Hepatic Clearance of Adenosine 3':5'-Cyclic Monophosphate from Plasma in the Rat

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Rats were given intravenous injections of cyclic [^3H]AMP and the disappearance of radioactivity from plasma and its appearance in bile were followed. Livers were removed and the cyclic [^3H]AMP content was measured. The binding of radioactivity to soluble proteins was measured after preparation of a cytoplasmic fraction. Experiments *in vitro* to determine the ability of hepatic cytoplasmic proteins to bind cyclic [^3H]AMP were also carried out. A role for cytoplasmic proteins in the clearance of cyclic AMP from plasma is discounted.

Several studies have shown that cyclic AMP (adenosine 3':5'-cyclic monophosphate) can pass from cells into the plasma (Broadus *et al.*, 1970; Blonde *et al.*, 1974). Several tissues, including liver (Liljenquist *et al.*, 1974; Exton *et al.*, 1972) and kidney (Kaminsky *et al.*, 1970), can release the nucleotide in the basal state and after hormonal stimulation. The mode of clearance of cyclic AMP from plasma is, however, not known. It has been shown that cyclic AMP in urine is derived both from plasma and from the kidney itself (Broadus *et al.*, 1970), and that the recovery of radioactivity in urine accounts for only 10% of a cyclic [^3H]AMP tracer (Coulson *et al.*, 1974). Broadus *et al.* (1970) also demonstrated that approx. 80% of an intravenous pulse of cyclic [^3H]AMP was cleared from the circulation by an extrarenal mechanism.

The uptake and subsequent excretion of certain anions is a well-recognized function of the liver. The presence of the nucleotide in bile (Levine *et al.*, 1969) and the ability of the liver to remove approx. 25% of the radioactivity of a tracer dose of cyclic AMP (Blonde *et al.*, 1974) suggest that the liver may be an important site of clearance of cyclic AMP from plasma. It has been postulated that certain soluble proteins that can be isolated from a liver cytosol fraction may have an important role in clearing anions from plasma (Levi *et al.*, 1969) and that one of these, 'Y' protein or ligandin (Litwack *et al.*, 1971), is a major determinant of the uptake phase of the overall clearance mechanism. The pK_a of cyclic AMP is 3.82 (White & Zenser, 1971), and the nucleotide is a univalent anion at neutral pH (Ramachandran, 1971).

The present paper describes experiments to determine the ability of the liver to remove the nucleotide from plasma and to investigate a possible role for cytoplasmic proteins in this uptake process.

Materials and methods

Male rats (250–300g) of an inbred Wistar strain were used in all experiments. Radioactivity was

measured in samples of plasma, liver, bile and in column eluates by liquid-scintillation counting with a toluene-based scintillant [2,5-diphenyloxazole, 7g; 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, 0.6g; naphthalene, 150g; 2-ethoxyethanol, 300ml; toluene to 1 litre]. Quench correction was carried out after internal standardization with a measured amount of cyclic [^3H]AMP in toluene. The protein concentration of column eluates was determined by a turbidimetric method (Meulemans, 1960). [^3H]Adenosine 3':5'-cyclic phosphate (30Ci/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Experimental

Clearance of cyclic [^3H]AMP from plasma. Rats were lightly anaesthetized with ether, and the bile duct and both femoral veins were cannulated with fine polyethylene tubes. Bile collections were made directly into scintillation vials for 10min periods. After a baseline bile collection, cyclic [^3H]AMP (10 μCi) in sodium-potassium phosphate buffer (100mmol/litre; pH 7.4; 0.5ml) was rapidly injected into one femoral vein and thereafter blood samples (0.2ml) were collected into heparinized syringes at 10min intervals from the second femoral vein. Each blood sample was taken at the midpoint of a bile collection period. The blood samples were centrifuged for 10min (1500g; 4°C) and portions of plasma (50 μl) were placed directly into scintillation vials.

After intravenous injection of cyclic [^3H]AMP, radioactivity quickly disappeared from the plasma. This was accompanied by the appearance of a small amount of radioactivity in the bile. Fig. 1 shows the result of one experiment, the results obtained in three other studies being very similar. The maximum amount of radioactivity appeared in the bile sample collected 10–20min after injection of cyclic [^3H]AMP. During the 60min experiment 0.03–0.05% of the radioactivity injected appeared in the bile and 10.7–16.2% in the whole liver homogenate. A similar

Fig. 1. Radioactivity present in plasma and bile after injection of cyclic $[^3\text{H}]\text{AMP}$ in the rat

Cyclic $[^3\text{H}]\text{AMP}$ ($10\mu\text{Ci}$) was injected intravenously at zero time. Bile was collected for 10 min periods and at the midpoint of each of these collections a blood sample was taken. Δ , Radioactivity in plasma; $\bullet$, radioactivity in bile.

percentage of radioactivity was found in the whole homogenate prepared from livers removed 15 min after injection of cyclic $[^3\text{H}]\text{AMP}$.

