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Adenosine-Mediated Inhibition of Glutathione Synthesis in Rat Isolated Hepatocytes

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ABSTRACT. The modulatory effect of adenosine on hepatic glutathione (GSH) synthesis has been investigated in an experimental model in which GSH synthesis from methionine was monitored in rat isolated hepatocytes previously depleted of GSH. Adenosine inhibited GSH synthesis in this system, with complete inhibition occurring at 1 mM. Studies with adenosine receptor agonists and antagonists and adenosine analogues devoid of agonist activity have indicated that this effect of adenosine is not receptor-mediated nor is it mediated through changes in ATP level or redox balance. Rather, the data are consistent with an inhibitory effect of adenosine on the methionine→cysteine transsulphuration pathway, probably at the level of the enzyme S-adenosylhomocysteine hydrolase. Submaximal inhibitory concentrations of adenosine produced an effect on GSH synthesis additive to that obtained with a maximal inhibitory concentration of adrenaline, which inhibits GSH synthesis at the level of γ-glutamylcysteinyl synthetase. Furthermore, exposure of hepatocytes to adenosine subsequent to brief treatment with the toxicant menadione precipitated cytotoxicity. These results suggest that adenosine-mediated inhibition of hepatic GSH synthesis may have a role in various pathological or toxicological states. BIOCHEM PHARMACOL 52;9:1423–1428, 1996. *Copyright © 1996 Elsevier Science Inc.*

KEY WORDS. hepatocytes; glutathione synthesis; adenosine; inhibition; S-adenosylhomocysteine hydrolase

GSH[†] is a tripeptide (γ-glu-cys-gly) that is present in all mammalian cells and that has a major cytoprotective function by virtue of its ability to act as a nucleophilic trap for electrophiles, as a reductant, and as a cofactor for certain antioxidant enzymes [1]. GSH is synthesised from its constituent amino acids in a two-step reaction sequence [1].

Mammalian hepatocytes maintain a high GSH content. This GSH is exported into the blood, where it acts as a circulating form of cysteine for use in other organs (in part for GSH synthesis) and as a direct cytoprotectant for other cells [2]. The majority of the cysteine required for GSH synthesis in hepatocytes is derived from methionine via activity of the trans-sulphuration pathway [2, 3].

There is evidence that levels of Ado in hepatocytes are increased with hypoxic challenge, the increased levels of Ado being exported out of the cells [4]. There is further evidence that Ado may in some cells act via Ado receptors to increase levels of GSH-dependent antioxidant enzymes and so provide protection from ischaemia-reperfusion injury [5].

In light of these observations, we wondered if Ado might regulate hepatocyte GSH status. The data presented in this paper indicate that Ado inhibits hepatocyte GSH synthesis by a mechanism that does not involve Ado receptors but is mediated by direct inhibition of the trans-sulphuration pathway. Furthermore, this inhibitory effect is additive to that produced by adrenaline and potentiates the cytotoxicity of menadione.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (150–180 g) were obtained from the University of Nottingham Medical School Animal Unit. They were housed at a constant room temperature of 22°C and had free access to standard laboratory diet and tap water.

Materials

Six-well cluster dishes were purchased from Fahrenheit Laboratory Supplies (Nottingham, UK). Culture medium, test chemicals, Trinder glucose assay kit, collagen and collagenase were purchased from Sigma Chemicals Co. (Poole, UK).

Hepatocyte Isolation and Culture

Hepatocytes were isolated by perfusion of liver lobes with collagenase, essentially as described by Reese and Byard [6].

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† *Abbreviations:* Ado, adenosine; 2CIA, 2-chloroadenosine; CPA, N^6-cyclopentyl-adenosine; DMPX, 3,7-dimethyl-1-propargylxanthine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; GSH, glutathione; MTT, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; SAH, S-adenosylhomocysteine.

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The hepatocyte suspension was washed with Hanks-Hepes buffer, pH 7.4 (137 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 4.16 mM $NaHCO_3$, 1.26 mM $CaCl_2$, 0.81 mM $MgSO_4$, 10 mM Hepes). Cells were resuspended in L-15 medium and centrifuged (50*g* for 10 min) in a 90% Percoll solution to improve the separation of viable and nonviable cells, as described by Kreamer *et al.* [7]. The cell viability as detected by trypan blue was typically greater than 95%. Hepatocytes were suspended in L-15 medium containing 10% v/v calf serum and dispensed into collagen-coated six-well plates at a density of 10^6 cells per well. After allowing 2 hr for attachment, cells were either used immediately or exposed to diethyl maleate (DEM; 0.6 mM, 30 min) to deplete GSH, followed by incubation in Hanks-Hepes buffer containing methionine (2 mM) to recover the cellular GSH level. Test chemicals were added either immediately after the 2-hr attachment or after GSH depletion.

