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UPTAKE AND METABOLISM OF S-ADENOSYL-L-METHIONINE BY ISOLATED RAT HEPATOCYTES

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SUMMARY. In the present study, direct evidence is given to SAMe capability of crossing the membrane of isolated rat hepatocytes. The kinetics of SAMe uptake is biphasic: a fast phase being completed in less than 15 sec and a slower one with an apparent K_m of 8.33 μM and a V_{max} of 10.6 pmol/min/mg protein. Both processes are pH and temperature dependent. Analysis of the fast phase by a Scatchard plot discloses two sets of binding sites of high and low affinity, respectively. Experiments carried out incubating isolated hepatocytes with double-labelled SAMe (methyl-^{3}H, carboxyl-^{14}C) have shown that about 70% of SAMe uptake by the cell is rapidly decarboxylated.

The uptake of S-adenosyl-L-methionine[+] (SAMe) by various cell systems has been the object of several reports (1, 2, 3). Direct evidence of this molecule crossing the cellular membrane has been obtained in studies with cells of Saccharomyces cerevisiae (4, 5, 6). More recently, SAMe uptake by mammalian cells was studied using rabbit erythrocytes (7). The present paper deals with SAMe uptake by isolated rat hepatocytes. Moreover, results are here presented which demonstrate that the intact molecule of SAMe crosses the cell membrane and is rapidly catabolized inside the hepatocyte.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats of 300 ± 20 g body wt were purchased from Charles River Breeding Laboratories, Inc.

[+]Abbreviations : SAMe, S-Adenosyl-L-methionine; NAS, N-(acetyl-^{3}H)-acetyl-serotonin; HIOMT, Hydroxyindole-O-methyl transferase; TCA, Trichloroacetic acid.

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(Wilmington, Massachusetts 01887, USA). The animals had free access to food and water.

Chemicals. Radioactive compounds were obtained from Radiochemical Centre (Amersham, England). N-(Acetyl-^{3}H)-acetylserotonin was prepared from serotonin creatinine sulfate (Regis Chemical Co., Chicago, Illinois) and ^{3}H-acetic anhydride (Radiochemical Centre, Amersham, England) as previously described (8). Hydroxyindole-O-methyltransferase (HIOMT) was partially purified from frozen ox pineal glands as reported elsewhere (9). Unlabelled disulfate-di-p-toluenesulfonate SAMe (10) was obtained from BioResearch Co., 20060 Liscate (Milan), Italy. Collagenase, purity grade 1, with less than 0.25% of protease contamination, was supplied by Boehringer (Mannheim, Germany). Other chemicals were of the highest grade commercially available.

1. Isolation of liver cells. Liver cells were isolated and tested for viability according to the procedure of Berry and Friend (11) as modified by Baur *et al.* (12). Only cell preparations satisfying the following requirements were used: a tripan blue stainability of 5 $\pm$ 0.94%; respiratory control ratio greater than 1.5; membrane potential greater than 30 mV. Furthermore, the very short incubation time used (up to a maximum of 15 min) ensures the maintainance of cell viability (12).

2. Incubation of isolated liver cells with $^{14}CH_3$ SAMe. Aliquots of the cell suspension were diluted in a saline standard medium to a final concentration of 2 mg cellular protein/ml. After 5 min pre-incubation at 37°C, the incubation was started by addition of $^{14}CH_3$ SAMe at a specific activity of 18 nCi/nmol for the experiments with higher concentrations (10; 15; 20; 45; 90 µM) and of 58 nCi/nmol for those with lower concentrations (0.5; 1; 2; 5 µM). Rapid separation of cells from the incubation medium at different times was obtained by the method of Baur *et al.* (12), where cells are quickly transferred, by centrifugation in a 152 Beckman microfuge, from the upper phase to a 3 M KOH solution through a silicone layer (density = 1.02). In order to evaluate the amount of the supernatant that remains adherent to the cells, 200 µl aliquots of cell suspension were centrifuged, immediately after addition of 50 nCi of $^{14}CH_3$ SAMe. The radioactivity in the cell bottom layer was counted in 6 ml of Bray's solution. Cellular proteins were determined by the method of Lowry (13).

