Journal of Chromatography, 429 (1988) 255–278
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4156

REVIEW

HYPOXANTHINE, XANTHINE AND URIDINE IN BODY FLUIDS, INDICATORS OF ATP DEPLETION

R.A. HARKNESS

Division of Inherited Metabolic Diseases, MRC Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ (U.K.)

(First received December 2nd, 1987; revised manuscript received January 29th, 1988)

CONTENTS

0378-4347/88/$03.50

LIST OF ABBREVIATIONS

ADP	Adenosine 5′-diphosphate
APRT	Adenine phosphoribosyl–transferase
ATP	Adenosine 5′-triphosphate
CSF	Cerebrospinal fluid
EDTA	Ethylenediaminetetraacetic acid
EHNA	*erythro*-9-(2-Hydroxy-3-nonyladenine)
HPLC	High-performance liquid chromatography
HPRT	Hypoxanthine (guanine) phosphoribosyltransferase
hyp	Hypoxanthine
K_M	Michaelis constant
MS	Mass spectrometry
NADH	Nicotinamide-adenine dinucleotide, reduced
NADPH	Nicotinamide-adenine dinucleotide phosphate, reduced
NMR	Nuclear magnetic resonance
REM	Rapkid eye movement
urd	Uridine
xan	Xanthine

1. INTRODUCTION

The major reason for measuring the purine bases hypoxanthine (hyp), xanthine (xan) and the pyrimidine nucleoside uridine (urd) in body fluids is to provide information on changes in intracellular nucleotides. Hypoxanthine is a central intermediate in purine nucleotide biosynthesis and uridine occupies a similar position in pyrimidine metabolism [1–6]. Since the purine nucleotide ATP is the energy currency of the cell and is therefore biologically and quantitatively the most important compound, the major emphasis in this review will be on hyp which is the extracellular compound most directly related to intracellular ATP. However hyp, xan and urd in extracellular fluid show similar changes during ATP depletion in tissues.

The metabolic interrelationships for the purine base nucleosides (bases combined with ribose) and nucleotides (nucleoside phosphates) are shown in Fig. 1 along with their metabolising enzymes (IUB abbreviations are used without preliminary definition throughout). Bases and nucleosides exist outside and inside cells whereas nucleotides are confined within cells. The formula of relevant purine bases, their ribose derivatives (nucleosides) and the nucleoside phosphates (nucleotides) and uric acid are shown in Fig. 2 with the conventional numbering of the purine ring.

The analytical methods generally used will be reviewed; there is agreement that

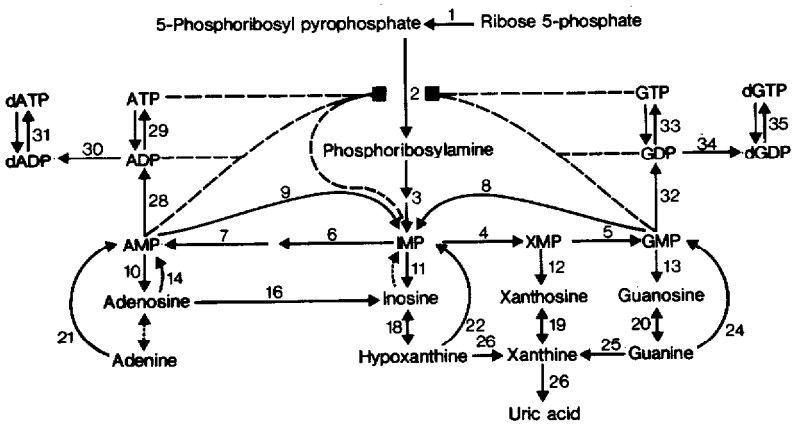

Fig. 1. Metabolic pathways of purine and pyrimidine metabolism. The compounds on the top four lines occur inside cells. The compounds on the bottom three lines can freely pass out of cells. (---) Negative feedback control on enzyme 2; (····) reactions not yet proven. Enzyme activities (reactions) are numbered; those important in this review are: (9) adenylate deaminase; (10, 11, 12, 13) 5′-nucleotidase; (14) adenosine kinase; (16) adenosine deaminase; (18, 19, 20) purine nucleoside phosphorylase; (21) adenine phosphoribosyltransferase; (22, 24) hypoxanthine guanine phosphoribosyltransferase; (25) guanase; (26) xanthine dehydrogenase/oxidase.

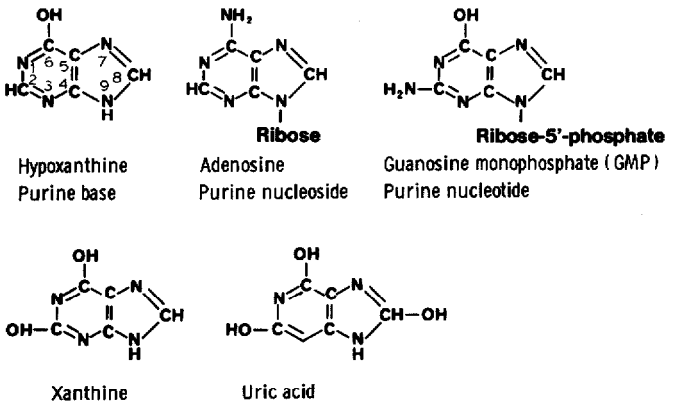

Fig. 2. Formulae of purine bases, nucleosides and nucleotides, with a numbered purine ring.

for the present phase of research and development high-performance liquid chromatography (HPLC) is the choice. There is also agreement on the HPLC-based analytical methods to be used for measuring hyp, xan and urd in body fluids. The development, evaluation and present status of these methods will be outlined and typical chromatograms reviewed. There are extensive reviews of the chromatographic aspects [7,8] covering the period from 1979 onwards. Regular reviews of all aspects of HPLC are available in this Journal and other publications [9]. Since methods have stabilised there are available sets of similar reference values

which are now usable in clinical practice. These values are assembled and discussed in this review. A substantial part of the review is concerned with the physiological basis for interpreting extracellular fluid concentrations of hyp, xan and urd and the excretion of hyp and xan. On the basis of this evidence suggestions are made for a variety of clinical uses of such measurements with some emphasis on situations in which chromatographic separations provide more information than considering hyp and xan together as total oxypurines.

2. ANALYTICAL METHODS

2.1. Early methods

Early methods measuring hyp and xan together as oxypurines have provided results which show similar trends to results from HPLC. For example, there was a good correlation between total oxypurine concentrations in plasma measured using an oxygen electrode and xanthine oxidase based method [10] and HPLC [11]. The raised concentrations in umbilical cord blood, about 5 μmol/l, can be measured well by both methods as shown in a recent comparison of results from seven centres [12]. However, normal plasma concentrations in children and adults are generally agreed to be about 1–2 μmol/l (see Table 1) which is below the sensitivity of the existing oxygen electrode–xanthine oxidase based method. A sensitive fluorimetric method has been described [13] but not tested in widespread use.

A spectrophotometric method for urine has provided results [14] which were about four times higher than HPLC [15]. Thin-layer chromatography has shown increased excretion in asphyxiated newborn infants [16], the trend again agreeing with HPLC data [15,17].

2.2. High-performance liquid chromatography

2.2.1. Extraction

Sample preparation by ultrafiltration with or without enzyme inhibitors has been used with good recoveries [18] and avoids degradation of labile compounds like NADH and NADPH by acids. All methods removing proteins from samples disinfect and detoxify samples as well as stabilising the analytes in solution. Protein precipitation with 5–15% (w/v) trichloroacetic acid is cheap and the most popular. Supernatants can be subsequently neutralised with freon [19] or serial extraction with diethyl ether [20]. Perchloric acid is also widely used with subsequent potassium hydroxide neutralisation of the extract but poor recoveries of nucleotides have been encountered. Contaminating peaks from buffer salts especially Tris bases are a common problem.