Identification of the radioactive compounds in the liver. The cyclic $[^3\text{H}]\text{AMP}$ content of the radioactivity present in the liver was measured as described by Blonde *et al.* (1974). Whole liver (1 g) was homogenized in trichloroacetic acid (6%, w/v; 10 ml) and cyclic $[^3\text{H}]\text{AMP}$ separated from other nucleotides on columns ($0.4\text{ cm} \times 6\text{ cm}$) of Dowex-50 W. The columns were eluted with distilled water and fractions (2 ml) were collected. Preliminary experiments carried out on pure solutions of ATP, cyclic AMP and AMP confirmed that ATP was eluted in fractions 1 and 2, cyclic AMP in fractions 3, 4 and 5 and AMP in a broad peak made up of fractions 6, 7, 8 and 9. To confirm that the radioactivity found in fractions 3, 4 and 5 was cyclic $[^3\text{H}]\text{AMP}$ and that this nucleotide did not appear in other fractions, ZnSO_4 (250 mmol/litre; 0.2 ml) and $\text{Ba}(\text{OH})_2$ (250 mmol/litre; 0.2 ml) were added to each fraction and after centrifugation a portion of the supernatant (0.1 ml) was counted (Krishna *et al.*, 1968). The cyclic $[^3\text{H}]\text{AMP}$ content of the liver 106000g supernatant preparation was also measured by the above methods.

After the addition of trichloroacetic acid extracts of whole liver homogenate or of liver supernatant, radioactivity was eluted from the Dowex-50 W columns as three peaks. The first corresponded to the elution volume of ATP (and ADP), the second to cyclic AMP and the third to AMP. In the whole liver homogenate 6.5% of the radioactivity appeared in

the cyclic AMP-containing fractions and no radioactivity was lost after precipitation with ZnSO_4 and $\text{Ba}(\text{OH})_2$. Of the remaining radioactivity, 30.8% appeared in the first peak and 62.7% in the third peak. All this radioactivity was lost after treatment with ZnSO_4 and $\text{Ba}(\text{OH})_2$. The cyclic AMP fractions contained 2% of the radioactivity of the 106000g supernatant after elution from the Dowex-50 W column and treatment with ZnSO_4 and $\text{Ba}(\text{OH})_2$.

Possible role of cytoplasmic proteins. The possibility that liver cytoplasmic proteins are involved in the passage of cyclic AMP from plasma into the liver was investigated in two ways.

First, supernatant (106000g) fractions were prepared from livers obtained 15 min or 60 min after injection of cyclic $[^3\text{H}]\text{AMP}$. The livers were perfused *in situ* with approx. 20 ml of ice-cold sodium-potassium phosphate buffer (100 mmol/litre; pH 7.4) via a portal-vein cannula. This buffer also contained theophylline (8 mmol/litre) to inhibit hepatic phosphodiesterase activity. The livers were then removed, gently swabbed dry, weighed and homogenized in a loose-fitting Teflon-glass homogenizer in (2:1, w/v) sodium-potassium phosphate buffer (100 mmol/litre; pH 7.4) containing theophylline (8 mmol/litre) and sucrose (250 mmol/litre). The homogenate was centrifuged (2500g; 4°C ; 15 min), the cellular debris discarded and the supernatant recentrifuged (106000g; 4°C ; 60 min). The supernatant fraction was harvested and, after removal of the lipid layer, portions (4 ml) were added directly to a Sephadex G-75-column.

Secondly, experiments were performed on 106000g supernatant fractions prepared from livers perfused as before but taken from animals which had not undergone any previous manipulation. Samples (4 ml) of supernatant fractions were mixed with cyclic $[^3\text{H}]\text{AMP}$ ($5\mu\text{Ci}$) and added directly to the Sephadex G-75 column. To eliminate the possibility that in these experiments some of the cyclic $[^3\text{H}]\text{AMP}$ tracer might be degraded by hepatic phosphodiesterase activity in spite of the presence of theophylline, further studies were carried out in which cyclic $[^3\text{H}]\text{AMP}$ and unlabelled cyclic AMP ($3.2\mu\text{g}$) were both added to liver supernatants. The mixture was then added to a Sephadex G-75 column.

To confirm the presence of the anion-binding proteins described by Levi *et al.* (1969) bromsulphthalein (4 mg) was added to samples (4 ml) of all the liver supernatant preparations described. After being mixed, the solutions were added to the Sephadex G-75 columns.

Gel-filtration experiments were performed on columns ($30\text{ cm} \times 2\text{ cm}$) of Sephadex G-75 which were equilibrated with sodium-potassium phosphate buffer (100 mmol/litre; pH 7.4) containing theophylline (8 mmol/litre). The columns were eluted at 4°C with the same buffer, the flow rate being 30 ml/h

Fig. 2. Elution pattern of liver supernatant fraction after intravenous injection of cyclic $[^3\text{H}]\text{AMP}$

An hour after the injection of a pulse of cyclic $[^3\text{H}]\text{AMP}$ the liver was removed and a post-106000g supernatant fraction was prepared. Supernatant (4.0ml) was applied to a Sephadex G-75 column and eluted at 4°C with phosphate buffer (100mmol/litre; pH 7.4) containing theophylline (8.0mmol/litre). Fractions (3.0ml) were collected and the protein concentration and radioactivity determined. ▲, Protein concentration; ●, radioactivity.

with 10 fractions/h being collected. The void volume of the column, determined by the use of Dextran Blue, was 35ml.

