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Methyl group transfer from exogenous *S*-adenosylmethionine on to plasma-membrane phospholipids without cellular uptake in isolated hepatocytes

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At external concentrations of 50 μM, L-methionine was rapidly taken up by hepatocytes, whereas almost no *S*-adenosylmethionine (SAM) was removed from the incubation medium. SAM did not enter the intracellular water space but equilibrated with a very small pool, which was most likely to be situated on the external side of the plasma membrane. Methyl groups from external L-methionine, but not from external SAM, were incorporated into total and nuclear RNA. A significant incorporation of methyl groups into phospholipids occurred not only with methionine but also with SAM. After subfractionation of hepatocytes it became evident that methyl groups from SAM were mainly incorporated into plasma-membrane phospholipids, and that phospholipid methylation in other cellular compartments resulted from contamination with plasma membrane. The pattern of methylation of the various phospholipid species with SAM as precursor was different from that obtained with L-methionine. In contrast with external L-methionine, external SAM did not enter the intracellular SAM pool. According to these results a transport system for SAM does not exist in rat hepatocytes, although methyl groups from external SAM can be incorporated into plasma-membrane phospholipids from the outside.

Studies concerning transmethylation processes in intact cells or tissues can be done by using [*Me*-^{3}H]- or [*Me*-^{14}C]-methionine. After entering the cell the labelled methionine is converted into the corresponding labelled SAM, which then mixes with the internal pool of unlabelled SAM. From this pool methyl groups are transferred by specific methyltransferases on to their physiological acceptors.

Since the early experiments of Stekol *et al.* (1958), who observed an increased formation of methylated products by rat liver slices in the presence of exogenous SAM, various groups have looked for a specific uptake system for SAM. This would offer the opportunity to label the internal SAM pool directly and allow measurements of transfer of methyl groups on to proteins without interference with the incorporation of labelled methionine via protein synthesis.

An uptake system for SAM has indeed been described for yeast cells by Svihla & Schlenk (1960) and by Spence (1971) and was characterized by Murphy & Spence (1972). The transport was temperature- and energy-dependent, had an apparent K_m for SAM of 3.3 μM and was inhibited by *S*-adenosylhomocysteine and *S*-adenosylethionine. Similar findings were obtained by Nakamura & Schlenk (1974).

Stramentinoli *et al.* (1978*a*,*b*) found a transport system for SAM in rabbit erythrocytes. Zappia *et al.* (1978) described the transport of exogenous SAM into isolated perfused rat livers, and Stramentinoli *et al.* (1978) claimed even that exogenous SAM was able to protect rat livers against galactosamine-induced liver damage. Hoffman *et al.* (1980) on the other hand, were unable to find any uptake of SAM or any transfer of methyl groups from exogenous SAM on to DNA or histones in isolated perfused rat livers.

In view of the contradictory reports we have

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; PtdEtn, phosphatidylmonoethanolamine; Ptd[Etn]$_2$, phosphatidyldiethanolamine; PtdCho, phosphatidylcholine; SAM, *S*-adenosyl-L-methionine; SDS, sodium dodecyl sulphate.

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re-evaluated this problem in experiments with isolated hepatocytes. From the results obtained, we conclude that hepatocytes are unable to take up exogenous SAM. Exogenous SAM can, however, act as substrate for methyl group transfer on to plasma-membrane phospholipids.

Material and methods

Rat hepatocytes were isolated by the method of Berry & Friend (1969) and suspended in Krebs–Ringer bicarbonate buffer containing 2% (w/v) bovine serum albumin and 15mM-glucose (termed the 'incubation medium'). About 900–1000mg wet wt. of cells was incubated in about 10ml of the incubation medium in a shaking water bath at 37°C and a shaking frequency of about 50/min. The incubation vessel was flushed with O_2/CO_2 (19:1) during the whole incubation. Unless otherwise mentioned the concentration of SAM and L-methionine was 50μM. The specific radioactivity of [*Me*-^{3}H]SAM and of L-[*Me*-^{3}H]methionine was 20Ci/mol. [U-^{14}C]Sucrose (0.1μCi/ml) was added as an indicator for the adherent water space.