Assays

Cellular GSH was measured by the method of Saville [8], which detects total nonprotein thiols, or the specific enzyme recycling method of Tietze [9]. MTT reduction, cellular LDH and the ATP level were measured as described previously [10]. Briefly, MTT reduction was measured by incubating the cells for 25 min in medium containing MTT (5 mg/mL), followed by desorption of the reduced dye and measurement of absorbance at the wavelength pair 570–655 nm. LDH activity was determined by conversion of lactate to pyruvate, and ATP was measured by bioluminescence. The glucose assay was done as described by the manufacturers of the assay kit.

Data Presentation and Statistical Analysis

Data are presented as mean ± SEM of at least three experiments, each value representing the mean of at least triplicate estimations. Statistical analysis was undertaken using unpaired t tests or analysis of variance (ANOVA)/Dunnett's test as appropriate. A value of $P < 0.05$ was considered significant.

RESULTS

In preliminary experiments, it was established that incubation of Ado (1 mM) with hepatocytes for up to 2 hr did not cause gross cell damage as judged by retention of cellular LDH and protein content in the monolayer (data not shown). Furthermore, Ado (1 mM) had no effect on the basal GSH level in cells after a 2-hr incubation (no Ado: 35.1 ± 1.6 nmol/well; with Ado: 32.1 ± 1.3 nmol/well; mean ± SEM; n = 4 experiments).

Treatment of cells for 30 min with 0.6 mM DEM produced a marked fall (>70%) in cell GSH, which then recovered in a time-dependent manner during incubation with a simple salt solution supplemented with methionine as a cysteine donor (Fig. 1), with complete recovery of GSH by approximately 3 hr. The increase in cell GSH following DEM treatment was taken to represent the GSH synthesis rate.

Ado, in the concentration range 10^{-5}–10^{-3} M, inhibited GSH synthesis in a concentration-dependent manner, with complete inhibition occurring at 10^{-3} M (Fig. 2). Similar results were obtained when total cellular glutathione (reduced + oxidised) was measured by the specific enzymic assay of Tietze (data not shown). The Ado receptor agonists, 2CIA and CPA, also inhibited GSH synthesis, although these agents were less potent than Ado (Fig. 2 and Table 1). The three deoxy-Ado isomers (2′-, 3′- and 5′-) also inhibited GSH synthesis, the 5′-isomer being less effective than the other isomers (Table 1).

2CIA (10^{-3} M) significantly increased glucose output from hepatocytes in addition to its inhibitory effect on GSH synthesis, whereas the same concentration of the deoxy-Ado isomers produced no stimulatory effects on glucose output; rather, in the case of the 3′-isomer glucose, output was significantly decreased. Inhibition of GSH synthesis mediated by Ado (10^{-4} or 10^{-3} M) could not be antagonised by the Ado receptor antagonists DPCPX or DMPX. GSH synthesis in the presence of 10^{-4} M Ado was 12.65 ± 1.15 nmol/well/2 hr; with the addition of 10^{-5} M DPCPX, this value was 11.45 ± 0.45 nmol/well/2 hr. In the presence of 10^{-3} M Ado, the GSH synthesis rate was 4.70 + 0.60 nmol/well/2 hr; with the addition of 10^{-5} M DPCPX, this value was 4.95 ± 0.15 nmol/well/2 hr. In a separate series of experiments, GSH synthesis in the presence of 10^{-4} M Ado was 8.78 ± 1.46 nmol/well/2 hr; with the addition of 10^{-5} M DMPX, this value was 9.14 ± 1.34 nmol/well/2 hr. In the presence of 10^{-3} M Ado, GSH synthesis was 4.18 ± 1.31 nmol/well/2 hr; with the addition of 10^{-5} M DMPX, this value was 3.93 ± 1.28 nmol/well/2 hr.