3. Total radioactivity determination and radioactivity associated with SAMe. An isolated liver cell suspension (30 mg protein/ml) was diluted in saline standard medium to a final concentration of 8 mg protein/ml and incubated (10 ml) shaking at 37°C for 2 and 15 min after addition of 45 µM $^{14}CH_3$ SAMe (specific activity 18.3 nCi/nmol). At the end of incubation time the isolated cells were collected by 2-min centrifugation at 50 x g and washed by suspension in 5 volumes of saline standard medium followed by 2-min centrifugation at 50 x g. Washings were then repeated till less than 0.1% incubated radioactivity was present in the supernatant. The cellular sediment was re-suspended with 3 volumes of 10% trichloracetic acid in 0.05 N HCl. The total

radioactivity present in the hepatocytes was determined counting the radioactivity in the TCA supernatant. On aliquots of the TCA supernatant, the analysis of radioactivity associated with SAMe was performed by the enzymatic method described by Baldessarini *et al.* (14) and based on determination of the $^{3}H/^{14}C$ ratio in the melatonin forming from $^{14}CH_3$ SAMe and N-(acetyl -^{3}H)-acetyl serotonin during enzymatic reaction catalyzed by HIOMT, without addition of labelled SAMe to the medium (15). The basal concentrations of SAMe in the hepatocytes were determined using Baldessarini's unmodified method (14), after incubation with 45 µM of unlabelled SAMe for 15 min at 37°C.

4. Fractionation of hepatocytes after incubation with (methyl-^{3}H, carboxyl-^{14}C)-SAMe. Isolated liver cells diluted with saline standard medium to a final protein concentration of 16 mg protein/ml were incubated with 45 µM (methyl-^{3}H, carboxyl-^{14}C)-SAMe at sp.act. 28 nCi/nmol and 9.8 nCi/nmol, respectively, under the same conditions as above described (see paragraph 3.). The hepatocytes collected after washing were homogenized and fractionated according to De Duve *et al.* (16). The radioactivity associated with cellular fractions was determined counting fraction aliquots containing about 6 mg protein.

5. Determination of $^{14}CO_2$ from (carboxyl-^{14}C)-SAMe. $^{14}CO_2$ released from the hepatocytes was evaluated incubating the liver cells in a Warburg flask for 15 min at 37°C with 45 µM (carboxyl-^{14}C)-SAMe (sp.act. 1 nCi/nmol). The central well contained 0.25 ml of 1 M Hyamine. At the end of incubation the flasks, after addition of 0.3 ml Na_2CO_3 (mg/ml) followed by 6 N sulfuric acid (0.15 ml), were shaken for 30 min at 40°C. The amount of $^{14}CO_2$ was determined by counting the hyamine solution.

RESULTS AND DISCUSSION

Experiments on SAMe uptake were carried out incubating isolated liver cells with various concentrations of $^{14}CH_3$ SAMe (0.5; 1; 2; 5; 10; 15; 20; 45 and 90 µM), for time intervals of 15 sec to 6 min. The results obtained are expressed by the lines drawn in fig. 1 (A and B). Two phases are apparent for SAMe uptake: a fast uptake process, which is already complete within a very short time (less than 15 sec), and a slower process, probably representing the rate of intracellular metabolism. Moreover, the uptake also evident at concentrations lower than 35 µM as found inside the cells may indicate an active transport of SAMe across the cell membrane. From the results shown in fig.1 (A and B), a K_m = 8.33 µM and V_{max} = 10.6 pmol/mg protein/min are calculated for the slow process. The data of the fast process,