For nucleosides specific extraction methods based on boron ester phase interaction with vicinal hydroxyl groups can be used [21]. The purification of urinary

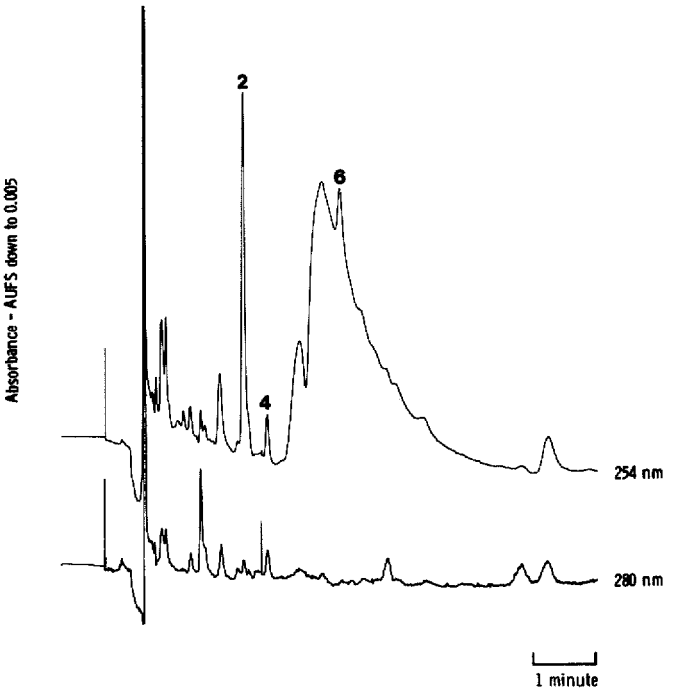

Fig. 3. Chromatogram of an extract of urine after preliminary ion-exchange chromatography [15,20] showing hypoxanthine (2), xanthine (4) and internal standard allopurinol (6) illustrating the persistence of contaminating compounds in urine extracts.

fractions is especially difficult and this is reflected in the limited amount of data available compared to plasma and cerebrospinal fluid (CSF) concentrations. Ion-exchange column extraction of the bases hyp and xan from urine using the cation exchanger Zerolit 225 (BDH) has been employed successfully on a wide variety of samples [15] but many impurities are still found in such extracts (Fig. 3). However, solid-phase extraction, especially with the wide variety of modern packing materials, could be further developed to solve the problems of adequate purification of urinary extracts. When high levels of compounds are present in urine as in some metabolic defects dilution, about 1:10–30, can be used to produce preliminary results but normal levels are difficult to secure in this way due to impurities.

2.2.2. Chromatographic techniques

Most groups have followed early leads [22,23] and use column packings of silica, particle size 3–5 μm, coated with a reversed phase of size C_{18} [20,24,25]. The widespread availability of column packing machines and materials has reduced prepacked column prices but it remains cheaper to prepare your own columns sharing a column packer with other users. Since HPLC is widely used,

suitable sharing groups can generally be assembled to justify such a facility. The manual repacking of the column top is a useful simple method of restoring performance.

The equipment from a number of manufacturers can be used reliably and cannot be reviewed here. High room temperatures cause compounds to be eluted earlier sometimes blurring separations of these poorly retained compounds. Column temperature control is worthwhile and can be achieved using a simple water jacket coupled to a standard thermostatically controlled and pumped water supply [20] or by manufactured special equipment.

Solvents and samples are filtered through 0.45-μm filters. Great care is needed especially with phosphate buffers to prevent crystalline precipitates forming by washing the system through with water and then aqueous methanol after use. Washing of injection systems is also periodically needed. Extensive degassing of the mobile phase is necessary with older systems but may be avoidable with modern systems.

In most cases on a C_{18} reversed-phase column, high-purity aqueous 0.0001–0.1 mol/l phosphate buffer of pH ca. 6 with 1–20% high-purity methanol is used. All-glass-distilled water is required. Resolution can be manipulated by pH change for

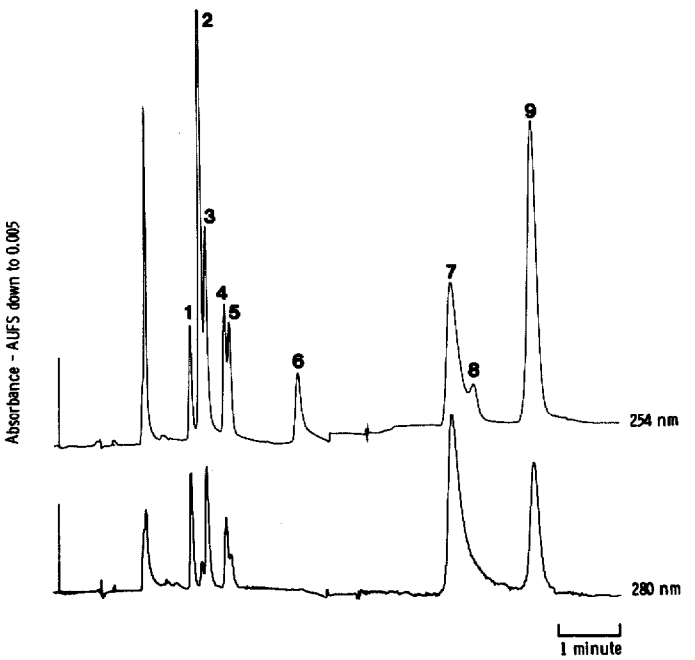

Fig. 4. Chromatogram of standards (approximately 100 pmol) containing cytidine (1), hypoxanthine (2), guanine (3), xanthine (4), uridine (5), internal standard allopurinol (6), 7-methylguanine (7), inosine (8) and guanosine (9). Conditions: 25 cm × 0.5 cm column Shandon ODS 5 μm; mobile phase, 0.001 mol/l KH_2PO_4, pH 4.0 with 1% (v/v) methanol; flow-rate, 1 ml/min.

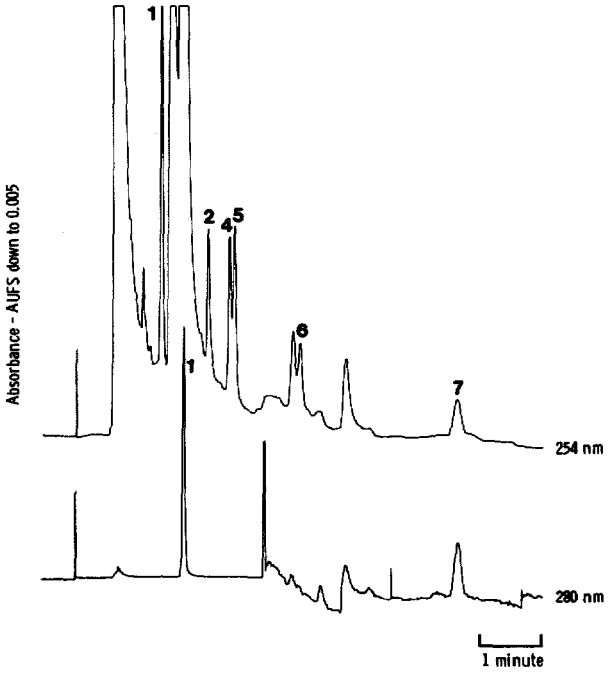

Fig. 5. Chromatogram of extract of amniotic fluid (20 μl) showing raised hypoxanthine (2). Peak 1 is urate. Conditions and other components as in Fig. 4 with 0.005 a.u.f.s. at 254 nm and 0.2 a.u.f.s. at 280 nm initially.

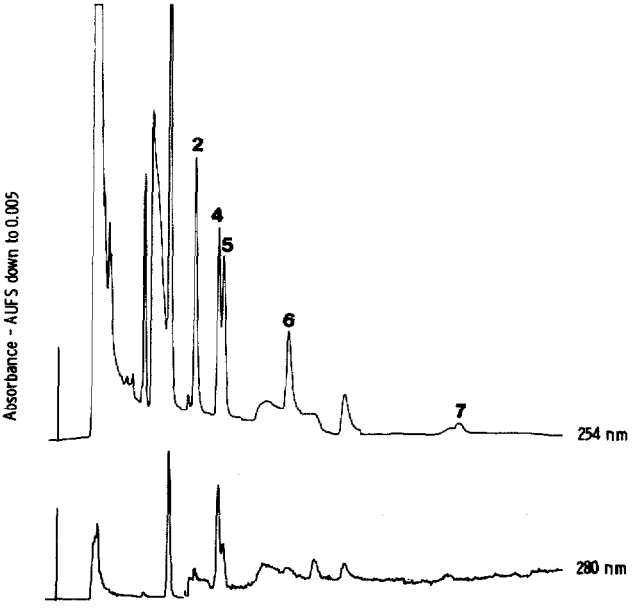

Fig. 6. Chromatogram of an extract of cerebrospinal fluid (20 μl) showing raised hypoxanthine (2), xanthine (4), and uridine (5) after brain damage. Conditions as in Fig. 4.

uric acid, hyp, xan and urd; urate is better above pH 6.0 as the more soluble anion. Sometimes organic phase changes to acetonitrile or tetrahydrofuran can alter selectivity [26].

