fractions between Nos 40 and 55. This position coincided with the position where the Ca^{2+}-dependent neutral protease of rat brain (mol. wt 84,000[1]) should be eluted[2]. By contrast, the fractions at the void volume decreased the preexistent protease activity, giving a large negative profile in figure 1. The results clearly indicated the occurrence of an endogenous, high mol. wt inhibitor(s) in the brain which could be separated from the Ca^{2+}-dependent protease upon gel filtration under the conditions employed.

The inhibitor fractions (Nos 26 through 40) were combined and concentrated to 7.4 ml (139 mg of protein) using Amicon PM-10 membrane. When rechromatographed on Sephadex G-200, the inhibitor was eluted near the void volume of the column, giving an approximate mol. wt of 3×10^5. The inhibitor was found to be markedly heat-stable: even after heating at 100 °C for 20 min, the inhibitor retained ist inhibitory potency as well as its large molecular size. This fact excluded the possibility of contamination of the present inhibitor preparation with protease inhibitors in serum such as α_2-macroglobulin and α_1-antitrypsin which are known to be heat-labile. Nevertheless, the inhibitor is of protein nature, since it lost the inhibitory potency by an incubation with one-hundredth ($^1/_{100}$) weight of trypsin at pH 7.5 and 37 °C for 40 min.

The inhibitor did not affect at all the caseinolytic activity of trypsin, chymotrypsin, and papain (data not shown), indicating that it is different from the inhibitor reported to be present in bovine brain and effective on trypsin[4]. The inhibition seemed to be specific to Ca^{2+}-dependent protease, but it was not due to the possible binding of the inhibitor with Ca^{2+} in the medium which is essential for the activity of the protease concerned. The evidence supporting this view is shown in figure 2.

A high mol. wt inhibitor of Ca^{2+}-dependent neutral protease was also found to be present in bovine cardiac muscle[5] and in rat liver[6]. We have recently found that the inhibitor from the liver was also effective on the protease from the brain, and vice versa. Since the biological significance of Ca^{2+}-dependent protease, widely distributed in various tissues, is at least partly understood in connection with cyclic nucleotide-independent activation of protein kinase[7], the present discovery of a unique and endogenous inhibitor must be of prime interest.

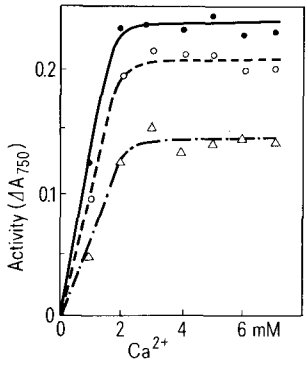

Fig. 2. Inhibition of Ca^{2+}-dependent protease as a function of Ca^{2+} concentration in the medium. The amounts of inhibitor added per tube were: ●, zero; ○, 4.3 µl; △, 13.3 µl. Each tube contained 50 µl of Ca^{2+}-dependent protease from rat brain. The assay was carried out at 37 °C for 30 min.

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Binding capacities of various analogues of S-adenosyl-L-homocysteine to protein methyltransferase II from human erythrocytes[1]

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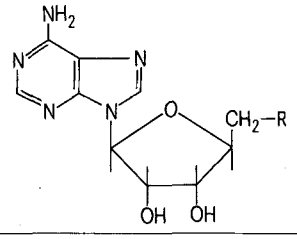
Summary. A series of analogues of S-adenosyl-L-homocysteine, modified mainly in the amino acid portion of the molecule, have been synthesized. All were found to be competitive inhibitors of protein methyltransferase II from human erythrocytes. S-adenosyl-L-homocysteine remains however by far the most effective inhibitor of the methylase.

5′-isobutylthio-5′-deoxyadenosine (SIBA) has been shown recently to inhibit cell transformation by Rous sarcoma virus[3], mitogen-induced blastogenesis[4], polyoma virus replication[5] and capping of herpes virus mRNA[6]. Furthermore, it possesses an antimalarial activity against *Plasmodium falciparum*[7].

SIBA, as well as other analogues, has been thought to act on the cells as inhibitors of methyltransferases. This remains to be proved, however. Earlier results obtained on various methyltransferases acting on t-RNA[8] or small metabolites[9-14] were not in favour of such an hypothesis. More recent reports have given evidence of a more powerful inhibition by SIBA of the methylation of the 5′-terminal 'cap' of m-RNA, as well as of arginine residues in proteins[6,15].

