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Metabolic conversion of N^6-substituted adenosines to their 5′-triphosphates in human blood

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RECENTLY it was reported[1] that MMPR-5′-P,* which had previously been thought to be inert to further phosphorylation,[2] is metabolized to MMPR-5′-DP and MMPR-5′-TP in man. A subsequent study *in vitro*[3] demonstrated that a sustained high level of intracellular MMPR-5′-P allowed a slow but measurable phosphorylation of this analog nucleoside 5′-monophosphate in several human tissues, apparently through the agency of adenylate kinase. The simplicity, speed and sensitivity of the methodology employed in this latter study has led to an examination of the potentiality of other purine analogs to be metabolized to 5′-nucleotides in human blood. A class of purine analogs of particular interest in this respect are the N^6-substituted adenosines. Several of these compounds have exhibited significant antiproliferative activity against a variety of animal cancers[4–8] and one member of this class, IPA, has been demonstrated to possess antileukemic activity in man.[9] This communication describes the results of a metabolic study *in vitro* of several of these N^6-substituted adenosines.

The analogs investigated were obtained from the following sources: N^6-methyladenosine, N^6-dimethyladenosine, N^6-furfuryladenosine and IPA, Sigma Chemical Co.; N^6-benzyladenosine, Aldrich Chemical Co.; zeatin ribonucleoside [N^6-(*trans*-4-hydroxy-3-methylbut-2-enyl)adenosine], Calbiochem. Other reagents were from sources identified previously.[3]

The purine analogs (0·73–0·87 mM) were incubated for 23 hr at 37° with fresh, heparinized human whole blood, inorganic phosphate, glucose and antibiotics as described previously.[3] Neutralized acid-soluble extracts of the blood samples were subsequently prepared and analyzed in the high-pressure liquid chromatograph according to the methodology detailed recently.[3] Ultraviolet spectra were recorded with a Beckman model Acta III spectrophotometer equipped with micro cell holders.

Incubation of human blood with various N^6-substituted purine nucleoside analogs led, in all cases examined, to the appearance of new peaks (i.e. peaks not present in the control extract from blood incubated in the absence of analogs), exhibiting distinct $A_{254\,nm}$:$A_{280\,nm}$ ratios, in the nucleotide region of the high-pressure liquid chromatograms (Fig. 1). Except in the case of N^6-methyladenosine, all of the extracts exhibited large new peaks in the nucleoside monophosphate region of the chromatograms (i.e. between AMP and ADP or, in the case of N^6-benzyladenosine, slightly later than ADP) and much smaller new peaks in the triphosphate region (i.e. beyond ATP). The somewhat enlarged peaks in the AMP region of these chromatograms appeared to represent either AMP or IMP on the basis of the observed $A_{254\,nm}$:$A_{280\,nm}$ ratios and the known retention times of these two compounds. The new peaks in the extract from N^6-methyladenosine-treated blood were eluted immediately after ADP and ATP, whereas in the other extracts the new peaks were better resolved from the adenine nucleotides. In general, new peaks in the diphosphate region (i.e. between ADP and ATP) were often not readily apparent, either because of their relatively small size or because of their co-elution with the large peak of ATP. The new metabolite peaks in the monophosphate and triphosphate regions were collected as they were eluted from the liquid chromatograph and were found to exhibit the same ultraviolet spectral characteristics as their corresponding N^6-substituted adenosine precursors. Treatment of these extracts with yeast hexokinase plus glucose caused each of the putative analog nucleoside 5′-triphosphate peaks to shift quantitatively to a retention time which was intermediate between those of the putative 5′-mono and 5′-triphosphate metabolites and which coincided with the retention time of the presumptive 5′-diphosphate metabolite present in the original extracts. In the case of N^6-benzyladenosine, the peak area of the putative 5′-diphosphate metabolite was readily measurable in the original extract; treatment of this blood extract with pyruvate kinase plus phosphoenolpyruvate caused a quantitative shift of the 5′-diphosphate metabolite to the retention time of the 5′-triphosphate metabolite. The relative retention times of these analog metabolites with respect to the adenine nucleotides, as well as the internal consistency of the enzymatic peak-shift experiments, strongly argue that human blood is capable of metabolizing all of these N^6-substituted adenosines to their corresponding 5′-mono-, 5′-di- and 5′-triphosphate nucleotides.