The elution pattern of the proteins and radioactivity in the supernatant fraction from a liver removed 60min after injection of the tracer is shown in Fig. 2. The patterns from supernatants prepared from other livers removed both 60min and 15min after injection of cyclic $[^3\text{H}]\text{AMP}$ were very similar. The amounts of radioactivity associated with the protein-containing fractions was small (<0.02% of the radioactivity injected) and was eluted in the fractions appearing immediately after the void volume of the column. The low radioactivity found, however, prevented further study of the identity of the radiolabelled material in the protein peak. Most of the supernatant radioactivity was present in fractions corresponding to the elution volume of free nucleotides (108–180ml). No radioactivity was found in fractions 54–78ml or 81–114ml in which the bromsulphthalein-binding proteins Y and Z described by Levi *et al.* (1969) were shown to be eluted.

In three experiments cyclic $[^3\text{H}]\text{AMP}$ was added directly to supernatant fractions prepared from rats which had not undergone previous manipulation. All the protein-bound radioactivity was eluted immediately after the void volume of the column and amounted to 40.5–57.0% of the total radioactivity added. The elution patterns were similar to that shown in Fig. 2, as was the pattern obtained when cyclic $[^3\text{H}]\text{AMP}$ was added to a supernatant preparation together with unlabelled cyclic AMP.

Discussion

Our results confirm the findings of Blonde *et al.* (1974) that the liver can remove cyclic AMP from the plasma. Blonde *et al.* (1974) infused cyclic $[^3\text{H}]\text{AMP}$ for 45min followed by a 90min infusion of NaCl. Samples of liver were then taken and the cyclic $[^3\text{H}]\text{AMP}$ content was determined. The design of these experiments, therefore, excludes determination of the speed of hepatic clearance. The cellular mechanisms involved in the uptake process and the importance of biliary excretion in hepatic removal are also unknown.

Our experiments show that radioactivity is rapidly removed from plasma and appears in the liver and bile within 15min of injection of the tracer. It is unlikely that radioactivity found in the liver could result from contamination with blood since all livers were extensively perfused with buffer before removal and appeared to be blood-free at the time of homogenization. Although specific-radioactivity measurements were not made, the rapid clearance of radioactivity from the blood (Fig. 1) would prevent substantial degradation of the nucleotide by erythrocyte phosphodiesterase. However, the intracellular degradation of cyclic AMP to AMP may explain the smaller amount of radioactivity bound to protein in experiments *in vivo* when compared with the experiments *in vitro* in which degradation of cyclic AMP was inhibited by theophylline. Accumulation of similar amounts of radioactivity in livers removed 60 and 15min after injection of cyclic $[^3\text{H}]\text{AMP}$ suggests that uptake is irreversible, probably because of conversion into nucleotides which are not so readily released. The small amount of radioactivity found in bile discounts a biliary route as a major mechanism of cyclic AMP excretion.

Litwack *et al.* (1971) have suggested that a cytoplasmic protein, ligandin or Y protein, may be involved in the removal from plasma of anions such as bromsulphthalein. This protein has a molecular weight of approx. 40000 and is eluted in fractions 54–78ml in the system described in Fig. 2. Although in experiments *in vivo* considerable degradation of cyclic $[^3\text{H}]\text{AMP}$ occurred in the liver, significant amounts remained both in the whole homogenate and in the supernatant preparation. Only a very small amount of radioactivity, however, was protein-bound and none was detected in the fractions containing Y or Z proteins. Further, when cyclic AMP was added *in vitro*, under conditions where degradation of cyclic $[^3\text{H}]\text{AMP}$ was prevented by the use of theophylline or by including unlabelled cyclic AMP, no binding to either Y or Z proteins was seen. Some radioactivity was associated with a protein of higher molecular weight. Hepatic cyclic AMP-dependent kinase is located almost entirely in the liver cytosol (Chen & Walsh, 1971) and partly consists of a cyclic

AMP-binding protein. The radioactivity in elution volumes 36–60ml may represent cyclic [^3H]AMP bound to this protein.

The Sephadex column described is a non-equilibrium system, and although no binding of cyclic AMP to Y protein was seen it is possible that cyclic AMP originally bound to this protein was eluted during passage down the column. However, we were able to show binding of bromsulphthalein to both the Y and Z protein fractions when this anion was added *in vitro*. Since no binding of cyclic [^3H]AMP could be demonstrated it appears unlikely that these soluble protein fractions are involved in the hepatic uptake of the nucleotide from plasma.

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