex and downslope spectra showed that the Hyp peak was pure (Fig. 4B). As little as 5% Gua in Hyp could be discerned in the upslope spectrum of the peak. Gua was present in four samples of placenta I (see Table II) and one sample of placenta II and all samples of placenta III (see Table III).

Concentrations of purines and β-NAD$^+$ in different lobules of term placentas

Concentrations of the purine metabolites in each of six samples of a term placenta are shown in Table II. Sampling of the placenta commenced 7 min after spontaneous delivery; two samples were taken from two peripheral lobules and two from a central lobule. With the exception of concentrations of Ade and β-NAD$^+$, concentrations of each metabolite varied widely, two- and four-fold, between the samples, but within each sample the concentration ratios of Ado/Ino and Ino/Hyp were relatively consistent, *i.e.* ≥ 1 (mean = 1.9) and ≤ 0.8 (mean = 0.6) respectively; ratios of concentrations of Ado/Guo and Hyp/Xan were ≥ 8 and ≥ 3, respectively. Similar profiles of metabolite concentrations were found in six samples from a second placenta in which sampling from two peripheral and one central lobule commenced 7 min after delivery by caesarian section. Fig. 5 compares concentrations of Ado, Ino and Hyp in these samples. The effect on metabolite levels of a longer ischemic period prior to sampling was examined in a third placenta in which sampling commenced 16.5 min after spontaneous delivery. The pattern of metabolites and concentration ratios were generally similar to those observed in the placentas subjected to a shorter period of global ischemia, although relatively high Ino levels in one sample gave a lower Ado/Ino concentration ratio (0.5), and in two other samples gave higher Ino/Hyp concentration ratios (0.9, 1.1). Overall, in the eighteen samples from these placentas, concentration ratios (mean ± S.E.M.) were: Ado/Ino, 1.49 ± 0.17; Ino/Hyp, 0.52 ± 0.05. Mean concentrations of the metabolites in each of the three placentas are compared in Table III, where the degree of intra-placental variation in purine levels is indicated by the R.S.D. Similar findings were obtained in two other term placentas in which sampling commenced 11 and 16 min after spontaneous delivery (results not shown).

TABLE III

CONCENTRATIONS OF PURINE NUCLEOSIDES, BASES AND β-NAD$^+$ IN THREE HUMAN TERM PLACENTAS

Values in parentheses are R.S.D. (S.D./mean × 100).

Analyte	Concentration (mean, n = 6) (nmol/g)		
	I[a]	II[a]	III[a]
Adenosine	60.8 (21)	44.0 (28)	57.2 (31)
Inosine	38.6 (46)	33.7 (46)	55.7 (32)
Guanosine	6.8 (29)	7.7 (23)	8.2 (13)
Adenine	54.3 (7)	34.8 (21)	47.5 (12)
Hypoxanthine	81.7 (59)	88.2 (43)	101.3 (40)
Xanthine	21.5 (64)	26.4 (27)	35.2 (28)
β-NAD$^+$	262.5 (7)	204.5 (13)	230.0 (8)

[a] Time from delivery to first and last sampling: 7–12.5 min for I, 7–11.5 min for II and 16–23.5 min for III.

Statistical analysis

Metabolite concentrations in the placentas indicated no anomaly of distribution. Metabolite levels did not differ significantly between placentas or between lobules (central *versus* peripheral) or with time to sampling within a placenta.

Identification of other nucleotide metabolites in placental extracts

The nucleotides ATP, ADP, NADP$^+$ and AMP were identified in chromatographic profiles of the placental extracts by comparison of retention times and peak apex purity parameters with those of standards (Fig. 2A and B; Table I).

