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UPTAKE OF S-ADENOSYL-L-METHIONINE BY RABBIT ERYTHROCYTES

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Abstract—S-[methyl-^{14}C] Adenosylmethionine was incubated with rabbit blood in order to demonstrate the uptake of the whole molecule of this compound by red blood cells. The results show that the radioactivity incorporated into the cells is partially associated with S-adenosyl-L-methionine. The methyl donor activity of exogenous S-adenosyl-L-methionine is also demonstrated.

During recent years much information has accumulated concerning the role of S-adenosyl-L-methionine* in transmethylation, transulfuration and aminopropylation reactions, and detailed discussions related to the biological significance of this compound have been reported in a number of reviews [1, 2]. Some work has also been devoted to the crossing of the cell membrane of yeasts by SAMe [3, 4] and mammal tissues [5]. Active transport of exogenous SAMe into cells of *Saccharomyces cerevisiae* has been demonstrated by various authors [6, 7]. Concerning the uptake by tissue cells of mammals, the incorporation of the methyl group of exogenous SAMe into tissue creatinine and choline of intact rat and the uptake of the methyl group by rat pancreas anlagen have been observed [8, 5]. However, as far as we are aware no direct evidence has been reported that the whole molecule of SAMe can cross the cell membrane of mammalian tissues. The present communication deals with the incorporation of SAMe into rabbit red blood cells (RBC). By incubation of S-[methyl-^{14}C] adenosylmethionine ($^{14}CH_3$ SAMe) with rabbit blood, the radioactivity was incorporated into these cells. An amount of that radioactivity was found associated with the molecule of SAMe, as determined both by isotopic dilution and the enzymatic method described by Baldessarini and Kopin [9]. Some of the radioactivity was also found into the cells was associated with phosphatidylcholine and with *N*-methyl- and *N,N*-dimethylaminoethanol.

MATERIALS AND METHODS

S-[methyl-^{14}C] Adenosyl-L-methionine ($^{14}CH_3$ SAMe), 55 mCi/m-mole, was obtained from the Radiochemical Centre, Amersham. *N*-Acetyl-[^{3}H] acetylserotonin was prepared from serotonin creatinine sulfate (Regis Chemical Co., Chicago, IL) and [^{3}H]acetic anhydride (Radiochemical Centre, Amersham, U.K.), as previously described [10]. Hydroxyindole-*O*-methyltransferase (HIOMT) was partially purified from frozen ox pineal glands, as reported elsewhere [11]. Unlabelled disulfate-di-*p*-toluenesulfonate of SAMe$^+$ [12] was obtained from BioResearch Co., 20060 Liscate, Milan, Italy. S-Adenosyl-homocysteine was obtained from Sigma Chemical Co., St. Louis, MO. Bovine phosphatidylethanolamine and egg phosphatidylcholine were purchased from BDH Chemicals Ltd, Poole, U.K., and picrolonic acid from Fluka AG, Buchs, Switzerland. 2,5-Dyphenyl-oxazole (PPO), 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP), Instagel and Soluene-100 were purchased from Packard Instrument Company, Inc. Chromatography of phospholipids was performed on 20 × 20 cm pre-coated silica gel 60 F_{254} plates 0.25 mm of thickness (Merck, Darmstadt).

Counting of radioactive samples. Radioactivity of the lysed RBC was determined as follows: 0.1 ml of lysed RBC were mixed in the counting vials with 1.5 ml of a soluene-100–isopropanol mixture (1:1, v/v) with gentle agitation. After digestion at room temperature for 10 min, 0.5 ml of 30–35% hydrogen peroxide was added, the mixture was left at room temperature for 10–15 min and then at 40° for 15 min. Finally, 15 ml of 0.5 N HCl–Instagel (1:9, v/v) mixture was added. The radioactivity of other aqueous samples was determined in 10 ml Instagel. SAM disulfate di-*p*-toluenesulfonate and samples containing phospholipids were dissolved in 1 ml of methanol and counted in 10 ml of a scintillation solution containing PPO (6.5 g), dimethyl POPOP (130 mg) and naphthalene (104 g) dissolved in 1 litre of dioxane–toluene (50:50, v/v).