Experiments with unfractionated cells

In experiments with unfractionated hepatocytes, the cells were separated from the incubation medium by silicone oil filtration centrifugation (Pfaff & Klingenberg, 1968). For this purpose 300μl of 7% $HClO_4$ ($d = 1.065$) was pipetted into Eppendorf tubes and overlayed with 500μl of silicone oil ($d = 1.041$). The hepatocyte suspension (800μl) was added on top of the silicone oil layer and the hepatocytes were sedimented into the $HClO_4$ phase by centrifugation for 10s at 10000*g* in a Beckman 152 Microfuge equipped with a home-made swing-out rotor.

Experiments with subcellular fractions of liver cells

The incubations were carried out as already described except that [U-^{14}C]sucrose was omitted, and that the incubation contained [*Me*-^{3}H]SAM (50μM; sp. radioactivity 100Ci/mol) plus L-[*Me*-^{14}C]methionine (50μM; sp. radioactivity 20Ci/mol).

At the end of the incubation the cells were diluted by adding a 3-fold volume of cold incubation buffer and spun at 100*g* for 150s. The sedimented cells were suspended in 10ml of buffer A (2.5mM-Hepes/0.75mM-$CaCl_2$, pH6.5) and homogenized by five strokes by hand in a tight-fitting Dounce homogenizer. The homogenate was spun at 150*g* for 10min to sediment nuclei and unbroken cells. The sediment was resuspended in 10ml of buffer A in the Dounce homogenizer, brought to 25ml by addition of buffer A in the Dounce homogenizer and spun again at 150*g* for 10min. The supernatant was combined with the first supernatant. The sediment represented almost pure nuclei. The combined supernatants were used for the isolation of plasma membranes, microsomes and cytosol. To this end, the 150*g* supernatant was centrifuged at 2000*g* for 10min and the sediment used for the isolation of plasma membranes by the method of Dorling & Le Page (1973). The final plasma-membrane fraction was diluted to a concentration of 2–3mg of protein/ml. The 2000*g* supernatant was spun at 10000*g* for 10min, which removed mitochondria and most of the lysosomes and peroxisomes. The resulting supernatant was spun at 100000*g* for 45min and the supernatant was designated 'cytosolic fraction'. The resuspended sediment was centrifuged again for 45min at 100000*g*. Resuspension and centrifugation were repeated a third time and the final sediment was designated 'microsomal fraction'.

Isolation and determination of SAM in the cytosolic fraction

To the cytosolic fraction ice-cold $HClO_4$ was added to a final concentration of 3.5%. Insoluble material was sedimented by centrifugation at 10000*g* for 15s. The supernatant was brought to pH6–7 with 5M-K_2CO_3 and the $KClO_4$ was removed by centrifugation. The neutralized supernatant was brought to 10mM-HCl and 0.5ml was loaded on to an SP-Sephadex G-25 column (0.5cm × 10cm) that had been equilibrated with 10mM-HCl. Methionine was eluted with 30ml of 50mM-HCl. The column was flushed with 30ml of 150mM-HCl, and SAM was finally eluted with 20ml of 500mM-HCl. The concentration of SAM was determined by measuring the absorption at 256nm. For control of the purity of this fraction, 2ml of the SAM containing eluate was freeze-dried, dissolved in 100μl of 10mM-HCl, and 50μl was analysed by t.l.c. on silica-gel plates (HPTLC Fertigplatten Kieselgel 60F 254; Merck, Darmstadt, Germany). The plates were developed with n-propanol/aq. 7M-NH_3 (7:3, v/v), and SAM was visually detected by u.v. light. The spots corresponding to SAM were scraped off, eluted with 2ml of 10mM-HCl and the absorption at 256nm as well as the radioactivity were measured.

