Journal of Chromatography, 426 (1988) 249-258
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4075

DEMONSTRATION OF THE ADENOSINE RESERVOIRS WITH NITROBENZYLTHIOINOSINE IN LIVER AND DIAPHRAGM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

RONALD L. JENKINS* and HUEY G. McDANIEL

*Department of Medicine, University of Alabama in Birmingham, Birmingham, AL 35294 (U.S.A.) and *VA Medical Center, 700 19th Avenue S. Birmingham, Birmingham, AL 35233 (U.S.A.)*

WILLIAM GRIZZLE

Department of Pathology, University of Alabama in Birmingham, Birmingham, AL 35294 (U.S.A.) and VA Medical Center, Birmingham, AL 35233 (U.S.A.)

S. WAYNE PARRISH

VA Medical Center, Birmingham, AL 35233 (U.S.A.)

and

HUEY B. McDANIEL

Department of Medicine, University of Alabama in Birmingham, Birmingham, AL 35294 (U.S.A.)

(First received August 24th, 1987; revised manuscript received December 1st, 1987)

SUMMARY

Purine nucleotides, nucleosides, nucleobases, dinucleotides and nucleosides derivatives from acid-extracted rat liver and diaphragm were separated and quantitated by reversed-phase ion-pair high-performance liquid chromatography with a mobile phase composed of 90 m*M* potassium phosphate, 15 m*M* tetrabutylammonium hydroxide and a 1-30% methanol gradient. During 5 min of ischemia, adenine and guanine nucleotides decreased along with significant declines in NAD and increases in adenosine, inosine, hypoxanthine, xanthine, NADP and adenylosuccinate. Nitrobenzylthioinosine by gavage (5 mg/kg per day for five days) increased adenosine levels but without any alteration in nucleobase levels. Adenosine was shuttled to every available intracellular reservoir which included in declining order of magnitude GDP > adenosylhomocysteine > adenosine > ADP > AMP > IMP = XMP = GMP.

INTRODUCTION

Accurate quantitation of cellular nucleotides, especially that of adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP) and adenosine 5′-monophosphate (AMP) are critical in the evaluation of the energy status of living tissues. High-performance liquid chromatographic (HPLC) techniques have en-

 © 1988 Elsevier Science Publishers B.V.

abled excellent separations by reversed phase [1], anion exchange [2] and normal phase [3]. Kamiike et al. [4] used cation-exchange columns to separate nucleotide metabolites and anion exchange to separate the nucleotides. The use of ion-pairing agents with reversed-phase columns has provided better resolutions of nucleotides; the larger the alkyl component (tetrabutyl > ethylbutyl > methylbutyl) of the ion-pairing agent the better the resolution [5].

With this in mind we have investigated the use of tetrabutylammonium (TBA) as an ion-pairing agent. With precise manipulation we demonstrate that not only can the adenine and guanine nucleotides be separated but also their metabolites, the dinucleotides (NAD and NADP), and various nucleotide derivatives (adenosylhomocysteine, adenylosuccinate and adenosylmethionine) from acid-extracted tissues. Schrader et al. [6] identified adenosylhomocysteine as a potential reservoir for adenosine following adenosine transport blockade with nitrobenzylthioinosine (NBMPR) [7]. This HPLC methodology was used to investigate the nucleoside reservoirs in liver and diaphragm in response to ischemia and adenosine transport blockade with NBMPR and to correlate the changes in nucleoside pools with nucleotide pools.

EXPERIMENTAL

Chemicals

Nucleotides, nucleosides, nucleobases, dinucleotides and adenosine derivatives which were used as standards were obtained from Sigma (St. Louis, MO, U.S.A.) and purity was verified by HPLC. Perchloric acid which was used in tissue extraction and the solvent consitutents, which were potassium phosphate monobasic, tetrabutylammonium hydroxide (TBAH) and methanol, were HPLC grade and were obtained from Fisher Scientific (Norcross, GA, U.S.A.). The purity of the water was of the highest grade (18 MΩ) and was produced in-house.

NBMPR administration

NBMPR was suspended in a 15% ethanol solution containing 25 m*M* phosphate buffer, pH 7.4. Male Fisher 334 strain rats received 5 mg/kg body weight NBMPR for five consecutive days by gavage. Male liter mates served as controls and received equal volumes by weight of the ethanol phosphate buffer lacking NBMPR.