Chemical and Radiochemical purity of SAMe. Unlabelled SAMe disulfate di-*p*-toluenesulfonate was analyzed by high pressure liquid chromatography using a Perkin-Elmer Mod. 601 chromatograph [13]. The compound was found to be 98 per cent pure and no trace amount of S-adenosyl-homocysteine was present. Since labelled SAMe is known to show a limited stability, the radiopurity of the commercially available $^{14}CH_3$

* Referred to in the text as SAMe.

SAMe was checked as follows. A solution was made up by dissolving radioactive $^{14}CH_3$ SAMe (10^5 d.p.m., spec. act. 55 mCi/m-mole) and 200 mg of the unlabelled compound (0.211 m-moles) in 1 mM sulfuric acid (20 ml). Picrolonate of SAMe was prepared by adding to the solution 320 mg of picrolonic acid in 3 ml of isobutyl alcohol [12]. The precipitate was separated by centrifugation and treated with 3 ml of a solution of sulfuric acid (0.1 N) and *p*-toluene sulfonic acid (0.1 N) and with 3 ml of methylisobutylketone. The aqueous phase was separated and washed with methylisobutylketone. The solution was stirred with charcoal to eliminate picrolonic acid completely, and methanol was added (20 ml). The obtained precipitate was dissolved in a 15% solution of *p*-toluenesulfonic acid in methanol. Precipitation of the disulfate di-*p*-toluenesulfonate was obtained by dropping the solution into 5 ml of diethyl ether. The solid compound was dried and the per cent content of $SAMe^+$ was determined by quantitative high pressure liquid chromatography. Weighed portions (2–3 mg each) of the salt were counted in order to establish the specific activity of the compound. No change of the spec. act. was observed after repeating the complete purification procedure. The amount of $^{14}CH_3$ SAMe in the commercial compound was calculated as follows:

$$\% \text{ Radioactivity as SAMe} = \frac{\text{act} \times 0.211}{10^5} \times 100$$

Where act = (d.p.m./m-mole) of the final salt. The percent radioactivity found as SAMe in the tested batch was 85 per cent.

Incubation of $^{14}CH_3$ SAMe with rabbit blood. Heparinized blood was collected from the lateral vein of rabbit ear, and 25 nmoles/ml of $^{14}CH_3$ SAMe (6.7 *n*Ci/nmole) was added as an isotonic solution. The mixture was shaken at 37° in stoppered flasks using a Dubnoff incubator. At the end of the incubation time, the RBC were collected by centrifugation at 2000 *g* for 10 min and washed by suspension in 2.5 vol. of saline followed by centrifugation. Washings were repeated until less than 0.1 per cent of the incubated radioactivity was present in the supernatant. Lysis of the washed cells was obtained by addition of a volume of distilled water equal to that of the plasma obtained from the first centrifugation. When radioactive SAMe was incubated, aliquots of the lysate were counted. For the determination of the radioactivity in phospholipids, the supernatant obtained by centrifugation of the lysate at 2000 *g* was used, and aliquots of it were counted for radioactivity as reported above.

Analysis of the incubation mixtures

(a) Lysed cells obtained from incubations with SAMe were analyzed for the content of this compound using the method described by Baldessarini *et al.* based on the determination of the $[^3H]$–^{14}C ratio in the melatonin formed from $^{14}CH_3$ SAMe and *N*-acetyl[^{3}H] acetylserotonin in the enzymatic reaction catalyzed by HIOMT [9]. The quantitative analysis of radioactivity associated with SAMe in the erythrocytes after incubation with the methyl labelled compound was carried out by the following methods.

(b) To an amount of lysed RBC (2.5×10^5 d.p.m.) 472 mg (0.5 m-moles) of unlabelled SAMe disulfate di-*p*-toluenesulfonate were added. The solution was worked up as described above for the determination of radiopurity of the labelled SAMe. The amount of radioactivity associated with SAMe in the erythrocytes was calculated as follows:

$$\text{d.p.m./ml RBC} = \frac{0.5 \times \text{spec. act.}}{V_{RBC}}$$

Where spec. act. = d.p.m./m-mole of SAM^+ disulfate di-*p*-toluenesulfonate purified through picrolonate formation; and V_{RBC} = volume (ml) of erythrocytes in the lysate used for the analysis.

