A radioisotopic method for the determination of S-adenosyl-L-homocysteine in tissues in the 10^{-7} M range[1]

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Summary. S-Adenosyl-L-homocysteine is able to bind to brain membranes. We used this characteristic to measure the level of S-adenosyl-L-homocysteine in rat brain tissue. The method is rapid, at the same time very sensitive (down to 10^{-7} M) and specific.

S-Adenosyl-L-homocysteine (SAH), formed during enzymatic transmethylations in which the methyl donor is S-adenosyl-L-methionine, is a potent inhibitor of this kind of reaction. Recently several methods have permitted SAH determination in tissues. Some of them are based on UV-detection after ion exchange or HPLC[2-4] and though rapid, they do not allow the determination of small amounts of SAH (at best 10^{-6} M concentrations can be determined). Another class of methods, based on radioenzymatic assay and using the inhibitory power of SAH on methylase activity[5,6], give higher sensitivity (in accordance with the SAH Ki for the enzyme) but are time-consuming; in fact they involve: 1. preparation of the extract to eliminate effectors of the enzyme; 2. preparation of the enzyme to obtain it in pure form in order to increase specificity, and 3. extraction of the labeled products formed.

In rat brain, we demonstrated SAH binding to a membrane protein with high affinity and specificity[7]. In the present study, we used this binding activity for the determination of SAH levels by a radioisotopic method; moreover, we verified the specificity of the method using SAH hydrolase. This method allowed SAH level determination in organs with a low content of SAH.

Methods. Membrane preparation. Membranes from OFA rat cerebral cortex were prepared as described previously[7]. Briefly, the P2 pellet was prepared according to Gray and Whittaker[8]; the cortex was homogenized in a 0.32 M sucrose solution, centrifuged (1000 × g, 30 min) and the supernatant was centrifuged 30 min at 50,000 × g; then the cell structure was destroyed in hypotonic Tris HCl buffer and the membranes resuspended in Tris HCl buffer 50 mM, pH 7.4.

SAH hydrolase preparation. The SAH hydrolase used was partially purified from rat liver by ammonium sulfate fractionation as previously described[9]. Fresh rat liver was cut into small pieces, washed 3 times with 3 vols of 0.01 M acetic acid and homogenized in 3 vols of this solution. The homogenate was centrifuged at 12,000 × g for 20 min and the supernatant precipitated between 40 and 50% saturation with ammonium sulfate. The final precipitate was centrifuged at 12,000 × g for 20 min and the resulting pellet dissolved in 0.25 M sucrose (0.5 ml per g of fresh tissue).

SAH binding determination. ^{3}H-SAH (100 µl) and cold SAH (1 ml) were incubated with 0.9 ml homogenate, for 10 min at 0 °C. The samples were then filtered on a GF/B glass filter (Whatman), the filter washed 3 times with cold Tris buffer and the radioactivity determined by liquid scintillation counting. A blank was performed with 10^{-5} M SAH solution (the nonspecific binding represents 10% of the total binding).

Products. SAH and ^{3}H-SAH were synthesized in our laboratory and analyzed by HPLC[10]. The purity of SAH was determined by physical analysis (elemental analysis, UV-spectrum, specific rotatory power) and by HPLC. The physical properties were in accordance with those described in the literature and identical with those of a sample from Boehringer Mannheim. HPLC analysis (UV-detection) showed no contamination within the limits of the sensitivity (the method allowed the detection of an impurity present at the 0.1% level). For the triatiated product the sensitivity of the method was lower (1%) but successive running on HPLC columns showed that the cpm/cold product ratio remained constant.

^{3}H-Adenosine (2-^{3}H-adenosine, sp.act. 23 Ci/mmole) was purchased from Amersham France; adenosine deaminase (calf intestinal mucosa) was from Sigma.

Results. SAH amount determination. The amount of SAH fixed on a membrane preparation (q) was, according to the mass action law,

$$q = \frac{n\,(SAH)}{Kd + (SAH)}$$

where (SAH) = free SAH concentration; n = number of fixation sites; Kd = dissociation constant.

The trapped radioactivity on the filter was: cpm = q × specific activity that is

$$cpm = \frac{n\,R\,(SAH)}{(Kd + (SAH))\,(SAH)}$$

where R was the radioactivity

$$\text{and } 1/cpm = \frac{Kd}{n\,R} + \frac{1}{n\,R}\,(SAH) \qquad (1)$$

External standard curve. We determined the relation between 1/cpm and SAH concentration for different SAH concentrations. The plot (fig.) showed the linearity of the phenomenon.

When submitted to the simultaneous action of SAH hydrolase and adenosine deaminase (SAH ⇄ adenosine + homocysteine; adenosine → inosine), for 2 h at 37 °C, the SAH was completely removed (fig.).