The injection volume using an analytical column, 100–250 mm×5 mm I.D., can be increased to about 50 μl for increased sensitivity but larger volumes lead to peak spreading [27].

The stationary phase differs between manufacturers even for a C_{18} coating; carbon loading and capping of residual silanol groups are variable. In general, for the required refined separations of the very low concentrations of endogenous purines and pyrimidines fine tuning of columns, to about 60 000 theoretical plates/m, is required which is demanding. Skilled operators are still needed.

An example of the practical difficulty is shown by the separation of standards 4 and 5 in Fig. 4. In body fluids guanine (3) is not present but xan (4) and urd (5) require separation which can be achieved with very similar conditions but pH 5.8 [20].

Most body fluids contain a variety of unresolved peaks which are eluted before peak 1 in Fig. 4. These components of the matrix can mask orotic acid, uric acid, cytosine, uracil and pseudouridine, all of which can be readily separated as standards in these systems. Uric acid, however, is present in sufficiently large amounts in plasma and amniotic fluid and has a high absorbance at 280 nm allowing measurement whenever required (Fig. 5). Plasma and most other body fluids provide similar chromatograms to those shown for CSF and amniotic fluid (Figs. 5 and 6) in which most unresolved components are eluted before hyp.

2.2.3. Detection

UV detection remains the method of choice provided that the absorbance of the compound is intense enough to measure physiological concentrations. Dual-detection interference filter-based assemblies are stable and sensitive. They can be independently controlled, thus urate in amniotic fluid can be measured from absorbance at 280 nm using 0.2 a.u.f.s. in that channel and 0.005 a.u.f.s. at 254 nm (Fig. 5). Absorbance at 254 and 280 nm provides a series of characteristic ratios for identification. Variable-wavelength machines are now almost as sensitive and diode array detectors allow extensive spectral checks of identity [28]. A cheap and practical method of checking identity is to react the peak with a 'pure' enzyme like xanthine oxidase to convert hyp and xan to urate or a specific reagent-like periodate to split vicinal glycols in the ribose residues of nucleosides. Unfortunately urd-metabolising enzymes are not available commercially possibly due to their instability [29,30]. Theoretically mass spectroscopy (MS) is available and has been used to identify urinary methylated nucleosides derived from tRNA [31]. For most problems of identification MS is very difficult possibly due to small quantities of unknown in the presence of large quantities of buffer.

For the high sensitivity required in the detection of the purine base adenine and its nucleoside adenosine in body fluids the fluorescent 1-N^6-etheno deriva-

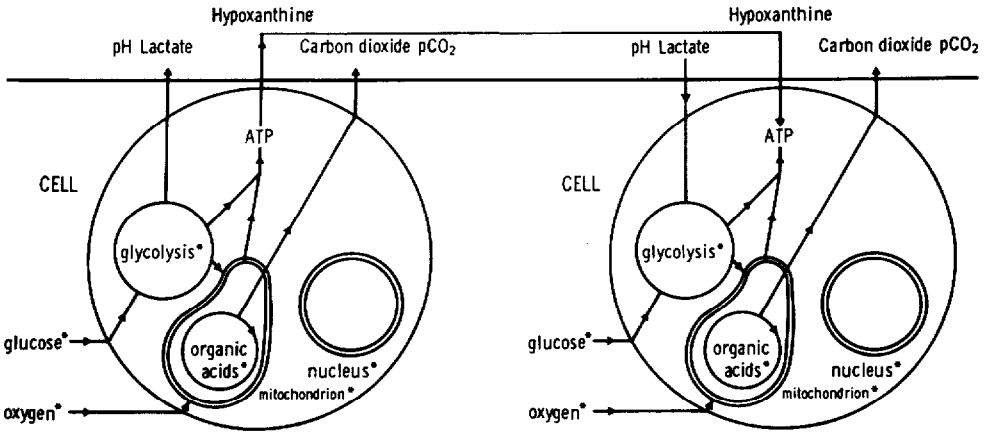

Fig. 7. Release of hypoxanthine by an ATP-using or -depleted cell and uptake by a cell restoring its nucleotide concentrations when glucose and/or oxygen supplies are restored. Asterisks indicate sites of known defects of energy supply, the cell nucleus is asterisked to indicate primary gene defects which can cause defects of energy supply.

tives using a pre-column reaction with chloroacetaldehyde are useable [32]. Post-column derivatisation with fluorescent detection is also used, for example in analysis of Zovirax, the antiviral drug from Wellcome in rat plasma [8]. Electrochemical detection of analytes is selective and sensitive [33] but unsuitable for routine use.

2.2.4. Applicability to large-scale clinical surveys

The problems of scaling up HPLC assays for large-scale clinical surveys for cancer detection has been recently discussed [34]; automation, on-line column switching and other developments have also been reviewed [8].

Published methods exist for hyp, xan and urd in plasma, serum, CSF, urine, amniotic fluid, saliva and vitreous humour [8]. However, there is no recorded experience of HPLC analyses of hyp, xan and urd in routine use in a clinical laboratory.

From this brief review of the analytical aspects it can be seen that current HPLC analyses of hyp, xan and urd are increasingly used but remain demanding. Existing methods could be maintained in a hospital reference laboratory but not in a busy routine area nor in a peripheral laboratory. Since measurements of hyp, xan and urd are biochemically closely connected with blood pH and gas concentrations (Fig. 7) further instrumental development appears probable.

2.3. Sampling and stability problems

These are related to biochemical mechanisms and can be systemically considered at this point. Intracellular nucleotides change very rapidly and freeze-clamped

samples of rapidly and cleanly dissected tissues must be used to obtain estimates corresponding to concentrations in vivo, but even with such precautions errors are large. Tissue degradation of ATP is fastest in brain with progressively slower changes in liver, kidney and placenta [35]. Erythrocytes are even slower with hyp release only becoming detectable after about 30 min [10,11,36]. Therefore centrifugal separation of erythrocytes from plasma within 30 min of sampling results in plasma concentrations of 1–2 μmol/l which seem to be truly physiological since they correspond to the apparent Michaelis constant (K_M) of the major metabolising enzyme, hypoxanthine (guanine) phosphoribosyltransferase (HPRT) [2,3], responsible for recycling and are below the apparent K_M of the major catabolic enzyme xanthine dehydrogenase [37].

In addition to hyp release from intact erythrocytes, losses from lysed erythrocytes are a potential problem. There is a significant linear correlation between extracellular ATP and haemoglobin in plasma sampled through standard syringe needles. Since ectonucleotidases and non-specific phosphatases are widespread and certainly exist in blood, blockage of their action is recommended by 4 mmol/l EDTA, chelating Mg^{2+} and acting as an anticoagulant. Valid results can be obtained from heparinised samples but EDTA samples are safer. Lysis of heparinised samples raises inosine rather than hyp concentrations in plasma because purine nucleoside phosphorylase activity is low in plasma. Thus a high inosine concentration relative to hyp is a warning of a haemolysed sample [38] even in the absence of visible haemolysis. If lysed blood contamination is a problem for example in CSF or amniotic fluid, then haemoglobin concentration can be monitored sensitively from the absorbance of the intense Soret band at about 410 nm. An absorbance of over 0.25 in a 10-mm cell may be associated with a raised hyp concentration. Obviously if normally virtually acellular fluids like CSF have a raised cell content the use of rapid (< 30 min) centrifugal separation and 4 mmol/l EDTA are worthwhile precautions in obtaining samples for analyses of hyp, xan and urd. Good results have been obtained with amniotic fluid and CSF using these precautions (see below) but for urine such precautions seem unnecessary.