(c) Aliquots of the lysate (10^4 d.p.m.) were treated with 1 vol. of 10% solution of trichloroacetic acid in 0.05 N HCl. Analysis of SAMe was then performed by the enzymatic method described by Baldessarini *et al.* [9], avoiding the addition of labelled SAMe to the medium [2]. The radioactivity associated with SAMe in RBC was calculated as follows:

$$\frac{^{14}C}{[^3H]} \times \text{spec. act.}_{NAS} \times \frac{\text{SAMe}}{V_{RBC}} = \text{d.p.m./ml RBC as SAMe}$$

Where:

$\frac{^{14}C}{[^3H]}$ = d.p.m. ratio in melatonin

spec. act.$_{NAS}$ = spec. act. of *N*-acetyl[^{3}H]acetylserotonin (d.p.m./nmole)

SAMe = nmoles of SAMe/ml of lysate determined as described in (a)

V_{RCB} = vol. of RBC in the lysate used for the analysis.

(d) For the determination of radioactivity in phospholipids the 2000 *g* supernatant obtained from the lysis of erythrocytes was extracted with 9 vol. of chloroform–methanol (2:1, v/v). Portions of the organic phase were evaporated to dryness and counted for radioactivity. Other portions (5000 d.p.m. each) were chromatographed on silica gel HF thin layer plates using chloroform–methanol–7 N ammonium hydroxide (60:35:5, v/v) as the eluting solvent [14]. The plates were then scanned for radioactivity using a Packard model 7201 radiochromatoscanner. Bands corresponding to radioactivity peaks were scraped off the plate and directly counted.

RESULTS AND DISCUSSION

The enzymes catalyzing the synthesis and the catabolism of S-adenosylmethionine are widely distributed in bacterial, plant and mammalian cells [1, 9]. On the other hand, due to the importance of SAMe in transmethylation, transulfuration and aminopropylation of various substrates, the uptake

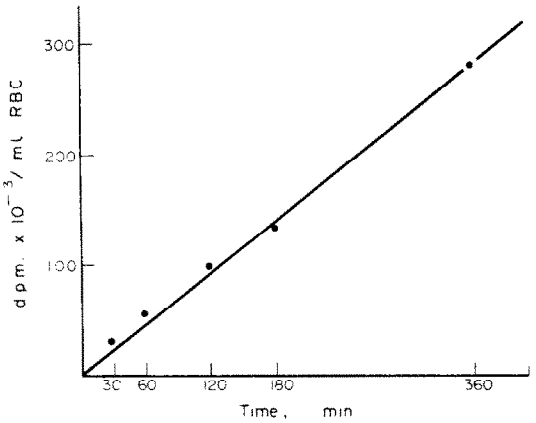

Fig. 1. Radioactivity in red blood cells after incubation of rabbit blood with $^{14}CH_3$ SAMe.* Incubation conditions: 30 ml of heparinized rabbit blood were incubated with 0.75 μmoles $^{14}CH_3$ SAMe (6.7 μCi/μmole).

* The data represent the average of three incubation experiments. Values did not differ from each other more than 5 per cent.

of this molecule by different cells has been considered. The crossing of the cellular membrane of *Saccharomyces cerevisiae* by SAMe has been demonstrated [6, 7]. Uptake from the medium by these cells is sufficient to permit the identification of the compound by ultraviolet micrography. Moreover, the cells of *Saccharomyces cerevisiae* grown in a methionine rich medium produce an amount of SAMe corresponding to about 3 per cent of their dry wt, and the compound is no further metabolized in the cell [15].