Extraction of nucleotides from animal tissues

Liver and diaphragm were excised from stunned male Fisher-334 rats (250–300 g) and frozen immediately in liquid nitrogen (−195°C) minimizing postmortem changes [8]. Ischemia was induced in excised tissues by incubation in phosphate buffer (pH 7.4) at room temperature prior to freezing in liquid nitrogen. Frozen tissues were thoroughly pulverized with frozen 6% perchloric acid and dry ice in a chilled mortar and pestle. Once thawed, samples were centrifuged to remove precipitated proteins and then again to remove perchlorate following addition of

potassium hydroxide 4 *M* to a pH of 6. Tissues were usually extracted to 100 mg wet weight per ml of final extract.

High-performance liquid chromatography

The equipment used (Rainin Instrument) consisted of two pumps which were controlled in unison by computer, an SSI injector and in-line filtration unions, and an ISCO UA 5 ultraviolet detector equipped with 254 and 280-nm filters. Integration of peak areas was automatically effected by a Hewlett-Packard 3380A integrator. Samples and standards (1.0 nmol) were delivered through a 20-μl loop at 1.0 ml/min flow-rate and separated on a standard prepacked 5-μm C_{18} column (10 cm$\times$4.6 mm) (Rainin Instrument, Woburn, MA, U.S.A.). Optimal ion-pairing solvent conditions for the adenine nucleotides and adenosine (Ado) were determined in an isocratic system in which phosphate, tetrabutylammonium, methanol and pH were systematically varied. Complex mixtures of standards and tissue extracts were separated by a 25-min linear gradient from 1 to 30% methanol in 90 m*M* phosphate and 15 m*M* TBAH.

Peak identification

Initial qualification of tissue extract constituents was based on comparison of peak retention times to that of standards. Positive peak identification in tissue extracts was by either (1) conversion of the unknown peaks in tissue extracts to peaks corresponding to the product(s) of enzymatic reactions from purified enzymes, and/or (2) 250 nm/260 nm absorbance ratios of collected peaks following HPLC elution. Enzymes used in peak identification included pyruvate kinase and adenylate kinase for the di- and triphosphate nucleosides, adenosylhomocysteine hydrolase for adenosylhomocysteine, adenylosuccinate synthetase for IMP, adenylosuccinate lyase for adenylosuccinate, and adenosine deaminase for adenosine.

RESULTS

Effect of the solvent parameters

Fig. 1A illustrates the dependency of the retention of adenine nucleotides on a C_{18} column on the concentration of the ion-pairing agent. In the absence of TBAH in 100 m*M* phosphate, none of the nucleotides were resolved. Increasing TBAH increases nucleotide retention: triphosphate > diphosphate > monophosphate. The monophosphate could not be resolved from the nucleoside with less than 5 m*M* TBAH in 100 m*M* phosphate and the best separation of nucleotides occurred in excess of 15 m*M* TBAH.

Decreasing phosphate levels in the solvents at a constant concentration of TBAH increased nucleotide retention but with little effect on the nucleoside. In 10 m*M* TBAH, AMP and Ado were not resolved above 130 m*M* phosphate while ADP and ATP were eluted with difficulty below 50 m*M* phosphate (Fig. 1B). Phosphate ions clearly compete with the nucleotide phosphate groups for the TBA ion.

In 100 m*M* phosphate and 15 m*M* TBAH, methanol can be used to systemat-

ically elute the adenine nucleotides. The resolution of Ado from AMP improved with methanol concentration less than 20%. However, ADP and ATP eluted poorly below 20% methanol (Fig. 1C). In isocratic conditions with a mobile phase composed of 100 m*M* phosphate, 15 m*M* TBAH and 20% methanol, optimal separations were obtained at a pH of 5.9 due to the p*K* of the phosphate system. Extremes toward pH 3 or 8 progressively worsened resolution (Fig. 1D). Increasing the pH above 5.9 increased the charges of the phosphate buffer and thus decreased the effectiveness of the TBA ion. Under more acidic conditions nucleotide retention was decreased by a poorer affinity between the protonated nucleotide and the TBA ion. At and beyond pH 3.7, protonation at the N-1 position within the adenine ring further repelled nucleotides and nucleosides from the TBA ion and thus further decreased retention times.