Of the N^6-substituted adenosines examined in this study, only N^6-methyladenosine has previously been reported[10] to be metabolized to 5′-di- and 5′-triphosphate nucleotides. Parks and Brown,[10] employing a 2-hr incubation with human erythrocytes, examined the metabolism of several other N^6-substituted

* Abbreviations used: IPA, N^6-(Δ^2-isopentenyl)adenosine; IPAMP, the 5′-monophosphate of N^6-(Δ^2-isopentenyl)adenosine; MMPR, 6-methylmercaptopurine ribonucleoside; MMPR-5′-P, MMPR-5′-DP and MMPR-5′-TP, the 5′-mono-, 5′-di- and 5′-triphosphates of 6-methylmercaptopurine ribonucleoside.

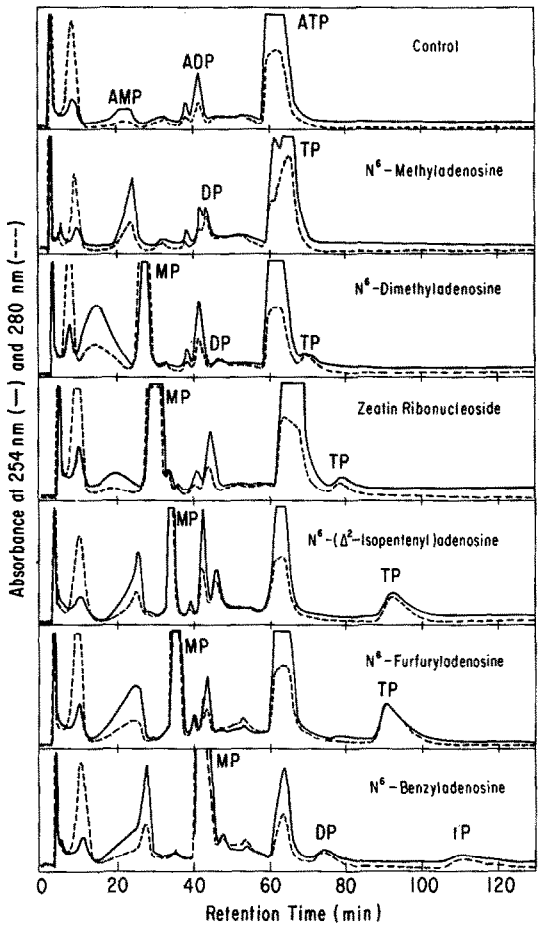

FIG. 1. High-pressure liquid chromatograms of extracts prepared from human blood which had been incubated with the specified N^6-substituted adenosines. Each incubation mixture consisted of 2·0 ml fresh, heparinized human whole blood, 100 μl of 1·0 M potassium phosphate (pH 7·4), 50 μl of 1·3 M D-glucose, 50 μl penicillin G (24 mg/ml), 50 μl streptomycin sulfate (24 mg/ml) and 100–600 μl of purine analog (4–20 mM, such that the final concentration of the analog in the incubation mixture was 0·73–0·87 mM). No purine analog was added to the control incubation. Incubations were carried out at 37° in the Dubnoff metabolic shaking incubator (85 oscillations/min) with air as the gas phase. After 23 hr, incubations were terminated by the addition of 10 ml of cold 0·5 M perchloric acid. Extracts were clarified, neutralized with KOH and concentrated to a final volume of 200 μl.[3] A sample (6–10μl) of each extract was analyzed in a Varian Aerograph model LCS-1000 high-pressure liquid chromatograph equipped with dual ultraviolet (254 and 280 nm) flow monitors and a Reeve Angel 1 mm × 3 m column containing the strong anion-exchange pellicular resin, AS-PELLIONEX-SAX, as described previously.[3] The full-scale absorbance was 0·16 optical density unit for both photodetectors. MP, DP and TP designate the 5′-mono-, 5′-di- and 5′-triphosphates, respectively, of the precursor N^6-substituted adenosine specified in each chromatogram.