Isolation of RNA

RNA was isolated from hepatocytes or isolated nuclei by a modification of the method of Nienhuis *et al.* (1974). When RNA from whole hepatocytes was analysed, the cells were isolated by silicone oil filtration centrifugation as described above, except that the bottom phase consisted of a 'lysing solution' (2mM-$MgCl_2$ / 1mM-dithioerythritol / 0.1mM-EDTA in deuterated water). After centrifugation, the buffer and silicone oil layers were removed and 650μl of the lysing solution was added, followed by addition of 1ml of 1% (w/v) SDS in 0.4M-NaCl. When the

material was completely lysed and dissolved, 2ml of a phenol solution (100ml of phenol/14ml of *m*-cresol/0.1g of 8-hydroxyquinolone/16ml of water) was added and RNA was isolated further as described by Nienhuis *et al.* (1974). Since the sedimented RNA could still contain traces of methylated polymers other than RNA (e.g., proteins), the isolated RNA was dissolved in 200μl of 5mM-Tris/HCl buffer, pH7.4. To 100μl of this suspension 250μl of 0.1M-sodium acetate (pH5.0) was added followed by addition of 100 units of bovine pancreas RNAase in 150μl of water or only of 150μl of water (control). After incubation for 30min at 25°C, the reaction was stopped with 500μl of ice-cold 0.7M-$HClO_4$. After standing in ice for 15min, insoluble material was removed by centrifugation; 1ml of the supernatant from the RNAase-treated and control samples was counted for radioactivity. Pilot experiments had shown that under these conditions RNA became sufficiently digested by RNAase to recover all u.v.-absorbing material in the supernatant. Concentrations of RNA were determined by the method of Fleck & Munro (1962).

Isolation of phospholipids and determination of phospholipid methylation

Phospholipids were isolated by the method of Skidmore & Entenman (1962). They were dissolved in 120μl of methanol/chloroform (2:1; v/v) and 100μl was transferred on to silica-gel t.l.c. plates. The chromatograms were developed with chloroform/methanol/aq. 7M-NH_3 (12:7:1, by vol.). The dried plates were scanned for radioactivity with a Berthold thin-layer scanner. Phospholipid fractions were scraped off and eluted by the method of Skipsi & Peterson (1964). The eluates corresponding to PtdEtn, Ptd[Etn]$_2$ and PtdCho were dried by rotary evaporation, dissolved in 150μl of methanol and analysed for radioactivity after addition of 5ml of a toluene-based scintillator. Lipid phosphorus was determined by the method of Skipski & Peterson (1964).

Other determinations

5′-Nucleotidase activity was measured by the method of Gerlach & Hiby (1974).

Protein was determined in the presence of SDS by using a modification of the method of Lowry *et al.* (1951) described by Markwell *et al.* (1978). Radioactivity was measured by liquid-scintillation counting in a Searle Analytic Mark III scintillation spectrometer. Aqueous samples were counted for radioactivity with Instagel (Packard Instruments, Frankfurt, Germany). $HClO_4$-precipitated material was first dissolved in 1ml of NCS tissue solubilizer (New England Nuclear, Dreieich, Germany) and counted for radioactivity after addition of 5ml of a toluene-based scintillator, which was also used for counting the radioactivity in the phospholipid fractions.

Calculations

Uptake of SAM was calculated from the silicone oil separation experiments in which [*Me*-^{3}H]SAM and [U-^{14}C]sucrose were used according to the following equation:

$$\text{SAM uptake (pmol)} = \frac{R_{PCA}(^3H) - \frac{R_T(^3H)}{R_T(^{14}C)} \cdot R_{PCA}(^{14}C)}{R_{SAM}(\text{spec.})} \cdot \frac{R_{SAM}}{R_{PCA}(^3H)}$$

where $R_{PCA}(^3H)$ and $R_{PCA}(^{14}C)$ represent total ^3H and ^{14}C radioactivity (d.p.m.) respectively in the $HClO_4$ phase, $R_T(^3H)$ and $R_T(^{14}C)$ the total ^3H and ^{14}C radioactivity (d.p.m.) respectively in the incubation medium, $R_{SAM(spec.)}$ the specific radioactivity (Ci/mol) of the added [*Me*-^{3}H]SAM, and R_{SAM} the total ^3H radioactivity of SAM in the $HClO_4$ phase after the removal of other ^3H-labelled compounds from this phase by SP-Sephadex G-25 chromatography.

Materials

Radioactive compounds, were purchased from the Amersham Buchler Co., Braunschweig, Germany. [*Me*-^{3}H]SAM was purified by SP-Sephadex G-25 chromatography before use as it contained about 10–15% contaminating radioactive material. SP-Sephadex G-25 was from Deutsche Pharmacia, Mainz, Germany; RNAase was from Boehringer, Mannheim, Germany. All other chemicals were from Merck.