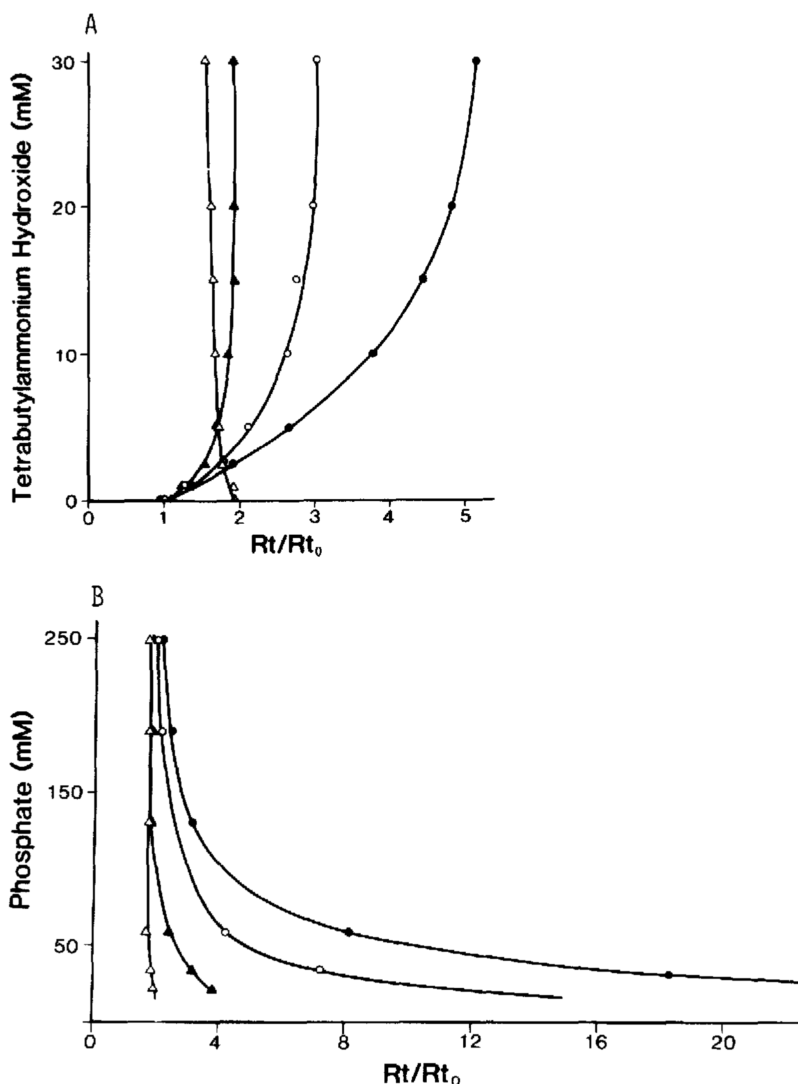

Fig. 1.