A further series of cellular changes, platelet action, is to be avoided to obtain valid estimates of plasma concentrations of hyp, xan and urd. Platelets contain and, on the clotting of blood, release large quantities of ADP. This ADP is degraded rapidly to inosine [38]. Serum samples are therefore to be avoided despite their widespread use for many analyses.

The nucleosides adenosine and inosine are precursors of hyp. Adenosine is of great interest because it possesses strong biological activities dependent on the 6-amino group (Fig. 2), producing vasodilatation and other actions after interaction with at least two groups of receptors [39]. The removal of the 6-amino group of adenosine to form inosine is rapid and, in order to observe adenosine release, from perfused organs for example, close, rapid catheter samples are necessary with inhibitors in the syringe and catheter blocking adenosine deaminase [5–10 μmol/l *erythro*-9-(2-hydroxy-3-nonyladenine) (EHNA)] [40] and aden-

osine uptake by cells (0.2 mol/l dipyridamole) [41]. Rapid separation of cells from plasma is also advisable. The blockage of nucleoside uptake by dipyridamole is also required for studies on inosine concentrations. Since there is normally rapid uptake and metabolism of nucleosides by cells, results using blockers of uptake and metabolism are overestimates [42]. It is advisable to regard inosine as reflecting adenosine concentrations since rapid intra- and extracellular deamination occurs. Once hyp is produced, it is stable unless recycled by energy replete cells. Thus hyp is the stable circulating intermediate in purine metabolism.

3. REFERENCE VALUES FOR CONCENTRATIONS OF HYPOXANTHINE, XANTHINE AND URIDINE IN BODY FLUIDS

3.1. Plasma

After the introduction of HPLC and the rapid separation of cells from samples of plasma, a series of groups have produced similar results using similar HPLC methods. Results from healthy adults are shown in Table 1. Our results on normal children are similar.

Permanent alterations are rare. The commonest changes are due to treatment with the xanthine oxidase inhibitor, allopurinol, which raises xan and hyp reducing urate. The naturally occurring equivalent enzyme deficiency causes similar

TABLE 1

CONCENTRATIONS OF HYPOXANTHINE, XANTHINE, URIDINE AND URATE IN BLOOD SAMPLES FROM HEALTHY ADULTS

Plasma was separated from cells within 30 min. Separation and measurement was carried out by HPLC using deproteinised extracts. n = number of individuals sampled.

Concentration; mean (S.D. or 95% range) (μmol/l of plasma)				n	Ref.
Hypoxanthine	Xanthine	Uridine	Urate		
0.5 (0.2)	0.4 (0.3)	4.5 (1.7)	276 (55)	-	84
1.0 (0.9)	4.9 (1.5)	-	-	10	85
2.2 (1.3)	0.2 (0.1)	-	-	6	86
1.5 (0.4)	0.5 (0.2)	3.2 (1.2)	-	4	20
1.2-2.0 (0.1-0.8)	0.6-0.9 (0.1-0.5)	3.0-4.4 (0.3-0.6)	250-230 (30-50)	4 (6)*	
1.6 (0.7)	0.3 (0.1)	-	-	4	58
2.5 (1)	1.4 (0.7)	-	-	-	36

*Number of samples for each individual.

changes but is rare, about 1 in 60 000 [43]. Both states are not generally associated with any other clinical abnormality. In contrast the very rare HPRT deficiency causes marked clinical abnormalities after the age of two years. These abnormalities include severe neural dysfunction, gout, growth failure and testicular atrophy [44]. These is abnormal biochemistry probably from conception. Nucleotide depletion in cells can occur in the absence of this enzyme [45]. Fourfold elevations of hyp and xan are found in plasma and more marked increases in urinary excretion; this condition also causes raised urate concentrations and excretion distinguishing it from xanthine oxidase deficiency and allopurinol treatment [46].

A reduction in plasma urate ($< 100\ \mu$mol/l) with normal levels of hyp and xan is generally due to aspirin administration. Very low, about 20 μmol/l, urate concentrations can be caused by an endogenous increase in renal urate clearance inherited as an autosomal recessive [47].

A variety of less directly linked enzyme defects may also be diagnosed, for example myoadenylate deaminase deficiency (reaction 9 in Fig. 1). In this condition after ischaemic muscle exercise plasma hyp only rises to 10, not a normal 40 μmol/l [48]. Urine can be used for this diagnosis. A cautionary note is that secondary myoadenylate deaminase deficiency is now being reported with mitochondrial defects in a variety of myopathies [49]. Carnitine palmitoyl transferase (a mitochondrial defect, Fig. 7) is diagnosable by a similar exercise test [50].

HPLC patterns of purine and pyrimidine and related compounds in a variety of diseases of this area of metabolism are available in the literature [51]. HPLC can also be used to assay the activity of enzymes like HPRT and adenine phosphoribosyltransferase (APRT), metabolising purines [52].

3.2. Cerebrospinal fluid

There is now agreement on concentrations (Table 2) although earlier estimates of total oxypurines were higher. A tendency for xan concentrations to fall with age has now been found by at least three groups although such a fall is not seen in normal plasma concentrations (Table 1).

The tendency for hyp concentrations to fall with age may be due to a greater number of entirely normal adults being sampled compared to children and to the difficulties of securing samples from newborn free from haemolysis. For example the bottom line of Table 2 shows results obtained from a series of samples obtained during myelography and not selected for those with essentially normal myelograms; no gross pathology was present. If selection for any definate abnormality was carried out the results for hyp fall into line with the other adult normal values. The lower limits encountered are similar at all ages. It is therefore possible that truly normal values for hyp but not for xan do not change with age. This is important because it is difficult to obtain age-matched normal controls in childhood.

TABLE 2

CONCENTRATIONS OF HYPOXANTHINE, XANTHINE, URIDINE, INOSINE AND URATE IN CEREBROSPINAL FLUID OF NORMAL NEWBORN CHILDREN AND ADULTS

Separation and measurement were carried out by HPLC using deproteinised extracts.

	Concentration: mean (S.D. or ±2 S.D. rnge) (μmol/l of CSF)					*n*	Ref.
	Hypoxanthine	Xanthine	Uridine	Inosine	Urate		
Newborn	3.6 (1.8-5.5)	5.0 (0.9-9.1)	3.3 (0.6-6.3)	0.7 (0-2.0)	30 (25)	18	53
	3.8 (0.9)	5.2 (1.3)			20.3 (11.6)	24	25
Children	3.6 (1.3-5.9)	3.1 (1.3-4.9)	2.3 (0.5-4.1)			9	Unpublished
	2.4 (0.6-4.1)	2.1 (0-4.2)	2.0 (0.3-3.7)	0.4 (0.2-0.6)	16 (0-52)	78	87
Adults	1.8 (0.6-5.1)	1.7 (0.6-4.7)	1.6 (0.3-8.2)	0.2 (0-1.0)		29	53
	2.6 (0.5)	1.7 (0.4)			12.8 (9.8)	26	
	3.2 (1.0)	2.5 (0.7)	3.4 (0.9)			8	Unpublished

Urate concentrations in CSF rise at puberty especially in males and are higher in aqueous and vitreous humour than in CSF; concentrations of adenosine and adenine nucleotides are very low [53]. As in plasma purines with the 6-amino group are too potent agonists [39] to be allowed to persist in biological systems.

3.3. Amniotic fluid

Concentrations of hyp, xan and urd are similar to those in plasma and CSF in the last three months of pregnancy (Table 3). This is surprising since the origin of most amniotic fluid late in pregnancy is the fetal kidney. A variety of evidence shows that amniotic fluid at this time is changed every 24–48 h [54]. Therefore amniotic fluid concentrations reflect fetal events over the preceding 24–48 h and thus provide a valuable cumulative record of ATP depletion. If information is needed on the 30 min immediately before sampling, that is the period before the fetal bladder has probably emptied, then fetal or umbilical cord blood samples can provide this information. In early pregnancy, when amniotic fluid is largely an ultrafiltrate derived from the fetus [54], concentrations of hyp are lower (Table 3) possibly due to more active salvage of hyp by live cells in close contact with the fluid. At this early stage, 15–24 weeks, somewhat higher concentrations of hyp have been found in some pregnancies in which the fetus aborts later in the pregnancy (R.A. Harkness and D.G.H. Brock, unpublished results) but there are analytical problems in measuring these low levels.