Results

Isolated hepatocytes removed rapidly L-methionine from the medium (Fig. 1). The uptake proceeded at a higher rate during the first 1–2min and reached an almost linear lower rate of about 3.8nmol·g^{-1}·min^{-1} after about 5min. SAM on the other hand was only very slowly removed from the medium. After 30min more than 90% of the initial SAM could still be recovered from the medium (Fig. 1).

Separation of cells from the medium by silicone oil filtration centrifugation showed that external SAM equilibrated with a cell-associated compart-

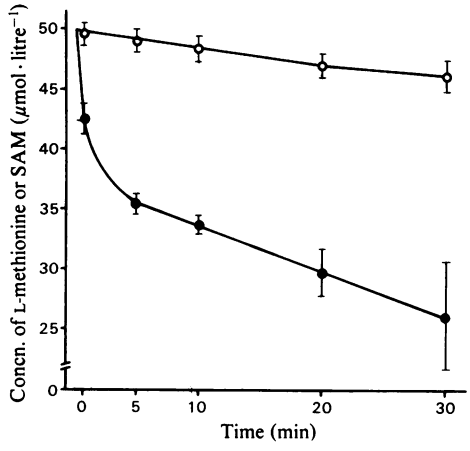

Fig. 1. *Removal of [Me-^{3}H]methionine (●) and [Me-^{3}H]SAM (○) from the incubation medium by isolated rat liver cells*

Rat liver cells (2g) were incubated in 20ml of Krebs–Ringer bicarbonate buffer containing 15mM-glucose and 2% bovine serum albumin. The initial concentration of L-methionine and of SAM was 50μM. The incubation temperature was 37°C. Results are means ± S.D. from four experiments in each group.

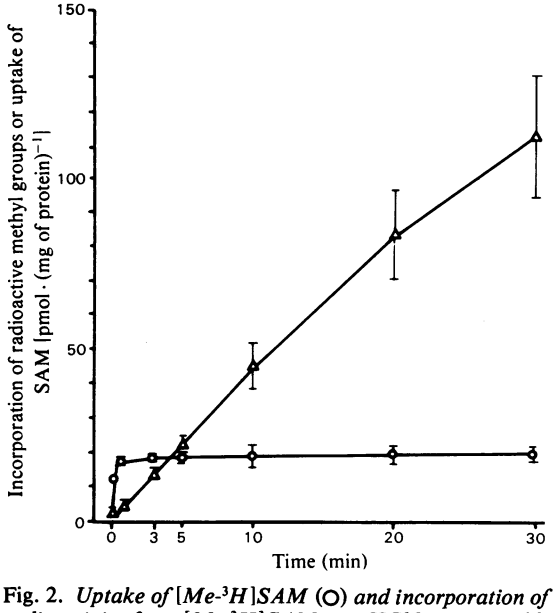

Fig. 2. *Uptake of [Me-^{3}H]SAM (○) and incorporation of radioactivity from [Me-^{3}H]SAM into $HClO_4$-precipitable material by isolated rat liver cells (△).*

The concentration of SAM was 50μM (sp. radioactivity 20Ci/mol). The incubation conditions were as given in the legend to Fig. 1. The uptake of SAM has been corrected for adherent water by measuring the sucrose space. Results are means ± S.D. ($n = 3$), represented by the bars.

ment within about 60s, whereas the incorporation of radioactive methyl groups from [*Me*-^{3}H]SAM into $HClO_4$-insoluble material occurred at an almost linear rate over the whole 30min incubation period (Fig. 2).

Formation of [^{3}H]SAM from L-[*Me*-^{3}H]methionine was linear during the first 5min, but slowed down after this time point (Fig. 3).