Ado, in the concentration range 10^{-5}–10^{-3} M, produced

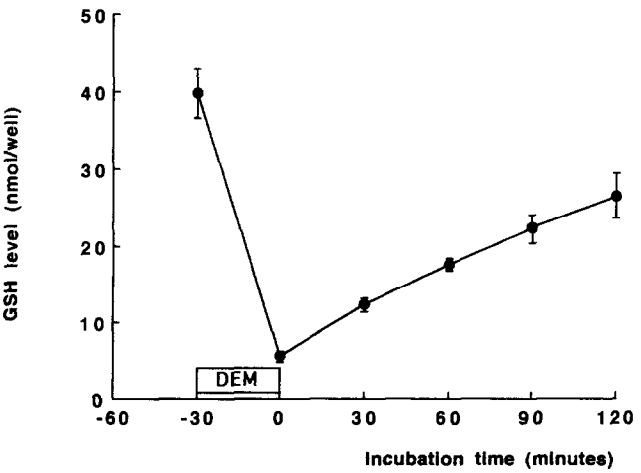

FIG. 1. Time dependence of GSH synthesis following DEM treatment. Rat hepatocytes were incubated with DEM (0.6 mM) for 30 min prior to washing and re-incubation in salt solution containing 2 mM methionine as the sulphur donor. Results are mean ± SEM of four experiments.

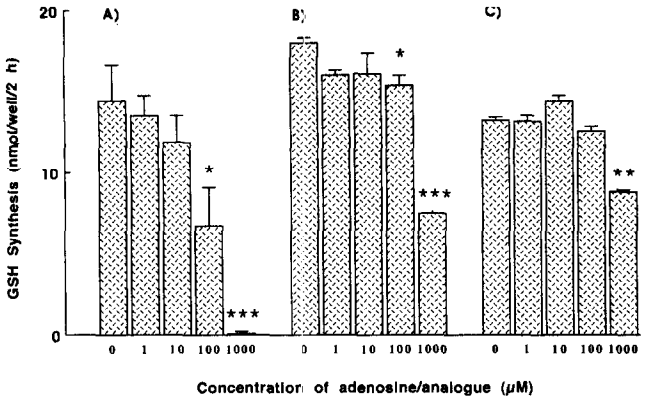

FIG. 2. Effect of adenosine and its analogues on GSH synthesis. The rate of GSH synthesis from methionine over a 2-hr period was measured in rat hepatocytes (after an initial depletion with DEM) in the absence or presence of (A) Ado, (B) 2ClA or (C) CPA in the concentration range of 1–1000 μM. Results are mean ± SEM of three to five experiments. Where indicated, values are significantly different from control at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

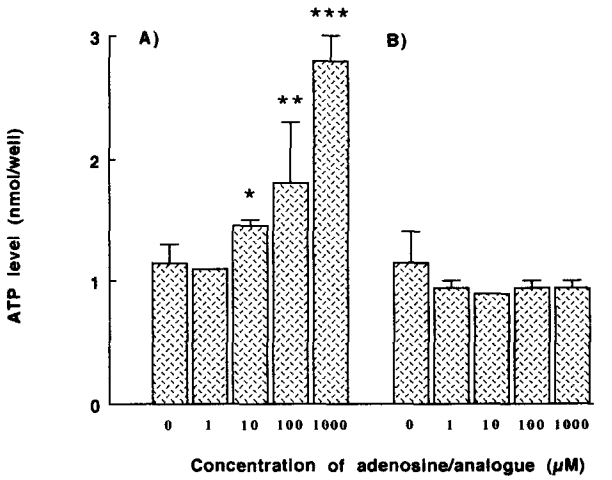

FIG. 3. Effect of Ado and 2ClA on ATP levels. Rat hepatocytes were depleted of GSH by treatment with DEM. Thereafter, cells were incubated in methionine-containing salt solution for 2 hr in the absence or presence of (A) Ado or (B) 2ClA in the concentration range of 1–1000 μM. Results are mean ± SEM of three experiments. Where indicated, values are significantly different from control at *$P < 0.05$, **$P < 0.01$, $P < 0.001$.

a concentration-dependent elevation of cellular ATP levels, an effect that was not mimicked by 2-chloro-A (Fig. 3). Ado produced a significant ($P < 0.01$) change in the cell redox balance as judged by an increase in the extent of MTT reduction; this effect occurred only at the highest concentration (10^{-3} M) tested (control: 0.130 ± 0.020 absorbance units/well; +Ado: 0.270 ± 0.070 absorbance units/well; n = 3 experiments). Similar responses were observed with 2CIA and CPA at 10^{-3} M.