The establishment of simple methods of sampling and handling amniotic samples [56] provided consistent results (Table 3). Methods for widespread clinical studies have thus been established. In the last three months of pregnancy the interpretation of amniotic fluid concentrations of hyp, $\geqslant 5$ μmol/l as indicative of fetal risk, appears justifiable [55,57].

TABLE 3

METHODS OF AMNIOTIC FLUID SAMPLING SUITABLE FOR THE MEASUREMENT OF HYPOXANTHINE, XANTHINE AND URIDINE CONCENTRATIONS

Concentrations from normal pregnancies.

Sampling method	*n*	Hypoxanthine	Xanthine	Uridine	Ref. for sampling method
Gestational age 36–41 weeks					
Drew Smythe Catheter	46 geometric mean	1.9	1.0	0.9	69
	Mean+2S.D. (log)	5.4	7.3	8.6	
Elective Caesarean section	15 geometric mean	2.1	2.7	1.4	55
	Mean+2S.D. (log)	4.1	4.9	3.1	
Clean forewater sample	57 geometric mean	1.7	2.1	1.0	56
	Mean+2S.D. (log)	4.1	16.4	6.5	
Gestational age 15–24 weeks					
Transabdominal with ultrasound control	16 geometric mean	0.52	1.50	0.74	Harkness and Brock, unpublished
	Mean+2S.D. (log)	0.86	1.90	1.06	

3.4. Urine

Reference values for the urinary excretion of hyp and xan corrected for body weight and concentration of hyp relative to creatinine are shown in Table 4 [58]. Urinary excretion especially of hyp is a sensitive method of studying nucleotide metabolism in man possibly because hyp has a high renal clearance approximating to the glomerular filtration rate [47] and because hyp is stable in urine; the cumulative record of events is preserved and not erased. Hypoxanthine excretion correllates well with that of creatinine whereas xanthine output does not correlate. The hyp/creatinine concentration ratio therefore appears to be a reasonable estimate of hyp output and correlates well with total excretion [59]. The renal clearance of xan can also exceed the creatinine clearance suggesting a tubular origin for some at least of the excreted xan. Hypoxanthine and xanthine excretion can alter independently and may have different biological origins [15]; thus initial research studies at least should use methods capable of separating hyp from xan.

3.5. Activity and hypoxanthine excretion

The results in Table 4 show that every day activities increase hyp excretion, the daytime output exceeding that at night. Since such activity does not involve

TABLE 4

EXCRETION OF HYPOXANTHINE AND XANTHINE BY NORMAL INFANTS AND ADULTS

Final separation and measurement were carried out by HPLC after preliminary ion-exchange chromatography [15].

	n	Excretion; mean (S.D. or 95% range) (nmol/h per kg body weight)		Concentration ratio hyp/creatinine; mean (S.D. or 95% range)
		Hypoxanthine	Xanthine	
Newborn				
Resting, intensive care	10	12.2 (2.1)	4.7 (2.2)	5.9 (2.0)
Active, ward cot	16	36.3 (14.1-58.5)	28 (13-41)	12.0 (5.3-27.5)
Adults				
Resting, night	7	53.4 (5.6)	31.2 (5.2)	6.2 (0.9)
evening	10	33.9 (3.2)	34.4 (4.8)	
Active, day	5	70.8 (38.5)	33.0 (56.5)	
Resting, night	5	45.5 (36.7)	19.5 (30.2)	
Hypothyroid*	8	28.9 (20.6)	21.5 (23.3)	
Hyperthyroid*	2	223, 68	163, 48	

*24-h urine samples.

ATP depletion in muscles, there appeared to be an effect of ATP turnover on hyp excretion. Metabolic recycling has also been described in cultured cells [60]. Subsequently a highly significant curvilinear relationship has been proved between hyp excretion and work done down to low levels equivalent to a gentle walk (Fig. 8); this relationship is only just detectable in xan and urate essentially due to changes at levels of exercise at or near fatigue. Increasing age decreases the hyp output and has related effects in rat muscle [45]. We are extending these observations below the baseline in Fig. 5 by examining output in hypo- and hyperthyroidism (Table 4). As expected, lower values are found in hypothyroidism and possibly higher values in hyperthyroidism compared to normal adults.

Our results in newborn in intensive care are consistent with the conscious efforts of nursing and medical staff to reduce the energy use of infants in intensive care.

4. PATHOPHYSIOLOGY OF HYPOXANTHINE, XANTHINE AND URIDINE IN BODY FLUIDS

This section will emphasise the changes in CSF, amniotic fluid and urine because these body fluids provide a record of events during the preceding 24–48 h. The rapid circulation of blood allows erasure of any record within hours of an event (Figs. 7 and 9).

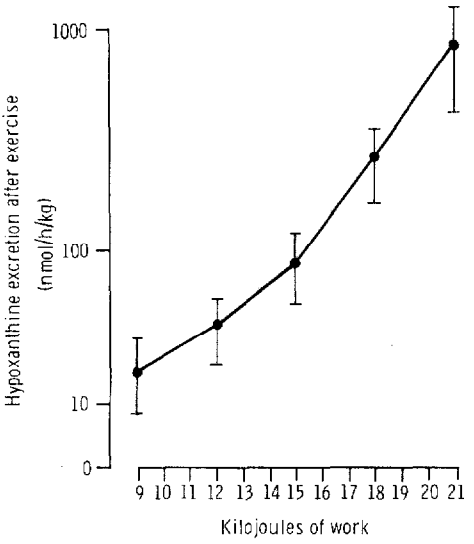

Fig. 8. Markedly increased hyp excretion with increased work from minimal to near fatigue levels in five normal men. There were no significant differences in response between sexes.

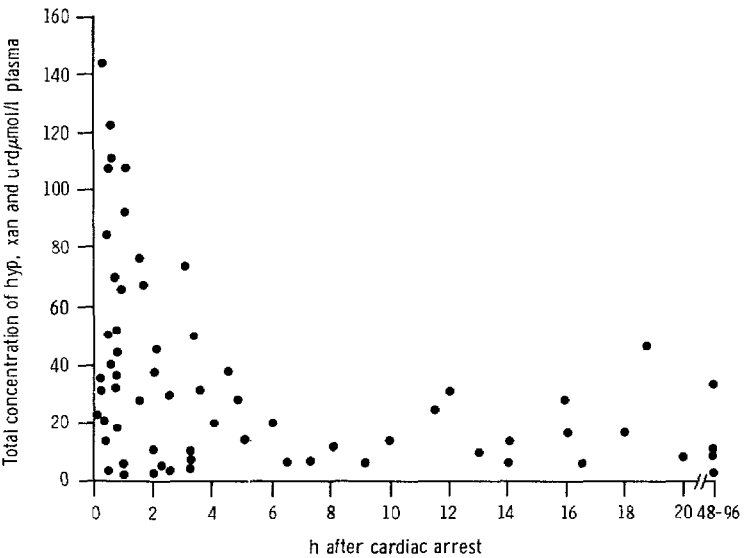

Fig. 9. Increased plasma concentrations of hypoxanthine, xanthine and uridine, shown as their arithmetic total after cardiac arrest in seventeen children. Note plasma concentrations were normal when the late urinary abnormalities after cardiac arrest start (cf. Fig. 11).

4.1. Cerebrospinal fluid

There is agreement using a total oxypurine method and HPLC that ATP depletion produces marked rises in hyp and xan for at least 24 h. The extent of the

rise is related to the brain damage assessed clinically [53] and in adults by imaging methods [61]. Raised levels were found in hydrocephalus by at least three centres [62–64]. In addition there are significant positive linear correlations between mean intracranial pressure during rapid eye movement (REM) sleep and CSF concentrations of hyp and of xan but not of urd [63]. In newborn the skull expansion measured by the occipitofrontal diameter clearly increases in parallel with the CSF hyp and xan (Fig. 10); pressure in newborn is low due to this expansion. These raised levels of hyp and xan fall after surgical relief of the raised pressure [64].

HPLC has shown that after ATP depletion [53] and in HPRT deficiency [46] hyp is raised markedly and xan little altered. In contrast in hydrocephalus hyp and xan are raised to a similar extent [63].

