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METHIONINE SYNTHASE ASSAY BASED ON COUPLING WITH THYMIDYLATE SYNTHASE REACTION

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Methionine synthase reaction may be coupled with thymidylate synthase-
catalysed tritium release from $[5^-3H]dUMP$ via non-enzymatic reaction of
formaldehyde with tetrahydrofolate. A convenient and sensitive assay of methionine
synthase activity, based on this principle, is described.

Methionine synthase (B12-dependent $N^5$-methyltetrahydropteroylglutamate:
homocysteine methyltransferase, EC 2.1.1.13) catalyses $S$-methylation of
homocysteine with methyl group derived from L(-)-$N^5$-methyltetrahydrofolate.
Methionine and tetrahydrofolate are the reaction products (Taylor & Weissbach,
1973). The described earlier and commonly used assay of the enzyme
activity, based on the determination of $[^{14}C]$methionine formed in the reaction
in which $N^5-[^{14}C]$methyltetrahydrofolate is used as a cofactor (Weissbach
et al., 1963) involves a rather tedious separation of $[^{14}C]$methionine from
$N^5-[^{14}C]$methyltetrahydrofolate.

We applied thymidylate synthase (methylene tetrahydrofolate:deoxyuridine-
$5'$-monophosphate $C$-methyltransferase, EC 2.1.1.45), catalysing methylation
of dUMP to dTMP, in order to determine tetrahydrofolate produced in the
methionine synthase reaction. It is possible to couple the reactions catalysed
by both enzymes via a non-enzymatic reaction of tetrahydrofolate with
formaldehyde (Blakley, 1960), (Scheme 1). The product of this reaction,
$N^{5+10}$-methylene tetrahydrofolate, is a cofactor in the thymidylate synthase
reaction, being consumed in an equimolar ratio with dUMP (Blakley, 1969).
Since methylation of each molecule of dUMP is accompanied by the release
of a protonium from its position 5 (Lomax & Greenberg, 1967), it is possible
to determine the consumption of $N^{5+10}$-methylene tetrahydrofolate (and thus
tetrahydrofolate production) by using $[5^-3H]dUMP$ and measuring tritium
Scheme 1. Mechanism of methionine synthase assay based on measurement of tritium released from [5-3H]dUMP. SAM, S-adenosylmethionine.

released. The latter is easy to achieve just by adding charcoal to the reaction mixture and counting tritium remaining in water (Roberts, 1966).

We present here a comparison of results obtained when methionine synthase activity was determined in crude extracts from mouse liver and Ehrlich ascites carcinoma cells by measuring [14C]methionine formation or tritium release from [5-3H]dUMP.

MATERIALS AND METHODS

N5-[14C]Methyltetrahydrofolate (59 mCi/mmol) and [5-3H]dUMP (12.7 Ci/mmol) were obtained from Amersham (Great Britain), Dowex 1 × 8 Cl−, 200 - 400 mesh, from Fluka AG (Switzerland), Norit A from Serva (F.R.G.), cyano B12 and DL-homocysteine-thiolactone-HCl from Sigma Chem. Comp. (U.S.A.) and S-adenosyl-L-methionine from Calbiochem (U.S.A.). N5-Methyltetrahydrofolate was synthesized according to Sakami (1963). DL-Homocysteine was prepared immediately before use from its thiolactone-HCl by hydrolysis in 6 m-KOH at room temperature for 30 min (Du Vigneaud et al., 1938) and, after neutralization, diluted to the desired concentration. Thymidylate synthase was purified from Ehrlich ascites carcinoma cells as previously described (Jastreboff et al., 1982).

Preparation of crude extracts. Livers of Swiss mice were homogenized with 3 volumes of ice-cold phosphate-buffered (pH 7) saline in a Potter-Elvehjem homogenizer and centrifuged at 200 g for 5 min at 4°C. The supernatant was centrifuged at 20000 g for 10 min at 4°C and the resulting pellet discarded.

Crude extract from Ehrlich ascites carcinoma cells was prepared as previously described (Jastreboff et al., 1982).

Extracts were assayed for protein by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Enzyme assay: determination of [14C]methionine formation. Slightly modified method of Kamely et al. (1973) was used. The reaction mixture in a total volume of 0.2 ml contained: 100 mM-K-phosphate buffer, pH 7.5, 250 mM-2-mercaptoethanol, 100 μM-ascorbate, 250 μM-homocysteine, 500 μM (0.25 μCi) N5[14C]methyltetrahydrofolate, 50 μM-cyano-B12, 250μM-S-adenosyl-L-methio-
nine, and the extract corresponding to 0.45 - 3.6 mg protein. Incubation was carried out at 37°C and the reaction was terminated by addition of 0.8 ml of ice-cold water. The resulting mixture was passed through a Dowex 1×8 (Cl⁻) column (0.5×4 cm) which retains N⁵[^14]Cl-methyltetrahydrofolate. The column was washed with an additional 1.0 ml of water and the[^14]Cl-methionine formed was measured in the pooled effluent by counting an aliquot with a liquid scintillation counter (Packard 2003). All assays were run in triplicate. Controls were not incubated.