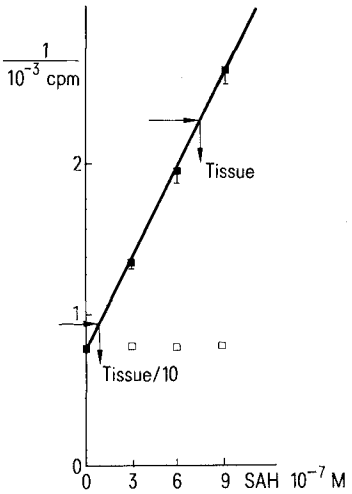
Rat brain tissue level determination. The tissue was homogenized in Tris buffer and the protein eliminated by heating for 10 min at 100 °C and centrifugation. This treatment did not destroy the SAH (samples heated 10 min at 100 °C did not show any metabolites), whereas 70–90% SAM was destroyed under the same conditions; the degradation products were adenine and other undetermined nucleosides but they did not inhibit the ^{3}H-SAH binding (no inhibition was observed with degradation products corresponding to a 10^{-4} M SAM concentration). The specificity of the measurement was given by the specificity of the SAH binding; we showed previously[7] that adenosine, adenosine triphosphate and other natural nucleosides exhibited no affinity (IC50 was at least 5000-fold greater than SAH IC50); we have shown also (using a series of 20 analogs) that analogs not provided with the amino acid characteristic groups ($-NH_2$ or $-COOH$) in their structure have no affinity[10]. The only exception was SAM, which exhibited an IC50 only 10-fold greater than SAH[7] and could thus interfere with the measurement. Therefore, we propose two different methods for increasing the specificity: 1. removing SAM by SEP-PAK cartridge purification[4]. 2. using the specificity of SAH hydrolase. An aliquot was directly

assayed for binding. Another aliquot was treated by SAH hydrolase/adenosine deaminase mixture and then assayed for binding. This 2nd aliquot was used as a tissue blank in order to show whether any endogenous product inhibited SAH binding; in the case of rat brain this blank was always zero, but it could be useful for tissues with a high level of SAM or where unknown interfering nucleosides might be present.

An internal standard curve performed with rat brain extract, where endogenous SAH had been removed, was identical with the external standard curve (results not shown).

The value of SAH level in rat brain was assayed using a concentrated (0.5 g/ml) or a 10-fold diluted (0.05 g/ml) homogenate; we observed respectively 1.52 ± 0.08 nmoles/g tissue or 1.66 ± 0.42 nmoles/g tissue. These values were compatible with the values previously determined by HPLC of 1 to 3 nmoles/g tissue[1,3].

In conclusion, the method described is more sensitive and specific than the previous methods because SAH binding protein (though undefined, and present in a crude extract) is as specific as any methylase; specificity could be further enhanced by using SAH hydrolase, the structural specificity of which differs from that of the binding. Our method is very rapid and 20-50 assays can be run simultaneously (in 1 day). The necessary materials, ^{3}H-SAH, SAH hydrolase and brain membranes (it is important to underline that calf brain can be used instead of rat brain) can be stored at least 3 months at $-20\,^\circ$C; thus, a large stock of the different materials can be prepared at 1 time. Finally, as we are presenting our results as a function of SAH concentration, the lowest measurable amount can be decreased using smaller volumes of reagents; for example, using a 100 µl final incubation volume, we measured 10 pmoles of SAH with satisfactory precision.



^{3}H-SAH binding to brain membranes in the presence of various amounts of cold SAH. 1 ml SAH solution was added to 100 µl ^{3}H-SAH (200,000 cpm per sample, $5 \cdot 10^{-9}$ M) and 900 µl brain homogenate. After 10 min incubation at 0 °C, the samples were filtered of GF/B glass filters and the radioactivity determined by liquid scintillation counting. Each point was the mean of 3 determinations ± SD. ■ Untreated assays; □ treated with SAH hydrolase (50 µl) + adenosine deaminase (4 units), 2 h at 37 °C. For tissue assay, brain tissue was homogenized in Tris buffer pH 7.4, 50 mM (0.5 g/ml) and the proteins were heat denatured (10 min at 100 °C). 1 ml aliquots were assayed: cpm = 438 ± 16 (3 determinations); other 1 ml aliquots were treated by SAH hydrolase, adenosine deaminase mixture and analyzed: cpm = 1240 ± 20 (3 determinations). In another experiment, the homogenate was diluted 10-fold; we obtained 1065 ± 14 cpm. The concentrations of SAH calculated from the radioactivity determination were 1.52 ± 0.08 nmoles/g tissue and 1.66 ± 0.42, respectively.

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A modified leaf chamber assembly to determine the rate of CO_2 exchange for upper and lower sides of leaves

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Summary. Using a simple leaf chamber and a differentially calibrated IR gas analyzer the photosynthetic CO_2 uptake rate has been determined separately for upper and lower sides of a leaf.

The rate of CO_2 exchange in plants has long been studied using either entire plants or leaves. At the present date no literature is available about the measurement of the CO_2 exchange rate separately for the upper and lower sides of a leaf[2,3]. Fluorescence induction studies indicate that the photosynthetic efficiencies of the upper and lower sides of a leaf differ significantly[4]. In this communication we describe a modified leaf chamber IR gas analyzer system to determine the CO_2 exchange rate separately for the upper and lower sides. The CO_2 exchange response in this system was relatively faster than those seen for whole leaves or seedlings.

Phaseolus mungo L. and *Ipomoea pentaphylla* Jacq. seedlings were grown in pots under field conditions. All experiments were performed on fully expanded leaves. The leaf chamber was bell shaped (25 mm diameter and 30 mm in height) with an air inlet and outlet (fig.). The broad mouth had a soft rubber ring to ensure complete sealing against air leakage when fixed on the leaf. The chamber was placed on one side of the leaf, the other side of which