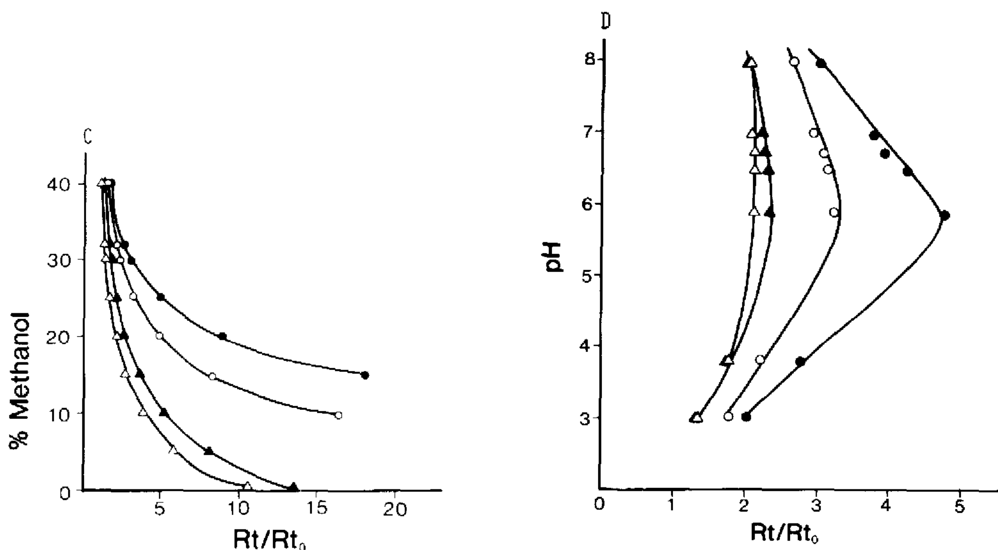

Fig. 1. Peak (1.0 nmol per injection) retention time/solvent front retention (Rt/Rt_0) of ATP (●), ADP (○), AMP (▲) and adenosine (△) in a C_{18}, ion-paired isocratic system under variable solvent conditions with a 1.0 ml/min flow-rate. (A) The concentration of tetrabutylammonium hydroxide was varied from 0 to 30 m*M* with 100 m*M* potassium phosphate and 20% methanol, pH 5.9. (B) Potassium phosphate (pH 5.9) was varied from 20 to 250 m*M* with 15 m*M* TBAH and 20% methanol. (C) Methanol series from 0 to 40% in the presence of 100 m*M* potassium phosphate (pH 5.9) and 15 m*M* TBAH. (D) The effect of solvent pH from 3.0 to 8.0 on the Rt/Rt_0 of adenine nucleotides and side in the presence of 100 m*M* potassium phosphate, 15 m*M* TBAH and 20% methanol.

Separation of nucleotides, nucleosides, nucleobases and nucleoside derivatives in liver

Adenine nucleotides from acid-extracted rat liver were separated with a solvent composed of 90 m*M* potassium phosphate, 15 m*M* TBAH and 20% methanol. Incorporation of a methanol gradient (1–30%) improved nucleotide separation and afforded complete separation of the purine nucleosides and bases (Fig. 2). NAD(H) and NADP(H) are simultaneously quantified. However, the reduced dinucleotides are largely destroyed during acid extraction of the tissues. Adenosylmethionine (AdM) and adenosylhomocysteine (AdH), which are important methyl donor and acceptor compounds, and adenylosuccinate (AdS), which is an intermediate in the (re)synthesis of AMP, are also separated and detected by UV absorbance.

The effects of 5 min of ischemia on the liver nucleotide and nucleoside levels are shown in Table I and Fig. 3. As reported elsewhere [4], ATP levels decreased by the dephosphorylation to AMP which in turn was eliminated as adenosine and subsequently inosine, hypoxanthine, xanthine and uric acid. Guanosine nucleotides followed this same route of degradation. NAD and NADP behaved independently during ischemia, with the former decreasing and the latter increasing significantly. Adenylosuccinate was significantly increased in ischemic liver but the other adenosine derivatives (AdM and AdH) were not altered during ischemia despite significant elevations of tissue AMP and adenosine levels.