adenosines and found evidence for the formation of the 5′-monophosphates of N^6-allyladenosine and N^6-ethoxyethyladenosine. Previous metabolic and enzymatic studies with IPA did not detect formation of nucleotide metabolites other than the 5′-monophosphate.[11–13]

The metabolism *in vitro* of these N^6-substituted adenosines in human blood appears to be very similar in nature to that described recently[3] for MMPR. All of these analogs appeared to be taken up nearly quantitatively and converted to their corresponding 5′-monophosphates by human erythrocytes during

the 23-hr incubation. This is consistent with the reported activity of these analogs as substrates for mammalian adenosine kinase.[12,14] With the exception of N^6-methyladenosine, all of the blood extracts contained high levels of the analog nucleoside 5'-monophosphates at the end of the 23-hr incubation, with relatively little formation of the 5'-di- or 5'-triphosphate metabolites. Subsequent metabolism of the analog nucleoside 5'-monophosphates to higher phosphates, therefore, appears to be limited primarily by their low rate of reactivity with a nucleoside 5'-monophosphate kinase, probably adenylate kinase. This interpretation is supported by the observation[10] that a shorter incubation period (2 hr) of human erythrocytes with N^6-methyladenosine yielded chiefly the corresponding 5'-monophosphate and relatively little of the 5'-di- or 5'-triphosphates. Once formed, the analog nucleoside 5'-diphosphates appeared to be efficiently converted to their corresponding 5'-triphosphates, presumably by either nucleoside diphosphokinase or pyruvate kinase.

The pharmacological significance of these results is not clear at this time. In the case of MMPR metabolism, the slow excretion (half-life > 24 hr) of this drug in man[15] and the long half-life (4–6 days) of MMPR-5'-P in human blood[16] allow for the slow but continuous formation of the corresponding 5'-di- and 5'-triphosphates *in vivo*.[1,3] However, the catabolism and elimination of IPA in man appear to be much more rapid, with a half-life in the body of a few hours;[17] under conditions of single daily dosage, it is uncertain whether the intracellular concentrations of IPAMP would be maintained sufficiently high or long enough to allow its subsequent phosphorylation to proceed significantly. Nevertheless, under conditions of chronic exposure of cells to these analog nucleosides, such as could take place in tissue culture, it would be possible to maintain high intracellular concentrations of the analog 5'-monophosphates and to form the corresponding 5'-di- and 5'-triphosphate metabolites. Little is presently known concerning the biological activity of these analog nucleoside oligophosphates. A number of N^6-substituted analogs of ATP have been shown to serve as efficient alternate substrates for hexokinase and glycerokinase.[18] Several N^6-substituted analogs of 3',5'-AMP, which are potential metabolites of the corresponding 5'-triphosphates, are effective in promoting release of pituitary hormones[19] and in activating a cyclic 3',5'-AMP-dependent protein kinase.[20]

These incubations of blood with purine analogs *in vitro* appear to be useful in revealing metabolic conversions not readily demonstrable with isolated enzyme preparations. This advantage may be attributed both to the relatively large amount of enzyme (e.g. adenylate kinase) present in 2 ml of whole blood and to the apparently increased stability of enzymes when incubated in their natural intracellular environment. These incubations also provide a convenient source of analog nucleotides with which to standardize chromatographic procedures which are to be employed in metabolic studies; in many cases, the desired retention time or elution position can be determined directly with the unpurified extract from such an incubation.