Where possible, assignment was confirmed by comparison of peak upslope, apex and downslope spectra. ATP and ADP were sharp prominent peaks but were not baseline-resolved from other components which also eluted before commencement of the acetonitrile gradient. Adenosine diphosphoribose (ADPR) had the same retention time and peak spectrum as ADP (results not shown) and could be present together with ADP. $NADP^+$ appeared as a shoulder or small peak on the trailing edge of the early cluster of unresolved peaks. In many extracts AMP was not resolved from a closely-eluting unknown component (*cf.* Fig. 2A and B); the spectrum of the downslope of the composite peak had no features above 200 nm, indicating that the contaminant was not a nucleic acid derivative. Uracil (Ura) and uridine (Urd) were well resolved homogeneous peaks. The retention time of uric acid was 2.3 min, and in chromatograms of some extracts the downslope spectrum of the peak with retention time 2.0-2.2 min was that of uric acid (λ_{max} 250 and 280 nm), indicating the presence of a trace amount of uric acid. A sharp peak (retention time 11.9 min) present in all chromatograms, was not identified. The peak spectrum was identical with that of Ado, λ_{max} 263 nm, and was unchanged at pH 3.3; the peak was not shifted by Ado deaminase. Purines that were not detected in the chromatograms were (retention times in parentheses): 2′-deoxyadenosine (11.8 min); 5′-deoxy-5′-methylthioadenosine (MTAdo, 13.8 min); S-adenosylhomocysteine (SAH, 10.5 min); S-adenosylmethionine (SAM, 5.6 min); and xanthosine (6.3 min).

DISCUSSION

In this report we describe the simultaneous quantitation of seven purine peaks in reversed-phase HPLC of placental extracts. The greatly different hydrophilic/hydrophobic properties of the nucleotide metabolites to be quantified and of other UV-absorbing components of the extract necessitated use of gradient elution in order to achieve resolution and timely elution of analyte peaks [9]. Gradient elution methods are generally not considered suitable for automated chromatographic analysis because changes in the mobile phase may result in baseline problems. In this study accurate solvent proportioning and reliable flow reproducibility minimized baseline problems [11], and thermostatting of the column minimized temperature effects on analyte capacity factors. The chromatograms contained complex arrays of peaks. Peak integration was routinely evaluated to ensure that the gradient method was robust. Peak width and signal-to-noise ratio of individual analyte peaks varied from sample to sample depending on their concentrations in the samples, and intra-sample proportions of the different analytes also varied. For these reasons, and because of the number of peaks to be quantitated, optimization of the integration of each individual analyte peak in a chromatogram was not appropriate. Automatic peak width recognition with relatively high sampling frequency was used and precision of integration was evaluated from placement of the peak event marks and reproducibility of replicate measurements. Storage and replaying of chromatograms allowed correction of any inappropriate integration.

Although the chromatographic method was optimized for resolution of the analyte peaks [9], it was possible that unknown contaminants might be present in different extracts and might co-elute with analyte peaks, as was observed with an impurity associated with the AMP peak in chromatograms of some extracts. Therefore, peak apex purity parameters were used to confirm homogeneity of the analyte peaks. An exception was the Hyp purity parameter, which could not be used to indicate Hyp peak purity because, when computed over the wavelength range used routinely, *i.e.* 220–311 nm, it was not modified by the presence of small amounts of co-eluting Gua [9]. The sensitivity of detection of Gua in the Hyp peak was increased when the Hyp purity parameter was computed over an optimized wavelength range, but this wavelength range was not suitable for routine measurement of purity parameters of the other analyte peaks. Purity of Hyp was established by post-run spectral analysis or extracts were chromatographed using the lower-pH mo-

bile phase to resolve Gua from Hyp and allow reliable quantitation of the latter.

It is noteworthy that peaks of Ura and Urd in chromatograms of the extracts were well resolved, homogeneous and quantifiable. Thus, this gradient-elution method allows automated analysis of a total of nine analytes from one chromatogram.