Furthermore the results in hydrocephalic newborn show marked differences in hyp and xan elevations between individuals; usually xan is greater than hyp but some patients show almost equal concentrations. Thus any detailed investigation of brain metabolism in hydrocephalus should measure hyp and xan separately. It can be suggested that in hydrocephalus there is more GTP generation and use than after a simple arrest of energy supply during acute anoxia-ischaemic change.

Since xan concentrations are higher in newborn a similar change may underlie this difference.

Data from four centres by two groups shows a slight increase in CSF hyp with fever in adults and in children [53,65,66]. Small changes in CSF hyp and xan are

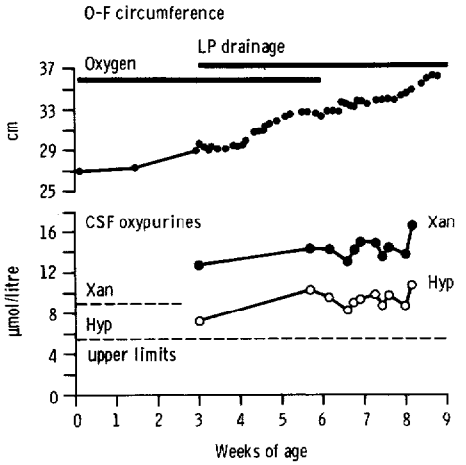

Fig. 10. Hydrocephalus in newborn: the relationship between increasing skull occipitofrontal circumference and increasing CSF concentrations of hypoxanthine and xanthine, with the upper (95%) limits indicated by the dotted lines. Oxygen indicates increased concentrations in inspired air and LP drainage shows serial removals of CSF to relieve pressure.

also linked to depressive symptoms [67]. A further application of CSF hyp, xan and urd concentrations may be to exclude the occurrence of marked ATP depletion in diseases in which such depletion is possible. For example continuous electrical activity by the brain should increase ATP turnover and could even deplete ATP concentrations in brain. However, the results show that ATP depletion does not occur in children with continuous electrical activity in their brains [68]. Similarly febrile convulsions do not cause raised CSF hyp concentrations.

In conclusion our ability to measure hyp, xan and urd in CSF allows a variety of studies of human brain metabolism which have already advanced our understanding sometimes by unexpected results.

4.2. Amniotic fluid

The most clinically important association of raised levels of hyp was with meconium staining [69]; this finding provided pathophysiological evidence justifying the use of this old clinical marker of fetal distress at a time when it was being questioned. Other epidemiologically defined risk factors, assessed independently, show similar relationships with raised levels [55] including an association with large (>4 kg) postmature infants [56].

A variety of evidence shows that during labour there is increasing danger to the fetus, a fall in arterial oxygen concentration, a rise in the hormone controlling erythropoiesis, erythropoietin, and increasing concentrations of hyp, xan and urd in serial amniotic fluid samples [69].

There is therefore extensive evidence that concentrations of hyp, xan and urd in amniotic fluid can provide quantitative evidence of fetal risk presumably from episodes of ATP depletion.

Existing clinical estimates may exaggerate the risk to the fetus. For example the intrauterine growth retardation of pre-eclampsia is associated with the removal by the fetus of more oxygen from fetal blood but little or no rise in erythropoietin production. There is little or no evidence of fetal stress or ATP depletion from studies of amniotic fluid [70]. Slowing fetal growth apparently allows the fetus to live within its reduced oxygen supply at a time when the mother is steadily deteriorating.

This survey suggests that analysis of amniotic fluid, at least for hyp and xan, will become part of a clinical laboratories work. The most cost-effective ways to employ such powerful methods have yet to be established. Confirmation of the diagnosis of fetal distress by on-line methods like fetal heart rate monitoring could be a start using samples obtained at consequent emergency Caesarian section.

4.3. Urine

Acute changes in plasma concentrations are reflected in urine, thus 1–2 h urine collections can be used in non-invasive studies in volunteers [47,71]. After car-

diac or ventilatory arrest, however, renal function might be reduced or even cease; for example, acutely asphyxiated newborn infants generally do not show raised urinary excretion of hyp and xan in the first 24 h.

There is an unexpected bonus from urinary studies. After tissue damage following ATP depletion there is a prolonged excessive excretion of hyp and xan starting about 24 h after the episode and lasting at least two to three days; this is illustrated in Fig. 11 which shows such a pattern after cardiac arrest. Similar patterns have been observed repeatedly in asphyxiated infants [15]. Contrasting these urinary changes starting about one day after cardiac arrest with the changes in plasma which are over in about 4 h (Fig. 9), it seems justifiable to conclude that they are due to different processes. The urinary changes are apparently a reflection of tissue damage which may follow marked ATP depletion. The urinary changes correlate with neurological and other damage assessed independently in two major studies each involving the asphyxiated infants from about 4000 deliveries [15,17]. Since hyp/creatinine concentrations ratios after moderate or severe asphyxia were four to eight times the levels after mild asphyxia such measurements appear more sensitive than urinary N-acetyl-β-D-glucosaminidase which only increased four-fold above normal in perinatal asphyxia [72].

There is some evidence in a variety of tissues for the intracellular events which may underlie this prolonged excretion. There is prolonged depression of intracellular nucleotide concentrations after an episode of ATP depletion although

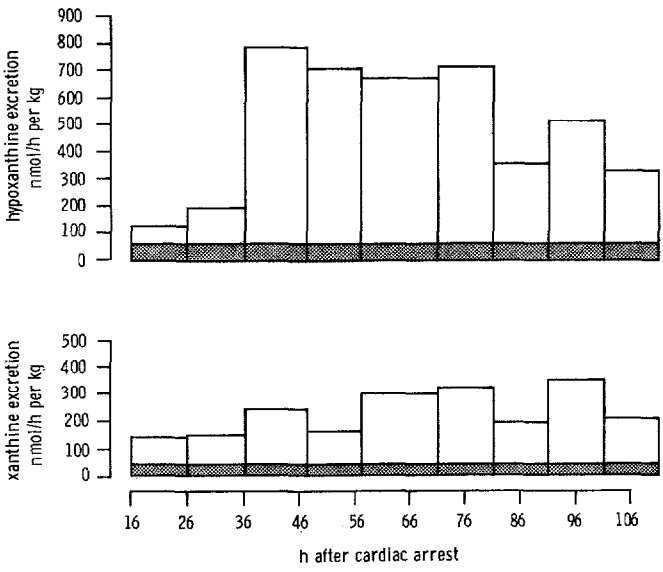

Fig. 11. Increased urinary excretion of hypoxanthine and xanthine after cardiac arrest in a child, 10 years of age, showing abnormal raised output for at least 110 h after the episode. The shaded area indicates normal excretion. There were persistent neurological signs with autopsy evidence of neuronal damage.

the adenylate energy charge returns rapidly to normal in kidney [73] and in heart. In man, ^{31}P nuclear magnetic resonance (NMR) provides similar evidence for brain. Changes are probably located in the plasma membrane and/or cytosol which are more sensitive than mitochondria and lysosomes to damage after ATP depletion [74–76].

4.4. Asphyxial damage and sources of problems in interpretation

Many of the apparent conflicts in the definition of asphyxia can be resolved by considering the chronological development of asphyxial damage. At the centre of the process is asphyxia itself; from first principles asphyxia can be defined at the molecular level as a failure of energy supply sufficient to cause cellular damage. As the energy currency of cells is the purine nucleotide ATP the definition is 'ATP depletion sufficient to cause irreversible or partially reversible cellular damage' [77]. Recent reviews [77–79] provide an entry to the extensive literature. The initial biochemical events occur rapidly (in seconds and minutes) but irreversible damage may only occur after 30 min or more [80].