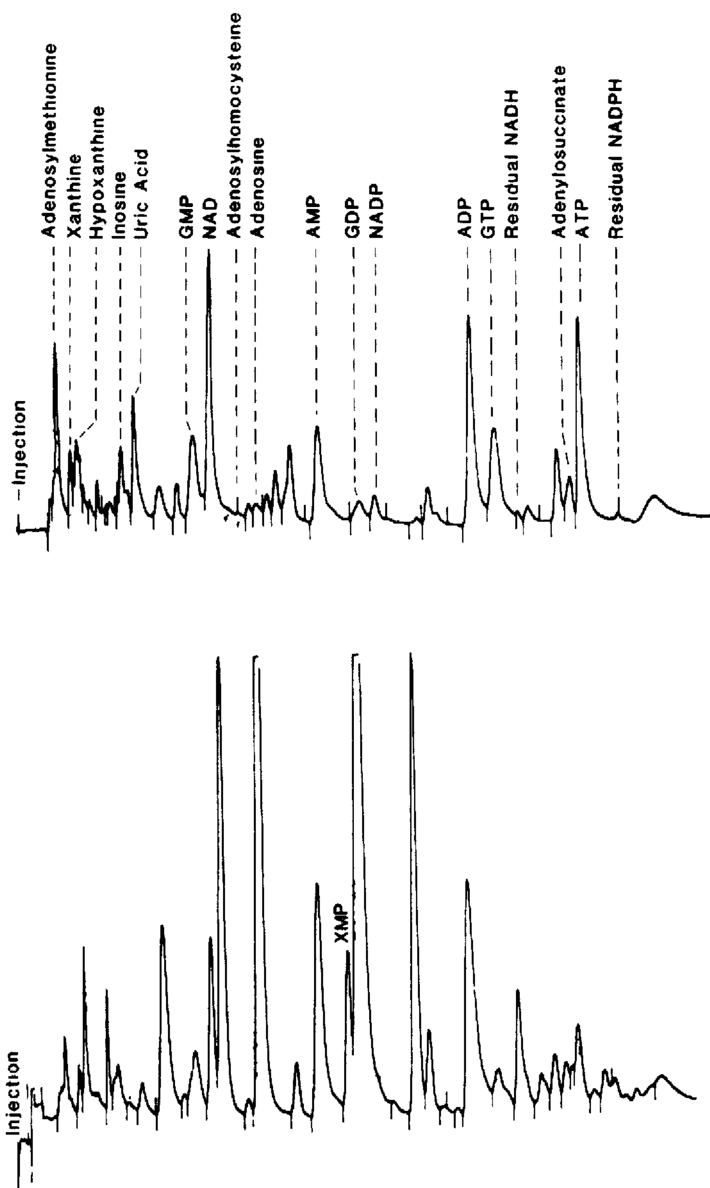

Fig. 2. Separation of adenine nucleotides, nucleosides, nucleobases, dinucleotides and adenosine derivatives from perchloric acid-extracted rat liver in a methanol (1 to 30% in 22 min) linear gradient with 90 m*M* potassium phosphate and 15 m*M* TBAH on a reversed-phase (C_{18}) (4.6×100 mm) column. The loop volume was 20 μl and the flow-rate 1.0 ml/min. Detection was at 254 nm, absorbance, 0.05 a.u.f.s. The upper chromatogram was from the liver of an untreated rat and the lower chromatogram was from a rat given oral nitrobenzylthioinosine (5 mg/kg per day for five days).

The metabolic inhibitor, NBMPR, which blocks adenosine transport [6], caused marked elevations of adenosine (Table II). This, in turn, expanded every possible reservoir for adenosine: adenosylhomocysteine from adenosylhomocys-

TABLE I

MEAN ADENOSINE, NAD, NADP, ADENYLOSUCCINATE, ADENOSYLMETHIONINE AND ADENOSYLHOMOCYSTEINE LEVELS IN RAT LIVER DURING 5 MIN OF ISCHEMIA

Analysis by ion-pair C_{18} HPLC (see text).

Ischemia (min)	Concentration (mean ± S.D., $n=5$) (nmol/g tissue, wet weight)					
	Adenosine	NAD	NADP	AdM	AdH	AdS
0.5	9 ± 10	637 ± 87	45 ± 13	38 ± 29	26 ± 27	21 ± 6
1.0	22 ± 9	620 ± 89	51 ± 18	39 ± 54	24 ± 24	31 ± 20
2.0	42 ± 24	577 ± 85	54 ± 15	35 ± 18	24 ± 31	28 ± 9
3.0	46 ± 42	562 ± 89	69 ± 24	31 ± 38	30 ± 22	34 ± 16
5.0	50 ± 27	516 ± 99	74 ± 33	33 ± 49	21 ± 24	59 ± 4
$P<0.05$*	S	S	S	NS	NS	S

*S = significant difference, NS = non-significant difference.

teine hydrolase activity, AMP from adenosine kinase activity, IMP from adenylate deaminase activity and ADP from adenylate kinase activity. Adenylosuccinate and adenosylmethionine levels were not affected by rising adenosine and thus did not serve as adenosine reservoirs. Moreover, the metabolites of adenosine, which included inosine, hypoxanthine, xanthine, and uric acid, were not elevated above the normal level despite a 200-fold increase in adenosine. Interestingly, while total adenine nucleotides (Σ AMP+ADP+ATP) were not significantly elevated, total guanine nucleotides (Σ GMP+GDP+GTP) were elevated over 7-fold. The energy expended to handle the accumulation of adenosine was manifested by a 20-fold drop in purine triphosphate/diphosphate ratio.