Analysis of purine metabolites from different lobules of three placentas showed that purine concentrations varied regionally within each placenta, by as much as two-fold for Ado and Guo, four-fold for Ino and five-fold for Hyp and Xan. However, the concentration ratios Ado/Ino and Ino/Hyp were consistent for all placental samples, indicating that metabolism of Ado was similar. Moreover the Ado/Ino concentration ratio was three times that for Ino/Hyp, suggesting that deamination of Ado is the rate-limiting step in the pathway from Ado to Hyp via Ino. This conclusion is in agreement with the finding that Ado deaminase activity in the placenta is low relative to AMP phosphatase activity [12,13]. Ino may also be formed from AMP via deamination of AMP to IMP followed by dephosphorylation, but this pathway in the placenta was shown to be less important than dephosphorylation of AMP to Ado under conditions where ADP and ATP levels are reduced, as in ischemia [14]. Hyp is the terminal metabolite of ATP catabolism in human placenta, which lacks detectable xanthine oxidase activity [12,15]. Xan present in the placenta must therefore be the product of GTP breakdown and be formed by the action of Gua deaminase on Gua produced by phosphorolysis of Guo. Gua deaminase activity has been demonstrated in human placental extract [16]. Gua nucleotide levels in tissues are generally 10–20% or less of the total free purine nucleotide complement [17]. Purine nucleotides were not quantitated in this study, but it is noteworthy that Guo levels were much lower than Ino levels, and that Gua was not present in all samples. Moreover, within any one sample, Xan concentrations were at least twice as high as Guo concentrations. Conversion of Guo to Gua may be the rate-limiting step in Xan formation.

The primary source of Ado in the ischemic placenta is AMP formed by ATP breakdown. AMP was present in all chromatograms of placental extracts. More than 90% of the total placental AMP phosphatase activity at neutral pH is contributed by membrane-bound, low-Michaelis constant, AMP-specific 5′-nucleotidase, which is potently inhibited by ADP and ATP, and less than 10% is contributed by soluble placental 5′-nucleotidase activity [18]. Soluble placental 5′-nucleotidases have been described [19]; these have broad substrate specificity and may be more important in normoxic pyrimidine nucleotide metabolism than in hypoxia- or ischemia-generated production of Ado from AMP. Under ischemic conditions the fall in ADP and ATP will result in disinhibition of AMP-specific 5′-nucleotidase with resultant hydrolysis of AMP to Ado. Another possible source of Ado is SAH produced from SAM by transmethylation pathways; S-adenosylhomocysteine hydrolase is present in human term placenta [20]. However, this route of Ado formation has been shown to be insensitive to hypoxia, at least in the isolated guinea-pig heart [21].

Placental Ade levels were generally similar in range to those of Ado, but showed less intra-placental variation. Ade is a metabolic by-product of polyamine biosynthesis, and the source of Ade in the placenta is most probably the polyamine pathway. The polyamines spermidine and sper-

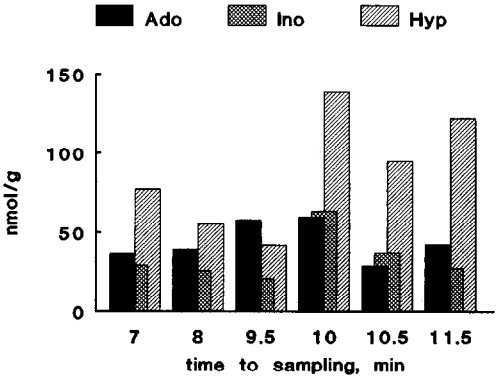

Fig. 5. Ado, Ino and Hyp content in six samples from different regions of a human term placenta *versus* time from delivery to sampling.

mine and MTAdo phosphorylase, which catalyzes the phosphorolysis of MTAdo to yield Ade and 5-methylthioribose-1-phosphate, are present in human term placenta [22,23]. The presence of relatively high levels of Ade in the placenta appears to be an unusual finding. Ade is present in plasma and urine, but few reports of measurable Ade levels in normal tissues have appeared, although rat liver, spleen and adrenals were shown to contain 13–20 nmol of Ade per g [24]. The concentration of Ade measured in this study may reflect the importance of polyamines in the term placenta. Polyamines accumulate in tissues undergoing rapid growth and differentiation, and it has been suggested that polyamine complexes may be involved in immunoregulatory activity of placental trophoblast [25].