Enzyme assay: determination of tritium released from [5-³H]dUMP. The reaction mixture in a total volume of 40 μl contained: 100 mm-K-phosphate buffer, pH 7.5, 250 mm-2-mercaptoethanol, 100 μm-ascorbate, 250 μm-homocysteine, 500 μm-N⁵-methyltetrahydrofolate, 50 μm-cyano-B₁₂, 250 μm-S-adenosyl-L-methionine, 8 mm-formaldehyde, 50 μm-[5-³H]dUMP (1.85 × 10⁸ cpn/μmol), thymidylate synthase (activity of 17 pmol/min: the presence of higher activity did not influence the results of the assay) and extract corresponding to 0.045 - 0.36 mg protein. The reaction was started by addition of the extract and terminated, after incubation at 37°C, by addition of 200 μl of charcoal suspension (Norit A, 100 mg/ml) in 2% solution of trichloroacetic acid. The mixture was centrifuged at 10000 g for 1 min. Samples of 100 μl were counted with a liquid scintillation counter. All assays were run in tightly closed 1.5 -ml polyethylene centrifuge tubes in triplicate. In controls the extract was added after addition of charcoal.

RESULTS

The specific activity of methionine synthase in mouse liver extracts was 0.064 ± 0.004 nmol/min per mg protein and 0.064 ± 0.002 nmol/min per mg protein (± SEM, n=4) when determined by measuring [¹⁴]Cl-methionine formation and release of tritium from [5-³H]dUMP, respectively. The dependence of both assays on the reaction time and protein content was very similar (Fig. 1), indicating that under conditions of the new assay the thymidylate synthase reaction (reflected by tritium release from [5-³H]dUMP) is tightly coupled with the methionine synthase reaction.

Both assays also showed similar requirements of the methionine synthase reaction. Only traces of the activity could be found when homocysteine or cyano-B₁₂ and S-adenosyl-L-methionine were absent from either reaction mixture (Table 1). Besides, the assay based on tritium release determination showed only traces of activity when no formaldehyde was added to the reaction mixture (Table 1). These facts present evidence that the new assay depends on the methionine synthase reaction and that formaldehyde is necessary to couple the latter with the thymidylate synthase reaction, as assumed in Scheme 1.
Fig. 1. Dependence of $[^{14}\text{C}]$methionine synthesis (upper panels) and tritium release from $[5^{-3}\text{H}]$dUMP (lower panels) on the reaction time and protein content of the reaction mixture. Mouse liver extract was used.

Table 1

Requirements of methionine synthase activity assayed in mouse liver and Ehrlich ascites carcinoma extracts by measuring either $[^{14}\text{C}]$methionine formation or tritium release from $[5^{-3}\text{H}]$dUMP

<table>
<thead>
<tr>
<th>Omissions from the reaction mixture</th>
<th>Methionine synthase activity (pmol/min per mg protein)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mouse liver</td>
</tr>
<tr>
<td></td>
<td>$[^{14}\text{C}]$Met</td>
</tr>
<tr>
<td>None</td>
<td>68</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>0.3</td>
</tr>
<tr>
<td>Cyano-B$_1$, and</td>
<td>0.1</td>
</tr>
<tr>
<td>S-adenosyl-l-Met</td>
<td></td>
</tr>
<tr>
<td>Formaldehyde</td>
<td></td>
</tr>
<tr>
<td>Enzyme extract</td>
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</table>
DISCUSSION

The assay presented here is easier to perform and much more sensitive than the assay based on measuring $^{14}C$-methionine formation ($[5^{-3}H]dUMP$ is available at specific radioactivity up to 15 Ci/mmol). Since thymidylate synthase purified from methotrexate-resistant Lactobacillus casei may be purchased from the New England Enzyme Center, Tufts University (Boston, Ma., U.S.A.), then the availability of this enzyme should not limit application of the assay.

Obviously, it should be possible to use the thymidylate synthase-catalysed tritium release from $[5^{-3}H]dUMP$ to measure activity of any enzyme producing or consuming tetrahydrofolate or $N^{5,10}$-methylene-tetrahydrofolate.

REFERENCES


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