The inclusion of lactate (10^{-2} M) or ethanol (2×10^{-2} M) during the GSH synthesis stage also produced significant increases ($P < 0.001$) in the level of MTT reduction (control: 0.119 + 0.005 absorbance units/well; +lactate: 0.166 ± 0.004 absorbance units/well; +ethanol: 0.171 ± 0.005 absorbance units/well; n = 4–5 experiments), but this was not accompanied by any impairment of GSH synthesis (data not shown).

The Ado-mediated inhibition of GSH synthesis was not relieved if serine (2 mM) was added with methionine during the GSH synthesis stage, but replacement of methionine by N-acetylcysteine abolished the inhibitory action of Ado (Table 2).

TABLE 1. Effect of adenosine analogues on GSH synthesis and glucose output of rat isolated hepatocytes

Adenosine analogue	GSH synthesis rate (nmol/well/2 hr)	Glucose output (μmol/ml/hr)
None	25.4 ± 1.2	46 ± 2
2 ClA	11.4 ± 1.2*	71 ± 4**
2′-deoxyadenosine	2.9 ± 0.9*	52 ± 3
3′-deoxyadenosine	0.0 ± 0.0*	24 ± 1**
5′-deoxyadenosine	14.6 ± 1.7*	57 ± 5

Each analogue was tested at 10^{-3} M. Values are mean ± SEM of four experiments. Where indicated, values are significantly different from control at *$P < 0.01$, **$P < 0.001$.

Adrenaline produced a modest (≈25%) reduction in the level of GSH synthesis, which was maximal at 10^{-7} M. This reduction was enhanced in an additive manner when a submaximal inhibitory concentration of Ado (10^{-4} M) was also present, such that more than 85% of the GSH synthesis rate was inhibited (Fig. 4).

Exposure to menadione (1 mM for 30 min) produced a marked fall in hepatocyte GSH levels, the magnitude of which was comparable to that observed with DEM (Fig. 5). Subsequent incubation in salt solution supplemented with methionine (2 mM) resulted in GSH synthesis, again similar to that observed following DEM treatment (Fig. 5). There was no significant loss of cell viability during this menadione–methionine incubation regimen as judged by retention of cellular LDH activity (Fig. 5). Inclusion of Ado (10^{-3} M) in the salt solution supplemented with methionine (2 mM) following menadione treatment led to a complete inhibition of GSH synthesis and development of cytotoxicity (Fig. 5).

DISCUSSION

The results presented herein demonstrate that Ado in the concentration range 10^{-5}–10^{-3} M produces a concentration-dependent inhibition of GSH synthesis in rat isolated hepatocytes when methionine is used as a sulphur donor. As far as we are aware, this effect of Ado has not been documented previously.

This property is shared, albeit with less potency, by two Ado analogues, 2CIA and CPA, which can act as nonselective A_1/A_2 receptor and selective A_1 receptor agonists, respectively. Although this might suggest involvement of an adenosine receptor in the Ado-mediated inhibition of

TABLE 2. Effect of adenosine on GSH synthesis in the presence of cysteine precursors

Treatment	GSH synthesis rate (nmol/well/2 hr)
Methionine (2 mM)	23.8 ± 1.9
Methionine (2 mM) + Ado (1 mM)	5.0 ± 1.4*
Methionine (2 mM) + Ado (1 mM) + serine (2 mM)	7.0 ± 2.1*
N-acetylcysteine (2 mM)	25.8 ± 1.7
N-acetylcysteine (2 mM) + Ado (1 mM)	23.2 ± 1.7

Values are mean ± SEM of four experiments. Where indicated, values are significantly different from appropriate control *$P < 0.001$.

GSH synthesis, this seems unlikely, given that (a) antagonists of the A_1 and A_2 receptors (DPCPX and DMPX, respectively) did not alleviate this inhibition, and (b) three deoxy-Ado isomers devoid of agonist activity, as judged by a lack of enhancement of glycogenolysis (an indicator of Ado-receptor activation [11]), also inhibited GSH synthesis.