Protein methyltransferase II (E.C. 2.1.1.24) catalyses the conversion of carboxylic functions in proteins into their methyl esters. These are particularly unstable[16] and the protein substrate is readily regenerated under physiological conditions. The enzyme responsible for this type of methylation was purified and isolated in an homogeneous form from the cytosolic fraction of calf thymus[17] and from equine red blood cells[18]. The latter preparation was well characterized from the molecular point of view. Also, the

Inhibition of protein methyltransferase II from human erythrocytes by S-adenosyl-L-homocysteine and analogues



Derivative	R	K_i(μM)
S-adenosyl-L-homocysteine	$S-CH_2-CH_2-CH(NH_2)-COOH$	0.1
Adenosine	OH	3500
1	Cl	650
2	$S-CH(CH_3)-CH_3$	> 300
3	$S-CH_2-CH_2-CH_2-CH_3$	> 6000
4	$S-CH_2-COOH$	> 1600
5	S-⟨O⟩	> 1100
6	$S-CH_2-CH_2-OH$	3000
7	S-⟨O⟩($-NO_2$, $-COOH$)	15
8	$S-CH_2-CH_2-NH_2$	500
9	$S-C(CH_3)_2-CH(NH_2)-COOH$	1600
10	S-⟨O, N⟩	⩾ 800
11	$S-CH_2-CH_2-CH(NH_2)-COOH$ (O)	7
12	(+)$S-CH_2-CH_2-CH_2-CH_3$ (CH_3)	38

amino acid side chains located in the active site of the enzyme were tentatively identified using a series of chemical modifications.

In order to characterize further this active site, analogues of S-adenosyl-L-homocysteine were synthesized and their binding to methylase II was studied. The results of this investigation are described in this communication.

Material and methods. Adenosine (lot 85C-0377), L-homocysteine thiolactone (lot 97C-3776) and 2-mercaptoethanol (lot 116C-0165) were provided by Sigma. Hexamethylphosphorotriamide, tri-n-butyl phosphin, dry pyridin (whose water content was less than 0.01%), 2,2′-dipyridyl disulfide and isopropyl mercaptan were purchased from Merck. Aldrich provided thionyl chloride and methyl iodide; Fluka, 5,5′-dithio-bis-(2-nitrobenzoic acid) and Baker, absolute methanol. The radiochemical centre Amersham supplied S-adenosyl-L-(methyl-^{14}C)-methionine (58 mCi/mmole). n-Buthyl mercaptan, thiophenol, D-penicyllamine, thioglycolic acid and cysteamine were generous gifts of Professors Fuks and Patriarche. 5-mercapto-2-nitrobenzoic acid was obtained by reduction of Ellman's reagent according to Degani and Patchornik[19]. 5′-chloro-5′-deoxyadenosine (derivative 1, see table) was synthesized according to Kikugawa and Ichino[20].

S-adenosyl-L-homocysteine and derivatives 2–9 were prepared by heating a mixture of 5′-chloroadenosine (3 mmoles), NaOH (30 mmoles) and the corresponding mercaptan (10 mmoles) in 15 ml water at 80 °C for 75 min[21]. Derivatives 2–5 were precipitated by adding glacial acetic acid to neutrality and recrystallized from absolute methanol. In order to purify S-adenosyl-L-homocysteine, as well as derivatives 6–9, after neutralization, the reaction mixture was submitted to a chromatography on a Biorex-70 (H^+ form) column (2.5 × 25 cm) and eluted using a gradient from 0 to 50 mM formic acid in water. Derivative 10 was obtained by reacting 2,2′-dipyridyl-disulfide (1 g), adenosine (265 mg) and tri-n-butyl phosphin (1 ml) in 5 ml dry pyridin for 24 h at room temperature[22]. To the reaction mixture was added water and diethylether (50 ml each) and the aqueous phase concentrated in vacuo. Water was then added and the resulting precipitate (derivative 10) filtered off and recrystallized from absolute methanol.

S-adenosyl-L-homocysteine sulfoxyde was synthesized according to Borchardt and Wu[9]. The methylation of 5′-buthylthioadenosine (150 mg) was performed using methyl iodide (0.5 ml) in a mixture of formic (2 ml) and acetic acid (0.5 ml) as described by Toennis and Kolb[23] for the methylation of methionine.