The levels of SAMe in animal cells are of a much lower order of magnitude, probably due to its activity as substrate in many enzymatic reactions [9]. Nevertheless, it has been demonstrated that SAMe administered i.p. to the rat acts as a better methylating agent in the synthesis of creatinine when compared to methionine [8]. The same result concerning the synthesis of both creatinine and choline has been obtained by experiments *in vitro* with rat liver slices [8]. Moreover, either the methyl group of SAMe or, at least, the hydrogen atoms of this methyl group has been shown to be incorporated into rat pancreas anlagen when SAMe is incubated with these isolated cells [5]. From these results, the question arises whether or not this behaviour is derived from the crossing of the cell membrane by the whole molecule of SAMe. In order to solve this problem, we performed incubation experiments of rabbit blood in the presence of SAMe. Incubations with $^{14}CH_3$ SAMe (25 nmoles/ml) were performed and increasing amounts of radioactivity were found in RBC during the incubation time, as reported in Fig. 1. However, these data cannot draw to the conclusion that the whole molecule of SAMe crosses the cell membrane. Either chemical degradation of the compound or its methyl donor activity could give rise to substances containing the labelled methyl group of SAMe which would be taken up by the cell. Measurement of the radioactivity associated with SAMe inside the red blood cells would

Table 1. Uptake of SAMe by rabbit RBC*. Incubation conditions as in Fig. 1

Incubation time (min)	Total radioactivity in RBC (d.p.m./ml RBC)	Radioactivity associated with SAMe in RBC (d.p.m./ml RBC)† Method b	Method c
30	29,300	2840	—
180	134,200	2990	3760

* The reported values are the average of duplicate experiments: single values did not differ from each other more than 10 per cent.

† For methods b and c see Methods.

clarify this aspect. Therefore, two independent methods were used allowing the specific analysis of the radioactivity bound to the molecule. The first one was based on the chemical purification of the labelled SAMe after dilution with unlabelled material, whereas the second one utilized the enzymatic methylation of *N*-acetylserotonin by SAMe [9]. The specificity of the enzymatic method was previously demonstrated by Baldessarini and Kopin [9], whereas that of the chemical purification is demonstrated by the purity of SAMe obtained by this method from yeast fermentations [12].

From the data shown in Table 1 it appears that some radioactivity in the erythrocytes is associated with SAMe and the results obtained by the two methods (methods a and b in Table 1) are of the same order of magnitude. It is noteworthy that the radioactivity associated with SAMe is the same at 30 and 180 min of incubation, whereas the total radioactivity increases from 29,300 to 134,200 d.p.m. Since SAMe levels in RBC determined as described in Methods (cf. method a), were also found unchanged during the incubation time, the spec. act. of the compound remains constant. From these data it might be concluded that no accumulation of exogenous SAMe occurs in the cells, and that the exogenous compound uptaken by RBC acts as a methylating agent as the endogenously synthesized compound. Therefore, the presence of radioactive methylated phospholipids inside the cell was determined. The 20,000 *g* supernatant of the lysed erythrocytes was used in order to eliminate membrane phospholipids [16]; 95 per cent of the radioactivity was in the supernatant (Table 2). Extraction with chloroform–methanol followed by thin layer chromatography showed that most of the

Table 2. Radioactivity (d.p.m.) incorporated into RBC phospholipids after incubation of ^{14}C H_3 SAMe with rabbit blood*. Incubation conditions as in Fig. 1; incubation time was 180 min

d.p.m./ml of lysate	57,000
20,000 *g* supernatant (d.p.m./ml)	54,000
Total radioactivity in phospholipids	5500
Distribution of radioactivity in phospholipids (%)	
MPE	38
PC	49

* As in Table 1.

extracted radioactivity was associated with compounds showing the R_f values of phosphatidylcholine and of the mono- and di-methylated derivatives of phosphatidyl-ethanolamine. Although it cannot be excluded that part of the radioactivity found in the cells is attributable to degradation products of SAMe, the present data lead to the conclusion that transport of the whole molecule of SAMe occurs through the cell membrane. Moreover, it may be argued that exogenous SAMe reaches the pool of the endogenously synthesized compound and follows its catabolic fate in the cell.

As regards the kinetics and mechanism by which SAMe is uptaken by the cell, further studies are in progress in our laboratories also utilizing some other experimental models as the isolated hepatocytes and perfused rat liver. The preliminary results suggest that SAMe uptake would occur through an active and saturable transport mechanism indicating the presence of a carrier, as already observed by other authors for *Saccharomyces cerevisiae* [6, 7].

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