In the presence of L-[*Me*-^{3}H]methionine there was a linear formation of [^{3}H]methylated RNA during the first 10min at 25°C as well as at 37°C. After this time point the rate of incorporation decreased (Fig. 4). In contrast incorporation of methyl groups from [*Me*-^{3}H]SAM into RNA was negligibly small. Methyl group incorporation into PtdCho occurred almost linearly over 30min, irrespective of whether L-[*Me*-^{3}H]methionine or [*Me*-^{3}H]SAM was the labelled precursor. The incorporation into PtdEtn and Ptd[Etn]$_2$ occurred mainly during the first 10min and represented only a small fraction of the radioactivity incorporated into the PtdCho fraction. After 30min the ratio PtdCho radioactivity/PtdEtn radioactivity was 32 and 71 with [*Me*-^{3}H]SAM and

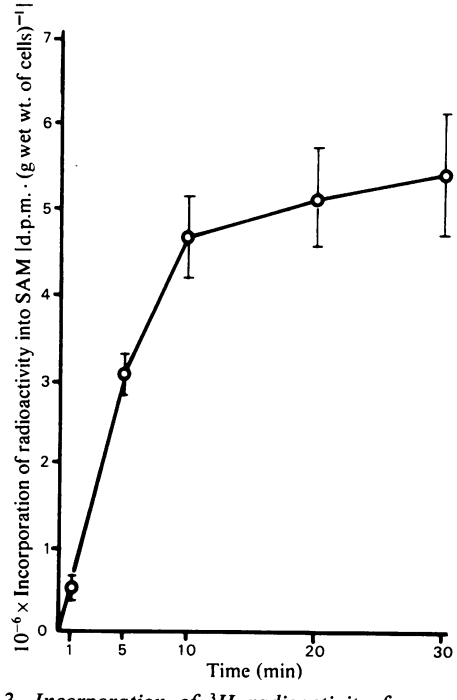

Fig. 3. *Incorporation of ^{3}H radioactivity from L-[Me-^{3}H]methionine into SAM by isolated rat liver cells*

The incubation temperature was 25°C, the initial concentration of L-methionine 50μM (sp. radioactivity 20Ci/mol). Otherwise the incubation conditions were the same as given in the legend to Fig. 1. Results are means ± S.D. (from three experiments), represented by the bars.

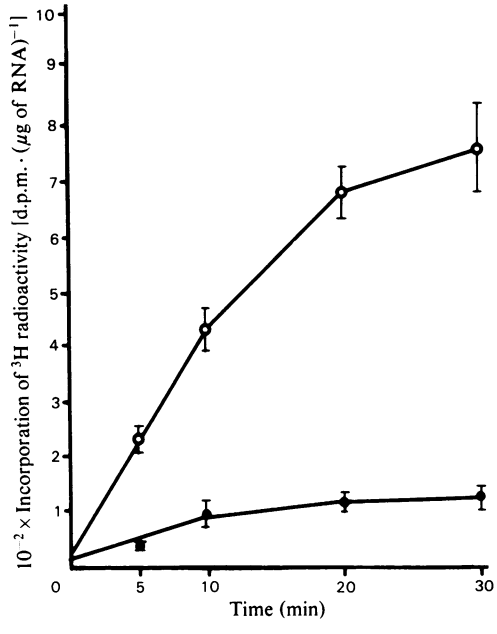

Fig. 4. *Time-dependent incorporation of radioactivity from* L-[*Me*-^{3}H]*methionine into total RNA by isolated rat hepatocytes*

The concentration of L-methionine was 50μM (sp. radioactivity 20Ci/mol). Results are means ± S.D. (from three experiments in each group), represented by the bars. ○, [*Me*-^{3}H]methionine, incubation temperature 37°C; ●, [*Me*-^{3}H]methionine, incubation temperature 25°C. The incorporation of radioactivity from [*Me*-^{3}H]SAM (50μM; sp. radioactivity 20Ci/mol) at 25°C or 37°C was below the limits of experimental error; up to 30min, incorporation did not exceed 7d.p.m./μg of RNA and 5d.p.m./μg of RNA at 25°C and 37°C respectively. The incorporation from [*Me*-^{3}H]methionine at zero time results from the fact that for this time point the reaction was started and immediately stopped by silicone oil layer centrifugation. The short time elapsing between the start of the experiments and migration of cells into the bottom phase was sufficient to allow for some incorporation into RNA.

L-[*Me*-^{3}H]methionine respectively. The corresponding values for the PtdCho/Ptd[Etn]$_2$ radioactivity ratios were 10 and 22 respectively.