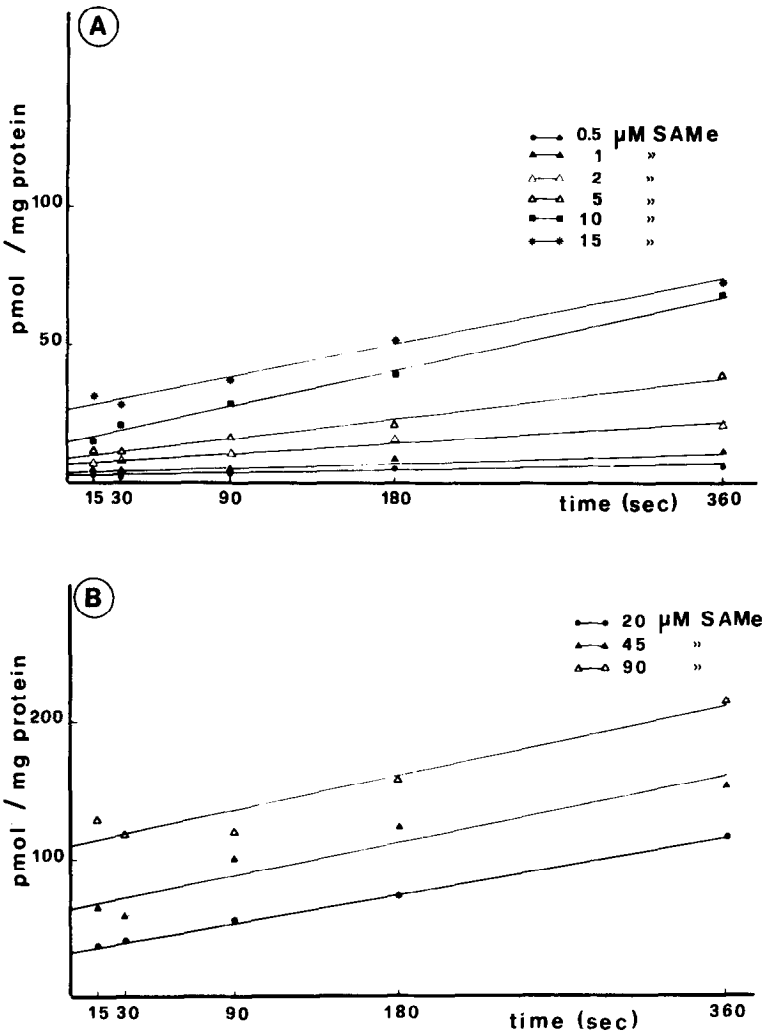

Fig. 1 (A and B) : *(Methyl-^{14}C)-SAMe uptake by isolated rat hepatocytes.* Each curve represents mean values from three different determinations.

obtained as ordinate section in fig.1 by extrapolation of the slow uptake curves to the zero time, can be analyzed by a Scatchard plot. Two slopes are obtained which are usually interpreted as indicative for two sets of binding sites: one of high affinity (K_1 = 4.6 x 10^{-6} M; n_1 = 19.1 pmol/mg protein), and the other of low affinity (K_2 = 16 x 10^{-5} M; n_2 = 303 pmol/mg protein). Yet, the latter may well derive from a fast penetration process via a saturable membrane carrier, since any saturable process would

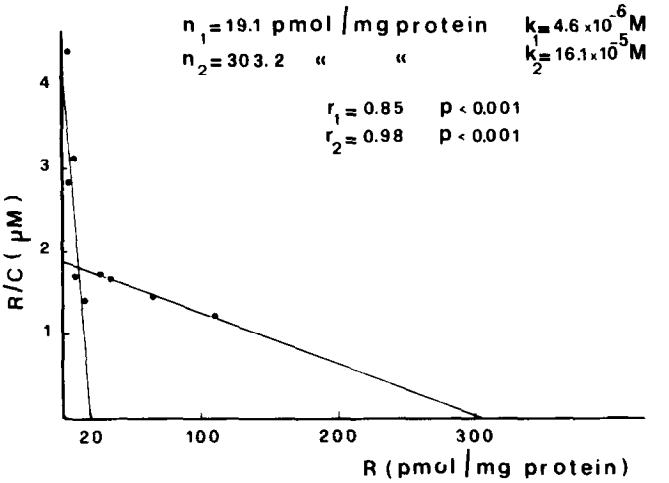

Fig. 2 : *Scatchard plot of fast uptake phase of (Methyl-^{14}C) SAMe.*
Data taken from the ordinate sections of fig. 1.

yield a straight line in a Scatchard plot. (This question will be the subject of a future study). The influence exerted by pH and temperature values on the cell conditions and SAMe uptake has been also considered. A significant increase in SAMe uptake seems to occur when the pH range is increased from 5-6 to 7-8 and temperature is raised from 15-25°C to more physiological values around 37°C.

