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A NOTE ON THE SYNTHESIS OF 5'-AMP ESTER OF TRIS (HYDROXYMETHYL)AMINOMETHANE BY RAT LIVER PLASMA MEMBRANE

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Summary

The alcohol-AMP synthesizing enzyme of rat liver plasma membrane also synthesizes the 5'-AMP ester of tris(hydroxymethyl)aminomethane as judged by the use of [α-^{32}P] ATP and [U-^{14}C] ATP. This synthetic process may decrease significantly the concentration of ATP during incubation.

Studying the properties of the alcohol-AMP synthesizing enzyme [1—5] of rat liver plasma membrane, the synthesis of an unknown nucleotide from [α-^{32}P] ATP was observed in the absence of alcohols. The present study was aimed at clarifying the structure of this compound and its possible interference with the study of other ATP consuming, membrane bound enzymes.

Female PVG/c rats (about 150—200 g body weight) were used. Liver plasma membranes were prepared according to the procedure devised by Neville [6] up to step 11. For the assay of Tris-AMP formation the assay mixture usually contained 3 mM [α-^{32}P] ATP ($1.5 \cdot 10^6$ dpm), 25 mM phosphocreatine, 60 μg creatine phosphokinase, 4 mM $MgCl_2$, 100 mM phosphate buffer (pH 7.5), 100 mM Tris-HCl (pH 7.5) and 50 μg of protein of rat liver plasma membrane in a final volume of 60 μl. Incubations were carried out at 37°C for 10 min and were terminated by heating the samples in boiling water for 2 min followed by centrifugation. Aliquots of the clear supernatants were applied to polyethyleneimine-cellulose F plates (Merck, plastic support). 60 mM sodium acetate (pH 5.4) was used as solvent. The R_F value for Tris-AMP was 0.66, while the R_F values for inorganic phosphorus and all the phosphorus-containing adenine nucleotides were less than 0.35. The spots corresponding to R_F 0.66 were scraped down, transferred to scintillation vials and counted for ra-

dioactivity in Unisolve, using a Nuclear Chicago liquid scintillation spectrometer. Protein was estimated by Lowry's procedure [7], with bovine serum albumin as standard.

The following sources were used for reagents: [α-^{32}P] ATP (21.5 Ci/mmol), [γ-^{32}P] ATP (3000 Ci/mmol) and [U-^{14}C] ATP (600 Ci/mol) (New England Nuclear Corp.); creatine kinase and creatine phosphate (Sigma); Tris and polyethyleneimine-cellulose F thin-layer plates (Merck).

Incubation of liver plasma membrane with either [α-^{32}P] ATP or [U-^{14}C]-ATP resulted in the same rate of the synthesis of the unknown nucleotide. [γ-^{32}P] ATP was not a ^{32}P precursor. When the ^{14}C-labelled unknown compound was purified by paper chromatography and reincubated by the plasma membrane, it was rapidly hydrolyzed enzymatically and the radioactivity was recovered in either 5'-AMP (30%) or adenosine (70%). Adenosine kinase and adenosine deaminase are absent from the plasma membrane. Therefore, the above results provide evidence that 5'-AMP was the first hydrolytic product (further transformed by 5'-AMPase to adenosine): thus, it was a constituent of the unknown compound.

When Tris-HCl buffer (pH 7.5) was replaced by phosphate buffer (pH 7.5), no synthesis of the unknown was observed, whereas glycerol-AMP synthesis was actually increased. Addition of different concentrations of Tris-HCl resulted in the synthesis of the unknown in a concentration-dependent manner (Table I). This indicated that the unknown nucleotide, besides 5'-AMP, also contained Tris linking the two parts of the molecule most probably by a phosphate-ester bond.

The conditions for the synthesis of Tris-AMP and glycerol-AMP [5] were the same, thus: (a) addition of a divalent cation was not obligatory: (b) the presence of enzyme-bound Zn^{2+} was required: (c) the pH optimum was 9.5; and (d) 6 mM NADH or 5 mM 5'-AMP gave 70—80% inhibition. These similarities between the synthesis of Tris-AMP and glycerol-AMP indicate that the same enzyme is involved.

TABLE I

SYNTHESIS OF Tris-AMP AT DIFFERENT CONCENTRATIONS OF TRIS

Results are the mean ± S.E. of four parallel incubations in one representative experiment

Concentration of Tris (mM)	Tris-AMP formation (nmol/mg protein per 10 min)
25	7.4 ± 0.6
50	13.9 ± 1.1
100	30.4 ± 0.4
200	58.5 ± 2.2

TABLE II

SYNTHESIS OF Tris-AMP AT DIFFERENT CONCENTRATIONS OF ATP

Besides standard components, the concentration of [α-^{32}P] ATP was 0.4—3.0 mM ($1.5 \cdot 10^6$ dpm). Results are the mean ± S.E. of four incubations in one representative experiment

Concentration of ATP (mM)	Tris-AMP formation (nmol/mg protein per 10 min)
0.4	16.8 ± 1.6
1.0	27.6 ± 2.0
2.0	35.2 ± 0.8
3.0	35.6 ± 0.4

Table II shows the rate of the synthesis of Tris-AMP at different concentrations of ATP. Accordingly, at 0.4 mM ATP and at 100 mM Tris and 50 μg plasma membrane protein, about 2.5% of ATP would be converted to Tris-AMP at pH 7.5 during 10 min. At the same concentrations of ATP, Tris and protein, at pH 8.0, 9.0 and 9.5 the rate of ATP conversion to Tris-AMP would be 5.5, 8.5, 12 and 15%, respectively, as calculated from a pH-dependence curve (not shown). However, the measurement of the radioactivity of the remaining ATP revealed that at 0.4 mM ATP the loss due to the presence of 100 mM Tris was 6% (instead of 2.5%).

Similar discrepancies were observed at all pH values. The approximately double difference between the real and 'theoretical' loss of ATP (due to Tris) can be attributed to the fact that Tris-AMP is rapidly hydrolyzed by a plasma membrane enzyme.

The present results indicate that the use of Tris-HCl as buffer at moderately high concentration may falsify some kinetic data with ATP-consuming plasma membrane-bound enzymes. This is especially true for pH dependence and time course studies. More important, however, for the study of the alcohol-AMP synthesizing enzyme, Tris buffer must be replaced by another buffer as Tris will certainly interfere unless its concentration be kept very low.

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