In practice, clinicians often use a variety of predisposing factors like maternal hypertension in pregnancy. Precipitating factors like ischaemia, hypoxia or hypoglycaemia are used by the physiologists. The body tissues and cells respond to such precipitating factors by a series of compensatory mechanisms like increasing the heart rate, vasodilatation, tachypnoea and hormonal release; inside tissues and cells phosphocreatine and glycogen mobilisation and metabolism occur with or followed by a reduction of ATP use by diminished function (seen perhaps as loss of consciousness or a reduced growth rate). Compensatory alterations of cellular metabolism occur reducing some functions like growth and related processes like protein synthesis; the switching of gene action in the acute phase and heat shock reactions also occurs. Such compensation may be adequate to avoid any marked fall in cellular ATP in which case no immediate damage may follow: this seems to be the case early in toxaemia of pregnancy [70]. If compensation is inadequate a marked fall in intracellular ATP occurs and hypoxanthine concentrations rise inside and outside the cell. Only if this fall is marked and prolonged will the next stage of cellular damage occur. It seems to be the duration of anoxia relative to the 'irreducible' energy requirements (related to temperature) which determine the reversibility of tissue changes. There is plasma membrane damage with leakage of cytosolic components like transaminases into plasma and loss of cellular volume control seen clinically as oedema especially in brain; such defects of osmoregulation may involve taurine [81]. This is the phase of the events described as reperfusion damage. Later cellular necrosis may excite an inflammatory reaction which will certainly produce oxidative and proteolytic damage. Healing may then occur or fibrosis or even cyst formation with total loss of tissue.

Such a defect may still be concealed by compensatory mechanisms especially in a resting nursed infant; the degree of residual function immediately after as-

phyxia is some guide to eventual recovery. All these steps are subject to a series of independent environmental and genetic controls. In addition the process can be halted at any stage.

Increased levels of hyp in extracellular fluids reflect ATP depletion a central process in the above sequence (Fig. 9) and the later increased excretion of hyp and xan (Fig. 11) seems to reflect tissue damage, a later consequence of marked ATP depletion. We can thus estimate changes at these two stages from hyp and xan measurements. However, the independent process of compensation for the defect will be a major factor in determining the eventual functional impairment. Prognosis is still very imprecise.

In future it seems probable that hyp and xan measurements may be used to definitively exclude asphyxia in newborn at risk and thus concentrate a search for other causes of abnormality.

4.5. Problems in complex situations

Increased oxypurine excretion and raised plasma urate concentrations occur after marked hypotensive episodes in adults although quantitative links were difficult to establish in one study [82]. Similar problems in interpretation occur in very ill newborn but the analysis of hyp and xan separately does simplify patterns. The complexity of the controls altering urate excretion [83] can make plasma urate changes difficult to interpret in complex clinical situations. The above failure to understand quantitative links in these complex situations was to be expected.

4.6. Blood

A future use of plasma hyp could be to regulate artificial ventilation or circulatory pumps as blood gas and pH concentrations are currently used. Since increased oxygen removal and anaerobic glycolysis might be effective in maintaining tissue ATP despite 'abnormal' pH and pO_2 and even pCO_2 measurements, ventilation and pumping rates might be justifiably decreased if plasma hyp were normal but other variables given above were abnormal.

Another possible application of plasma hyp is in defining the concentration of glucose in plasma which is adequate to maintain cellular energy stores. There is a problem in defining hypoglycaemia in newborn who seem to tolerate lower levels than adults. A working definition of hypoglycaemia could be 'concentration of glucose in plasma which is just not capable of maintaining tissue (brain) ATP concentrations'. Practically, if the plasma hyp rises then the glucose concentration is inadequate. However, such applications remain for the future.

5. SUMMARY

Measurements of hyp, xan and urd in body fluids can provide evidence of energy, ATP, depletion in the body, in organs or in cells. Such information is clinically useful in the many diseases in which cellular energy supplies cannot be maintained like perinatal asphyxia, hydrocephalus and vascular insufficiency in brain, heart, limbs, kidneys or other organs.

Similar HPLC methods using reversed-phase C_{18} columns and quantitation by UV absorption have been employed in a variety of centres to yield almost identical results. These have been assembled in this review to form a series of reference values. The current analytical problems are reviewed. Since concentrations of hyp and xan may alter independently situations are discussed in which separate measurements rather than their summed, total oxypurine concentrations are needed.

The biochemistry and physiology underlying the use of such analyses is examined to guide sampling of the appropriate body fluid at a relevant time and to avoid oversimplified interpretation of results as well as unnecessary arguments.

Specifically:

(1) Intracellular concentrations of hyp and xan are inversely related to adenylate energy change and therefore to the energy currency of the cell ATP. Uridine in tissues is similarly 'controlled'.

(2) There is extensive evidence that large increases in hyp, xan and urd in body fluids indicate ATP depletion.

(3) Small changes in hyp probably reflect alterations of ATP turnover.

(4) Xanthine arises mainly from guanine and can change independently of hyp.

(5) Clinically useful information is obtainable from hyp and xan concentrations in CSF, amniotic fluid, urine and plasma. Extensive clinical correlations are reviewed.

At present we are in a development phase for which HPLC is ideal but the most efficient way to perform and use such analyses in routine clinical practice remains to be established.

REFERENCES

1 D.E. Atkinson, Cellular Energy Metabolism and its Regulation, Academic Press, New York, 1977.
2 A.W. Murray, Ann. Rev. Biochem., 40 (1971) 811.
3 J.E. Seegmiller, in P.K. Bondy and L.E. Rosenberg (Editors), Metabolic Control and Disease, Saunders, Philadelphia, PA, 1980, p. 777.
4 R.O. McKeran and R.W.E. Watts, in J.L.H. O'Riordan (Editor), Recent Advances in Endocrinology and Metabolism, Churchill-Livingstone, Edinburgh, 1978, Ch. 10, p. 219.
5 R.W.E. Watts, in S.S. Brown, F.L. Mitchell and D.S. Young (Editors), Chemical Diagnosis of Disease, Elsevier, Amsterdam, 1979, Ch. 21, p. 1059.

6 J.B. Stanbury, J.B. Wyngaarden, D.S. Frederickson, J.L. Goldstein and M.S. Brown, The Metabolic Basis of Inherited Disease, McGraw-Hill, New York, 1983, Part 7, pp. 1043–1226.
7 R.C. Simpson and P.R. Brown, J. Chromatogr., 379 (1986) 269.
8 R.A. Harkness and R.J. Simmonds, in A.M. Krstulović (Editor), Handbook of Chromatography: Nucleic Acids and Related Compounds, CRC Press, Boca Raton, FL, in press.
9 J.C. Giddings, E. Grushka and P.R. Brown, Advances in Chromatography, Vols. 1–27, Marcel Dekker, New York, 1965–1987.
10 O.D. Saugstad, Pediatr. Res., 9 (1975) 575.
11 M.C. O'Connor, R.A. Harkness, R.J. Simmonds and F.E. Hytten, Br. J. Obstet. Gynaecol., 88 (1981) 381.
12 O.D. Saugstad, Perinatol. Neonatol., 1 (1987) 25.
13 D. Gardiner, Anal. Biochem., 95 (1979) 377.
14 M.H. Jensen and S. Jorgensen, Biol. Neonate, 32 (1977) 197.
15 R.A. Harkness, A.G.L. Whitelaw and R.J. Simmonds. J. Clin. Pathol., 35 (1982) 999.
16 H.K. Manzke, K. Dorner and J. Grunitz, Acta Paediatr. Scand., 66 (1977) 713.
17 I. Laing, J.K. Brown and R.A. Harkness, J. Clin. Pathol., (1988) in press.
18 K. Nakano, S.P. Assenza and P.R. Brown, J. Chromatogr., 233 (1982) 51.
19 R.A. Hartwick, D. Van Haverbeke, M. Mikeoy and P.R. Brown, J. Liq. Chromatogr., 2 (1979) 725.
20 R.J. Simmonds and R.A. Harkness, J. Chromatogr., 226 (1981) 369.
21 K.W. Gehrke, G.E. Kuo, Q.E. Davis, R.D. Suits, T.P. Walker and E. Borek, J. Chromatogr., 150 (1978) 455.
22 R.A. Hartwick and P.R. Brown, J. Chromatogr., 126 (1976) 679.
23 P.R. Brown, Cancer Invest., 1 (1983) 527.
24 J.F. Kuttesch, F.C. Schmalstieg and J.A. Nelson, J. Liq. Chromatogr., 1 (1978) 97.
25 F. Niklasson, Acta Univ. Ups., (1983) 473.
26 J.L. Glajch, J.J. Kirkland, K.M. Squire and J.M. Minor, J. Chromatogr., 199 (1980) 57.
27 G.A. Taylor, P.J. Dady and K.R. Harrap, J. Chromatogr., 183 (1980) 421.
28 F. Overzet, A. Rurvak, H. van der Voet, B.F.H. Drenth, R.T. Ghijsen and R.A. de Zeeuw, J. Chromatogr., 267 (1983) 329.
29 O. Skold, J. Biol. Chem., 235 (1960) 3273.
30 T.A. Krenitsky, M. Barclay and J.A. Jacquez, J. Biol. Chem., 239 (1964) 805.
31 J.E. Mrochek, S.R. More and J.T.P. Waalkes, J. Natl. Cancer Inst., 53 (1974) 1553.
32 D. Perrett, J. Chromatogr., 386 (1987) 289.
33 A.M. Krstulović, L.M. Bertani-Dziedzic, S.E. Gitlow and K. Lohse, J. Chromatogr., 164 (1979) 363.
34 R.C. Simpson and P.R. Brown, Int. Lab., 17 (1987) 20.
35 R.A. Harkness, S.B. Coade, R.J. Simmonds and S. Duffy, Placenta, 6 (1985) 199.
36 R. Boulieu, C. Bory, P. Baltassat and C. Gonnet, Anal. Biochem., 129 (1983) 398.
37 T.A. Krenitsky, T. Spector and W.W. Hall, Arch. Biochem. Biophys., 247 (1986) 108.
38 R.A. Harkness, S.B. Coade and A.D.B. Webster, Clin. Chim. Acta, 143 (1984) 91.
39 G. Burnstock, Purinergic Receptors, Chapman and Hall, London, 1981.
40 M.H.N. Tattersal, P. Slowiaczer and A. Defazio, J. Lab. Clin. Med., 109 (1983) 411.
41 J. Ontyd and J. Schrader, J. Chromatogr., 307 (1984) 404.
42 H. Gewirtz, P.R. Brown and A.S. Most, Proc. Soc. Exp. Biol. Med., 185 (1987) 93.
43 R.A. Harkness, G.M. McCreanor, D. Simpson and I. MacFadyen, J. Inher. Metab. Dis., 9 (1986) 407.
44 R.W.E. Watts, R.A. Harkness, E. Spellacy and N.F. Taylor, J. Inher. Metab. Dis., 10 (1987) 206.
45 M.G. McCreanor, R.A. Harkness and R.W.E. Watts, Biochem. Soc. Trans., 15 (1987) 555.
46 R.A. Harkness, G.M. McCreanor and R.W.E. Watts, J. Inher. Metab. Dis., 11 (1988) in press.