The effects of NBMPR on the diaphragm were markedly different from the liver. Adenosine was not elevated above controls nor were the conventional nu-

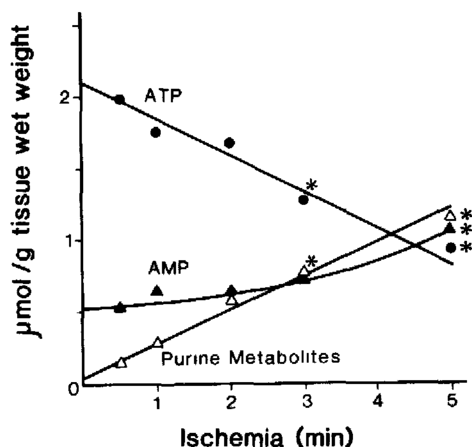

Fig. 3. ATP (●), AMP (▲) and Σ purine metabolites (adenosine, inosine, hypoxanthine, xanthine and uric acid) (△) in rat liver during 5 min of ischemia. Each point is the mean of five samples. Asterisks indicate significant differences ($P<0.05$) from the extrapolated value at 0-min ischemia.

TABLE II

EFFECTS OF NITROBENZYLTHIOINOSINE (NBMPR) (5 mg/kg PER DAY FOR FIVE DAYS) ON RAT LIVER AND DIAPHRAGM NUCLEOTIDES, NUCLEOSIDES, NUCLEOBASES AND ADENOSINE DERIVATIVES

Values (mean ± S.D.) are expressed as μmol/g tissue wet weight. N.D. = not detectable; AdS = adenylosuccinate; AdHc = adenosylhomocysteine; AdM = adenosylmethionine; ΣI, Hx, X U = Σinosine, hypoxanthine, xanthine, uric acid.

Compound	Liver		Diaphragm	
	Control	NBMPR	Control	NBMPR
ATP	2.1 ±0.4	1.7 ±0.7*	4.6 ± 0.7	3 1 ±0.9
ADP	0.7 ±0.2	2.4 ±0.8*	0.7 ± 0.09	0.7 ±0.07
AMP	0.5 ±0.2	1.7 ±0.2*	0.4 ± 0.2	0.4 ±0.06
Adenosine	0 02±0.01	3.3 ±1.6*	0.04± 0.02	0.06 ±0.02
AdHc	0.04±0.04	3.7 ±1.6*	N.D.	0.05 ±0.11
AdM	0.78±0.24	0.15±0.04*	0.07± 0.004	0.27 ±0.09
AdS	0.04±0.01	0.03±0.02	0.01± 0.004	0.012±0.006
ΣI, Hx, X, U	0.52±0.38	0.61±0.51	0.41± 0.18	0.52 ±0.17
GTP	0.98±0.27	0.26±0.06*	0.2 ± 0.06	0.2 ±0 09
GDP	0.13±0.16	7.53±2.60*	0.04± 0.013	0.07 ±0.05
GMP	0.13±0.11	0.30±0.11*	0.03± 0.04	0.03 ±0.02
IMP	0.07±0.04	0.30±0.04*	0.40± 0.16	1.07 ±0.50*
XMP	0.02±0.02	0.50±0.24*	N.D.	N.D.
Total Ad/Gu Nt	2.80±0.87	0.67±0.13*	27.21±13.5	20.34 ±6.01
Tri/dinucleotides	3.71±0.63	0.15±0.02*	6.40± 0.74	4.05 ±0.51*

*$P < 0.05$.

cleoside reservoirs of liver observed in the diaphragm. The primary adenosine reservoir proved to be IMP, which increased 2.5-fold at the expense of ATP probably from the activity of adenosine kinase to yield AMP, which was deaminated to IMP by adenylate deaminase (Table II).

DISCUSSION

HPLC separation of nucleotides on a C_{18} column with ion pairing is gaining popularity among nucleotide studies. Solvent conditions were presented which allowed the separation of the purine nucleotides, nucleosides and nucleobases. Additionally, AdM, AdH, and AdS, essential in the analysis of potential adenosine reservoirs, were also quantified.