Experiments with subfractionations of cells

When cells were simultaneously incubated with [*Me*-^{14}C]methionine and [*Me*-^{3}H]SAM for 30min, followed by subfractionation of cells, a significant incorporation of ^{14}C radioactivity, but not of ^{3}H radioactivity into nuclear RNA could be measured [^{14}C radioactivity incorporated, 2712 ± 224d.p.m./μg of RNA; ^{3}H radioactivity incorporated, 106 ± 115d.p.m./mg of RNA (means ± S.D., $n = 4$)]. As the specific radioactivity of [*Me*-^{3}H]SAM was five times that of [*Me*-^{3}H]methionine the incorporation of methyl groups from external L-methionine into nuclear RNA was almost 130-fold higher than that from exogenous SAM, indicating that external SAM had almost no access to the intracellular SAM pool.

The distribution of 5′-nucleotidase activity and total phospholipids between the subcellular fractions tested is given in Table 1. The plasma-membrane-enriched fraction showed a 9.2-fold increase in specific 5′-nucleotidase activity indicating a reasonable purification of plasma membranes.

Table 2 gives the incorporation of radioactive methyl groups into PtdEtn, Ptd[Etn]$_2$ and PtdCho of the total homogenate and the two subcellular fractions under consideration. There occurred a significant incorporation of methyl groups not only from L-[*Me*-^{3}H]methionine but also from [*Me*-^{3}H]SAM. However, whereas the incorporation from L-[*Me*-^{3}H]methionine was high in the microsomal, and low in the plasma-membrane fraction, the opposite was the case for [*Me*-^{3}H]SAM, indicating that labelling from exogenous [*Me*-^{3}H]SAM did occur via a SAM pool different from that labelled by L-[*Me*-^{14}C]-methionine. This was further supported by the fact that the PtDCho/PtdEtn and PtdCho/Ptd[Etn]$_2$ radioactivity ratios differed considerably with the different precursors.

Table 1. *Distribution of 5′-nucleotidase activity and phospholipid content in the plasma-membrane fraction and the 100000g sediment in comparison with the total homogenate from isolated rat liver cells*

Results are means ± S.D. ($n = 4$). The numbers in parentheses represent relative enzyme activity with the activity in the total homogenate set to 1.0.

Fraction	5′-Nucleotidase [μg of P_i · (mg of protein)$^{-1}$ · 30min^{-1}]	Total phospholipids [μg of lipid phosphorus · (mg of protein)$^{-1}$]
Total homogenate	29.1 ± 8.1 (1.0)	102 ± 15
Plasma membranes (37–41% sucrose interphase)	266.9 ± 30.2 (9.2)	240 ± 30
Microsomes (100000g pellet)	64.1 ± 10.3 (2.2)	223 ± 22

Table 2. *Incorporation of radioactivity from [Me-^{3}H]SAM (a) and [Me-^{14}C]methionine (b) into phospholipids of total homogenates, plasma membranes and microsomes from isolated rat liver cells*

The incubation was carried out at 37°C for 30min in the presence of 50μM-SAM (sp. radioactivity 100Ci/mol) and 50μM-L-methionine (sp. radioactivity 20Ci/mol). Results are means ± S.D. ($n = 4$).

Fraction	Radioactivity (d.p.m./μg of lipid phosphorus)			Radioactivity ratio	
	PtdEtn	Ptd[Etn]$_2$	PtdCho	PtdCho/PtdEtn	PtdCho/Ptd[Etn]$_2$
(a) Plasma membranes	338 ± 84	1066 ± 173	15833 ± 3274	46.8	14.9
Total homogenate	146 ± 24	398 ± 103	6391 ± 329	43.8	16.1
Microsomes	60 ± 22	254 ± 60	2719 ± 547	45.6	10.7
(b) Plasma membranes	23 ± 17	55 ± 35	2522 ± 1050	109.7	45.9
Total homogenate	23 ± 17	101 ± 10	4193 ± 914	182.3	41.5
Microsomes	16 ± 6	95 ± 7	5398 ± 977	337.4	56.8

Table 3. *Equilibration of exogenous methionine or SAM with the intracellular SAM pool in isolated rat hepatocytes*

Isolated hepatocytes were incubated in the presence of either 50μM-L-[Me-^{14}C]methionine or 50μM-[Me-^{3}H]SAM for 30min. They were separated by silicone oil layer centrifugation from the medium and the specific radioactivity of the intracellular SAM pool was determined as given in the Materials and methods section. Results are means ± S.D. from three experiments. The values in parentheses show the relative radioactivity (ratios *a/b* and *c/d*, as appropriate).