47 R.A. Harkness, S.B. Coade, K.R. Walton and D. Wright, J. Inher. Metab. Dis., 6 (1983) 114.
48 S.P.T. Sinkeler, E.M.G. Joosten, R.A. Wevers, R.A. Birkhorst, F.T. Oerlmans, C.A. van Bennekom, M.M. Coerwinkel and T.L. Oei, Clin. Sci., 70 (1986) 399.
49 H.R. Scholte, H.F.M. Buoch, A.E.F.H. Meijer, I.E.M. Luyt-Houwen, M.H.M. Vaandrager-Verduin, Klin. Wochenschr., 65 (Suppl. X) (1987) 26.
50 I.H. Fox, J. Lab. Clin. Med., 106 (1985) 101.
51 L.D. Fairbanks, H.A. Simmonds and D.R. Webster, J. Inher. Metab. Dis., 10 (1987) 174.
52 J.C. Crawhall, K. Itiaba and S. Katz, Biochem. Med., 30 (1983) 261.
53 R.A. Harkness and R.J. Lund, J. Clin. Pathol., 36 (1983) 1.
54 D.V.I. Fairweather and T.K.A.B. Eskes, Amniotic Fluid Research and Clinical Application, Excerpta Medica, Amsterdam, 1978.
55 R.A. Harkness, R.T. Geirsson and I.R. McFadyen, Br. J. Obstet. Gynaecol., 90 (1983) 815.
56 R.A. Harkness, P.M. Cotes, H. Gordon, N. McWhinney and P. Sarkar, J. Obstet. Gynaecol., 8 (1988) 235.
57 R.A. Harkness, P. Purkiss, S. Duffy, R.A. Chalmers and M. Jones, J. Inher. Metab. Dis., 11 (1988) 103.
58 R.A. Harkness, R.J. Simmonds and S.B. Coade, Clin. Sci., 64 (1983) 333.
59 R.A. Harkness, R.J. Simmonds, S.B. Coade and C.R. Lawrence, Br. J. Obstet. Gynaecol., 90 (1983) 447.
60 J. Barankiewicz, E.W. Gelfand, A. Issekutz and A. Cohen, J. Biol. Chem., 257 (1982) 11597.
61 R. Hallgren, F. Niklasson, A. Terent, A. Akerblom and E. Widerlov, Stroke, 14 (1983) 382.
62 R. Bejar, O.D. Saugstad, H. James and L. Gluck, J. Pediatr., 103 (1983) 44.
63 S.D. Levin, J.K. Browm and R.A. Harkness, J. Neurol. Neurosurg. Psychiatry, 47 (1984) 730.
64 M. Castro-Gago, S. Lojo, R. Del Rio Rodriguez, I. Novo and S. Rodriguez-Segde, Child's. Nerv. Syst., 2 (1986) 109.
65 G. Ronquist, T. Callerud, F. Niklasson and G. Firiman, Infect. Immun., 48 (1985) 729.
66 J.H. Livingston, J.K. Brown, R.A. Harkness and G.M. McCreanor, Dev. Med. Child Neurol., (1988) in press.
67 H. Agren, F. Niklasson and R. Hallgren, Psychiatr. Res., 9 (1983) 179.
68 J.H. Livingston, J.K. Brown, R.A. Harkness and G.M. McCreanor, Dev. Med. Child Neurol., (1988) in press.
69 M.C. O'Connor, R.A. Harkness, R.J. Simmonds and F.E. Hytten, Br. J. Obstet. Gynaecol., 88 (1981) 375.
70 J.H. Smith, K.J.S. Anand, P.M. Cotes, G.S. Dawes, R.A. Harkness, T.A. Howlett, L.H. Rees and C.W.G. Redman, Br. J. Obstet. Gynaecol., (1988) in press.
71 G.M. McCreanor and R.A. Harkness, Biochem. Soc. Trans., 15 (1988) 1060.
72 T. Kojima, T. Kobayashi, S. Matsuzaki, S. Wrase and Y. Kovayashi, Arch. Dis. Childh., 60 (1985) 908.
73 C.T. Warnick and H.M. Lazarus, Can. J. Biochem., 59 (1981) 116.
74 D.A. Whalen, D.G. Hamilton, C.E. Granote and R.B. Jennings, Am. J. Pathol., 74 (1984) 381.
75 R.A. Kloner, C.E. Granote, D.A. Whalen and R.B. Jennings, Am. J. Pathol., 74 (1974) 399.
76 J.W. Parce, P.I. Spach and C.C. Cunningham, Biochem. J., 188 (1980) 817.
77 R.A. Harkness, J. Clin. Pathol., 40 (1987) 1128.
78 S.P. Soltoff, Ann. Rev. Physiol., 48 (1986) 9.
79 D.P. Jones, Ann. Rev. Physiol., 48 (1986) 33.
80 D.J. Hearse, Contrib. Gynaecol. Obstet., 13 (1985) 48.
81 R.W. Chesney, Adv. Pediatr., 32 (1985) 1.
82 J.O. Woolliscroft and I.H. Fox, Am. J. Med., 81 (1986) 472.
83 F. Lang, in R. Greger, F. Lang and S. Silbernagel (Editors), Renal Transport of Organic Substances, Springer, Berlin, 1981, p. 234.
84 A. McBurney and T. Gibson, Clin. Chim. Acta, 102 (1980) 19.
85 W.E. Wung and S.B. Howell, Clin. Chem., 26 (1980) 1704.
86 E. Harmsen, J.W. de Johl and P.W. Serruys, Clin. Chim. Acta, 115 (1981) 73.
87 P. Gerrits, A. Haagen, R. DeAbreu, L. Monnens, F. Gabreels, F. Trijbels and A. Theeuwes, Klin. Wochenschr., 65 (Suppl. X) (1987) 16.