Following 5 min of ischemia, liver nucleotides were lost with a 2-fold accumulation of adenosine. This nucleoside was rapidly deaminated to inosine which was further degraded to hypoxanthine, xanthine and uric acid [4]. It is most probable that adenosine formation from 5′-nucleotidase is closely linked to its transport out of the hepatocyte. Thus, during ischemia adenosine does not accumulate within the hepatocyte but rather extracellularly or within endothelial or erythrocytes where abundant catabolic enzymes (adenosine deaminase, nucleoside phosphorylase, and xanthine oxidase) reside [9].

In contrast to ischemia, the oral administration of NBMPR elevated adenosine levels by 200-fold. As previously proposed [10–12], NBMPR binding is highly variable between species and tissues but it affects intracellular adenosine by inhibiting adenosine transport out of the cell. Thus, the rise in intracellular adenosine is the result of normal adenosine turnover. The fact that adenosine catabolites were not elevated indicated that adenosine accumulation was intracellular and not extracellular, as during ischemia. Moreover, this adenosine was not degraded to uric acid but rather dispersed to every possible storage site. The noted sites for adenosine storage in declining order of magnitude were guanine nucleotides (GDP) > adenosylhomocysteine > adenosine > ADP > AMP > IMP = XMP = GMP. The fact that guanine nucleotides rose so markedly in NBMPR-treated livers indicated either that guanosine transport was affected by NBMPR as is adenosine transport or that elevated AMP levels are converted to XMP and then to GMP through the activities of IMP dehydrogenase (EC 1.2.1.14) and GMP synthetase (EC 6.3.4.1) [13]. Significant elevation in XMP but not guanosine indicated an active AMP–IMP–XMP–GMP interconversion. Adenosylhomocysteine levels were increased by the activity of adenosylhomocysteine hydrolase and would be limited by the availability of L-homocysteine [14]. The rise in ADP, AMP and IMP levels following NBMPR was related to the activity of adenosine kinase and adenylate deaminase.

The results obtained from the diaphragms of NBMPR-treated rats indicated that either the affinity of NBMPR for the nucleoside transport was much less than in liver or that the obligatory rate of adenosine turnover was much less. In either case, the adenosine reservoir was limited to IMP and AMP, respectively. A meager and variable rise of adenosylhomocysteine indicated that adenosylhomocysteine hydrolase, although present, was limiting.

ACKNOWLEDGEMENTS

Acknowledgement is made to the American Diabetes Association and to the Veterans Administration for their financial support.

REFERENCES

1 E.A. Hull-Ryde, R.G. Cummings and J.E. Lowe, J. Chromatogr., 275 (1983) 411.
2 J.R. Miksic and P.R. Brown, J. Chromatogr., 142 (1977) 641.
3 K. Sugino, K. Yamada and T. Kawasaki, J. Chromatogr., 361 (1986) 427.
4 W. Kamiike, F. Watanabe, T. Hashimoto, K. Tagawa, Y. Ikeda, K. Nakao and Y. Kawashima, J. Biochem., 92 (1982) 1349.
5 T F. Walseth, G. Graff, M.C. Moos and N.G. Goldberg, Anal. Biochem., 107 (1980) 240.
6 J. Schrader, W. Schutz and H. Bardenheuer, Biochem. J., 196 (1981) 65.
7 A R.P. Patterson, S. Yang, E Y Lau and C.E. Cass, Mol. Pharmacol., 16 (1979) 900.
8 A. Wollenberger, O. Ristau and G. Schoffa, Pfluegers Arch. Gesamte Physiol. Menschen Tierre, 270 (1960) 399.
9 F.L. Belloni, P L. Elkins and B. Giannotto, Br. J. Pharmacol., 85 (1985) 441.
10 S M. Jarvis, J.R. Hammond, A.R.P. Paterson and A.S. Clanachan, Biochem. J., 208 (1982) 83.
11 R.D. Bukoski and H.V. Sparks, J. Mol. Cell. Cardiol., 18 (1986) 595.
12 E.F. Williams, P.H. Barker and A.S. Clanachan, Can. J. Physiol. Pharmacol., 62 (1984) 31.

13 J.P. Manfredi and E.W. Holmes, Ann. Rev. Physiol., 47 (1985) 691.
14 A.M. Svardal, R. Djurhuus, H Refsum and P.M. Ueland, Cancer Res., 46 (1986) 5.