In order to demonstrate that unmodified SAMe is taken up by the hepatocytes without previous modifications, some incubation experiments were carried out with 45 μM $^{14}CH_3$ SAMe. After 2 and 15-min incubation, total radioactivity was measured and the radioactivity associated with unmodified SAMe was determined by isotopic dilution method described by Baldessarini and Kopin (14) as modified by Lombardini and Talalay (15). Table 1 shows that only part of the intracellular radioactivity is associated with unmodified SAMe, i.e. 12.5% and 2.8% for the two incubation times considered, respectively. The specific activity is constant both after 2 and 15 min of incubation as

Table 1 : *Total radioactivity and radioactivity associated with unmodified SAMe in isolated rat hepatocytes after incubation with (methyl-^{14}C)-SAMe.*

Incubation times	Total radioactivity (dpm/mg protein)	* Radioactivity associated with SAMe (dpm/mg protein)	** Specific radioactivity of SAMe in the cells dpm/nmole
2'	2000	250	2590
15'	9100	260	2720

Values represent the mean of two determinations.
The radioactivity associated with SAMe in the cell and expressed as dpm/mg protein was calculated as follows:

$$\frac{\text{nmol SAMe/ml TCA supernatant} \times {}^{3}H/{}^{14}C \times \text{dpm/nmol NAS}}{\text{mg protein/ml TCA supernatant}}$$

The specific radioactivity of SAMe in the cell as dpm/nmol was calculated as follows:

$$\frac{\text{dpm/nmol NAS}}{{}^{3}H/{}^{14}C}$$

where $^{3}H/^{14}C$ was the ratio in melatonin.

already observed for rabbit erythrocytes (7), indicating that once in the liver cell, SAMe is rapidly metabolized.

A further proof of the rapid metabolism of exogenous SAMe is given by examination of the $^{3}H/^{14}C$ ratio in different subcellular fractions of the hepatocytes after incubation with 45 µM of (carboxyl-^{14}C, methyl-^{3}H)-SAMe for 15 min. These results are reported in Table 2. It can be observed that the $^{3}H/^{14}C$ ratio in the homogenate is four times higher than that of SAMe in the incubation medium. Considering the SAMe amount taken up by the cell on the basis of the ^{3}H present in the homogenate, 40 pmol SAMe/mg protein result to be decarboxylated. This finding was confirmed by the measure of $^{14}CO_2$ produced by incubation of the hepatocytes with 45 µM (carboxyl-^{14}C)-SAMe for 15 min at

Table 2 : Radioactivity distribution in subcellular fractions of isolated rat hepatocytes after incubation with (carboxyl-^{14}C, methyl-^{3}H)-SAMe.

Fraction	$^{3}H/^{14}C$	dpm ^{3}H %	dpm ^{14}C %	Protein %
Homogenate	12.2	–	–	–
Debris	12.4	100	100	100
Cytoplasmic extract	10.6			
Mitochondria	36.9	39.3	11.7	14.9
Microsomal	24.2	14.0	6.4	10.3
Soluble	2.8	15.0	58.1	20.8

The $^{3}H/^{14}C$ ratio of incubated SAMe was 2.9.

Table 3 : Decarboxylation of (carboxyl-^{14}C)-SAMe by incubation with liver cells.

	dpm $^{14}CO_2$	pmol/mg protein
Samples	1893	30
Boiled hepatocytes	30	0

Values are the mean of two determinations.

37°C; 30 pmol SAMe/mg protein were produced and the enzymatic nature of this reaction was confirmed incubating boiled cells under the same experimental conditions (Table 3). This fast metabolism of SAMe makes it impossible to evaluate its intracellular localization by the radioactivity found in the subcellular fractions.

In conclusion, the present work indicates that SAMe can be taken up by the hepatocytes and metabolized rapidly by the cell enzymatic system. Further experiments would be required in order to specify the transport system and define the molecule localization after SAMe uptake by the cell.

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