Pathways in human term placenta leading to Ado, Hyp, Xan and Ade, the major purine catabolites, are indicated in the schematic shown in Fig. 6. Under normoxic conditions *in utero*, when ATP and PRPP (5-phosphoribosyl 1-pyrophosphate) availability is not limiting, Ado, Hyp, Gua and Ade can be salvaged to the nucleotide level via action of the salvage enzymes Ado kinase, HGPRT (Hyp-Gua phosphoribosyltransferase) and APRT (Ade phosphoribosyltransferase). Ado kinase is present in human term placenta [13,26], and levels of HGPRT and APRT activities in supernatants of placental homogenates were 2–4 and 1–2 μmol per min per g, respectively [27]. There appears to be no evidence as yet for interconversion of Ade and Gua nucleotides in human term placenta (indicated by the broken lines in Fig. 6). IMP metabolism to GMP via XMP and to AMP via AMPS (adenylosuccinate) requires nucleoside triphosphates ATP and GTP, respectively, and may occur in the placenta *in utero* under normoxic conditions. Adenylosuccinase activity is present in the term placenta [27].

In conclusion, reversed-phase HPLC using a robust gradient-elution method which allows facile quantitation of seven peaks from one chromatogram has established the levels of six purines and β-NAD^+ in samples from human term placenta. In agreement with earlier findings [9], concentrations of Ado and its metabolites varied

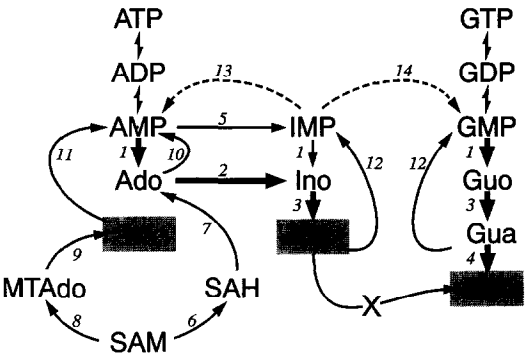

Fig. 6. Schematic diagram of pathways of purine metabolism in human term placenta. The reactions and enzymes are as follows: 1, 5′-nucleotidase; 2, Ado deaminase; 3, purine nucleoside phosphorylase; 4, Gua deaminase; 5, AMP deaminase; 6, SAM-dependent transmethylations; 7, SAH hydrolase; 8, aminopropyl transfer from decarboxylated SAM (polyamine biosynthesis); 9, MTAdo phosphorylase; 10, Ado kinase; 11, APRT; 12, HGPRT; 13, AMPS synthetase and AMPS lyase; 14, IMP dehydrogenase and GMP synthetase. Broken lines indicate reactions which have not been demonstrated in human term placenta. Bold arrows indicate reactions enhanced by ischemia.

in samples taken from different lobules of the same placenta. However, relative concentrations of Ado, Ino and Hyp were similar between samples, indicating that metabolism of Ado was similar in the different lobules. One explanation for the regional differences in purine levels may be that in different lobules of the placenta, ischemic challenge induces a different degree of ATP breakdown to AMP and Ado with resultant differences in levels of Ado. It is well known that the uterine contractions of late pregnancy and labor impair functioning of the spiral arteries which deliver maternal blood to the placental cotyledons, and may completely inhibit spurting of maternal blood into the intervillous spaces of the placenta [28], resulting in foci of ischemia. Although on delivery the placenta becomes globally ischemic, our findings suggest that the effect of regional differences in maternal perfusion remain over and above the effects of several minutes of global ischemia. Ideally, pre-ischemic, control levels of purines should be determined in tissue samples frozen within seconds of cessation of circulation. Such samples of human term placenta cannot be obtained. Even when caesarian section is per-

formed, clinical obligations preclude immediate sampling of the placenta. Although it is not possible to determine control normoxic levels of purines in human term placenta, this study indicates the importance of Ado as a major ATP metabolite under ischemic or hypoxic conditions.

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