It is pertinent to note that N^6-substitution of adenine nucleotides resulted in varying increases in the affinity of these compounds toward the anion-exchange resin (Fig. 1). This enhancement of affinity toward the resin appears to be primarily dependent upon the hydrophobicity of the N^6-substituent, N^6-benzyladenine nucleotides exhibiting the longest retention times of any of the analog nucleotides examined. Failure to recognize this extraordinary retention of particular N^6-substituted adenine nucleotides by this resin, and possibly by other resins also, may lead to incomplete results of metabolic studies conducted with these and similar compounds.

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Effects of Mg^{2+} and Ca^{2+} on soluble and membrane-bound acetylcholinesterase from *Electrophorus electricus*

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NUMEROUS investigators have studied the effects of monovalent and divalent cations on acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7; AChE).[1–7] Most of the enzyme preparations used for such studies have been either soluble[1–4,7] or some undefined mixture of soluble and membrane-bound AChE.[5,6] Fries,[2] Nachmansohn[1] and others[3–8] have reported that $CaCl_2$ and $MgCl_2$ increase the maximal rate of hydrolysis of acetylcholine (ACh) by AChE. The extent of increase in the maximal rate, however, is not a consistent finding among investigators;[1–8] the reported variations are most probably due to the different sources of AChE (red blood cell membranes and electric tissue of *Torpedo marmorata* and *Electrophorus electricus*) used, as well as to the different assay conditions and methods. No comparative study has been made of the effect of $MgCl_2$, $CaCl_2$ and NaCl on membrane-bound and soluble AChE obtained from the same source.

We have prepared both membrane-bound and soluble AChE from the electroplaque of *Electrophorus electricus*. The K_m for ACh using membrane-bound AChE is nearly twice as great, and the amount of inhibition by excess substrate, half that of the soluble enzyme.[9] We report here the effects of $MgCl_2$, $CaCl_2$ and NaCl on the kinetics of membrane-bound and soluble AChE.

The two states of the enzyme respond differently with respect to at least one of the kinetic parameters studied (K_m, V_{max} and inhibition by excess substrate) when the concentration of any one of the above inorganic ions is varied.

Preparation of soluble and membrane-bound AChE. Membrane-bound and soluble AChE were prepared as previously described.[9] Electric organ from live eels, *Electrophorus electricus*, was homogenized at 4° for 15 sec in a Sorvall Omni-mixer and then again with ten up and down strokes of a Potter–Elvehjem apparatus operating at 5700 rev/min in a solution containing 180 mM NaCl, 5 mM KCl, 6 mM $CaCl_2$, 1·5 mM $MgCl_2$, pH 7·2. The homogenate was filtered through a stainless steel sieve (96 μm diameter openings), rehomogenized and centrifuged at 20,000 *g* for 30 min. The pellet was washed three times with the above salt solution; the third wash had less than 1 per cent of the AChE activity of the pellet. The pellet was resuspended in a volume of the salt solution equal to that of the original wet weight of the tissue; the suspension was stored at 4° for 48 hr and then centrifuged at 20,000 *g* for 30 min. The pellet was resuspended in the salt solution described above and this suspension will be referred to as the membrane-bound enzyme preparation; its supernatant was centrifuged at 100,000 *g* for 2 hr and the enzyme activity in the resulting nondialyzed supernatant will be called soluble AChE. The ratio of soluble AChE activity to soluble plus membrane-bound AChE activity, the per cent solubilization, reaches 50 per cent after storing the membrane preparation for 48 hr at 4° from the time of initial preparation and remains constant for at least another 7 days.[9]

Assay of AChE. AChE activity was determined by measuring the rate of hydrolysis of AChI by a pH-stat (Radiometer Corp.). Assays were done under a nitrogen atmosphere at 30° in 1·0 ml of a salt solution