	Specific radioactivity (Ci/mol)
(a) [Me-^{14}C]Methionine in the medium	20 (20.6)
(b) Intracellular [Me-^{14}C]SAM	0.97 ± 0.09
(c) [Me-^{3}H]SAM in the medium	100 (188.7)
(d) Intracellular [Me-^{3}H]SAM	0.53 ± 0.11

It seems likely that the incorporation from [Me-^{3}H]SAM into microsomal phospholipids was due to contamination with plasma membranes. In line with this is the constancy of the PtdCho/PtdEtn and PtdCho/Ptd[Etn]$_2$ radioactivity ratios in the total homogenate and the subcellular fractions in the experiments with [Me-^{3}H]SAM, whereas labelling with L-[Me-^{14}C]methionine clearly resulted in different PtdCho/PtdEtn ratioactivity ratios in the different fractions (Table 2).

Although the radioactivity incorporated into plasma-membrane phospholipids was higher with exogenous [Me-^{3}H]SAM, one has to take into account that the specific radioactivity of [Me-^{14}C]methionine was almost 20-fold diluted in the endogenous SAM pool (see Table 3), whereas incorporation from exogenous [Me-^{3}H]SAM most likely occurred from a pool that had almost the same specific radioactivity as the [Me-^{3}H]SAM in the incubation medium. As the initial specific radioactivity of [Me-^{3}H]SAM was 100Ci/mol, and that of L-[Me-^{14}C]methionine only 20Ci/mol, one can estimate that the total incorporation of methyl groups from the endogenous SAM pool (i.e. from the inside) was about 15 times higher than the incorporation from the exogenous SAM pool (i.e. from the outside).

Data on the specific radioactivities of the intracellular SAM pool are given in Table 3. Even after incubation for 30min the specific radioactivity of the cellular SAM pool was only about 5% of that of the exogenous L-[Me-^{14}C]methionine. When [Me-^{3}H]SAM was the substrate the specific radioactivity of the intracellular SAM pool was only about 0.5% of that of the exogenous SAM pool, indicating that exogenous SAM had practically no access to the intracellular SAM pool.

Discussion

The rapid equilibration of exogenous radioactive SAM with a cell-associated pool (see Fig. 2), together with the fact that even after an incubation for 30min almost no radioactive SAM appeared in the intracellular pool (Table 3), indicates that exogenous SAM does not penetrate the plasma membrane but equilibrates with a small sucrose-inaccessible compartment on the outer side of this membrane. This is further supported by the following two findings. (1) There is almost no methyl group transfer from exogenous SAM on to total or nuclear RNA. (2) There is a significant incorporation of methyl groups from exogenous SAM into plasma-membrane phospholipids, whereas incorporation into phospholipids of intracellular membranes is significantly less and most likely due to contamination with plasma membranes.

This fits well with the recent data of Hoffman *et al.* (1980), who observed neither an uptake of exogenous SAM by isolated perfused rat livers nor a measurable incorporation into DNA or histones. Previous reports on uptake of SAM by various cell

systems (see the introduction) may be partially explained by omission of appropriate corrections for adherent water, and partially by the fact that commercially available radioactive SAM contains considerable amounts of breakdown products (in our hands about 14% of total radioactivity), which may have entered the cells.

Our results nevertheless indicate that plasma-membrane phospholipids of isolated hepatocytes (and possibly of other cells) can be methylated as well from the outside (exogenous SAM) as from the inside (via the endogenous SAM pool). This might be of importance for the interpretation of experiments where phospholipid methylation has been measured with exogenous SAM as substrate in isolated cells, cell ghosts or membranes. The data in Table 2 indicate that the pattern of phospholipid methylation in the plasma membrane (as represented by the PtdCho/PtdEtn and PtdCho/Ptd[Etn]$_2$ radioactivity ratios) may be different, depending on whether SAM is coming from the outside or from the inside. Experiments with exogenous SAM therefore may lead to erroneous results.

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