The purity of the synthesized compounds was checked using TLC on silica gel plates. 4 solvent systems with the following composition were used: Solvent A, 1-butanol-acetic acid-water (12:3:5), solvent B, 2-propanol-water (16:9) and solvents C and D, ethyl acetate-methanol (8:2) and (9:1). Furthermore, mass spectra and (^{13}C)-MNR-spectra were shown to confirm the structures listed in the table (Looze et al., unpublished).

Protein methyltransferase II was grossly purified from human erythrocytes according to the procedure described by Hildesteim et al.[8]. Only the first 3 steps of the purification procedure were performed. At this stage of the purification, the protein(s) substrate is present in the enzyme preparation and no exogeneous methyl accepting protein is to be added.

Enzymatic activity was measured in triplicate by the method outlined by Jamaluddin[24], S-adenosyl-L-methionine was used at 5 different concentrations between 6 and 50 μM. The inhibition constant (K_i) was determined graph-

ically by plotting $1/v$ versus the concentration of the inhibitor (only competitive inhibitions were indeed found).

Results and discussion. Protein methyltransferase II from human erythrocytes was found to be inhibited by several analogues of S-adenosyl-L-homocysteine (the natural inhibitor of the methylases). All were competitive inhibitors and thus bound to the site normally occupied by S-adenosyl-L-methionine. The results of our investigation have been summarized in the table.

For derivatives 2–5, no inhibition could be observed at concentrations respectively of 30, 600, 160 and 110 μM. As a consequence, the K_i-values were estimated to be greater than 300, 6000, 1600 and 1100 μM. A 80 M solution of derivative 10 decreased by 10% the enzymatic activity. Its inhibition constant was thus estimated to range around 800 μM. More accurate results could not be obtained for these compounds due to their poor solubilities in aqueous solvents.

Examination of the table reveals that S-adenosyl-L-homocysteine remains by far the most effective inhibitor of protein methyltransferase II. Modification at the level of the sulphur atom typified by the conversion into the sulfoxyde results in a decrease of the K_i-value. More important losses of inhibition are observed after the replacement of the amino acid portion of S-adenosyl-L-homocysteine. Most of these compounds so obtained however remain inhibitors, and it may be surmised that all of them would be so if it is taken into account that adenosine itself inhibits the methylase. Thus if the amino acid part of S-adenosyl-L-homocysteine contributes to the binding to the enzyme, a non-negligible contribution of the base and/or ribose moieties is to be awaited. The results and the conclusions obtained here with protein methyltransferase II are different from those published recently concerning a protein (arginine) N-methyltransferase[15]. This enzyme seems indeed to accomodate quite well to any change in the amino acid portion of S-adenosyl-L-homocysteine.

Surprising results have been obtained with derivatives 7 and 12. The binding constant of the latter is to be considered with caution since its methyl donor capacity has not yet been measured (under study).

Derivative 7 was also shown to be a good inhibitor of the protein methyltransferase(s) from *Echerichia coli* responsible for the methylation of the ribosomial protein L 11 of this microorganism (unpublished). It should be pointed out that a good inhibitor of protein methylases may be of practical use for in vitro studies. S-adenosyl-L-homocysteine cannot be utilized for that purpose, being rapidly metabolized in the cells. Finally, it should be emphasized that the pharmacological properties of SIBA (cited in the introduction) do not seem to be related to an inhibition of protein methyltransferase II.

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Inversal relationship between basal adenylcyclase activity and maximal degree of stimulation in membranes of red blood cells from rats[1]

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Summary. The degree of stimulation of adenylcyclase activity, in membranes from immature red blood cells from rats, brought about by isoprenaline, guanylyl-imidodiphosphate and sodium fluoride is strongly dependent on the basal activity of the enzyme. The inversal relationship between basal activity and the maximal degree of stimulation by (—) isoprenaline, shows an apparent seasonal dependence.

It becomes obvious from the results published by numerous authors[2–5]that in membranes of different origin the degree of hormonal stimulation of adenylcyclase (AC) activity, and also the 'basal activity' of the enzyme (i.e. cAMP synthesis in the absence of any stimulant), show extreme variations. Experimental procedures, however, to obtain membrane preparations with constant 'basal AC activities' and constant degrees of hormonal stimulation by standardized methods, were unsuccessful[5,6]. During our recent investigations with membrane preparations from immature red blood cells from rats[7,8], we observed also such extreme variations of basal and stimulated AC activities. Our obser-