In agreement with the findings of others [12], incubation of hepatocytes with Ado also led to a concentration-dependent elevation in the cellular ATP content but was not observed when cells were incubated with 2CIA. Incubation of hepatocytes with Ado, 2CIA or CPA also produced an alteration in the redox balance of the cells. However, this effect was apparent only at the highest concentration tested, and the comparable change in redox balance

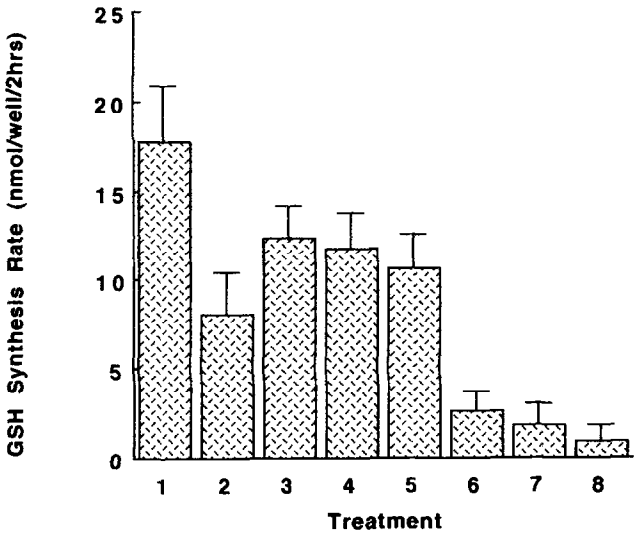

FIG. 4. Effect of Ado alone or with adrenaline on GSH synthesis in rat hepatocytes. Rat hepatocytes were depleted of GSH by treatment with DEM. Thereafter, cells were incubated in methionine-containing salt solution with various additions for 2 hr and the rate of GSH synthesis was determined. Treatment conditions were: 1, no addition (control); 2, with 10^{-4} M adenosine; 3, with 10^{-7} M adrenaline; 4, with 10^{-6} M adrenaline; 5, with 10^{-5} M adrenaline; 6, with 10^{-4} M adenosine + 10^{-7} M adrenaline; 7, with 10^{-4} M adenosine + 10^{-6} M adrenaline; and 8, with 10^{-4} M adenosine + 10^{-5} M adrenaline. Results are mean ± SEM of 4 experiments. All values were significantly different from control ($P < 0.001$).

elicited by lactate or ethanol was not accompanied by a corresponding inhibition of GSH synthesis. Collectively, these data suggest that the alterations in ATP level or redox balance elicited by Ado were not causal factors in the observed impairment of GSH synthesis.

The pathways of hepatic methionine metabolism are indicated in Fig. 6. The first step in the conversion of methionine to cysteine is the production of S-adenosylmethionine, which, under the action of various methyltransferases, is demethylated to S-adenosylhomocysteine. SAH is metabolised by SAH hydrolase with the production of homocysteine and adenosine. The former combines with serine to yield cystathionine, which, under the influence of γ-cystathionase, is finally converted to cysteine and thence to GSH. The possibility of a reduced supply of serine being the cause of the Ado-mediated impairment of GSH synthesis was ruled out by the failure of exogenous serine to reverse the impairment of GSH synthesis. However, the inhibition of GSH synthesis was clearly located prior to production of cysteine, as Ado was without effect on GSH synthesis when a stable form of cysteine, N-acetylcysteine, replaced methionine as the sulphur donor.

Ado is an inhibitor of purified human liver SAH hydrolase, as are 2CIA and the deoxy-Ado isomers, with 2′-deoxyadenosine being the most potent inhibitor [13]. The rank orders of potency of inhibition of GSH synthesis (present study) and of the purified human liver SAH hydrolase were as follows:

inhibition of GSH synthesis: 3′-deoxy > 2′-deoxy ≫ 2-chloro > 5′-deoxy
inhibition of SAH hydrolase: 2′-deoxy > 3′-deoxy = 2-chloro > 5′-deoxy

It is apparent that there is broad similarity in the rank orders; the discrepancies may be accounted for by species differences and/or different rates of cellular metabolism of these analogues.

From these data we suggest that inhibition of SAH hydrolase could account for the observed inhibition of GSH synthesis by Ado. In support of this suggestion is the recent finding that incubation of rat hepatocytes with 10^{-3} M Ado does inhibit SAH hydrolase, as measured by accumulation of SAH and S-adenosylmethionine [14].

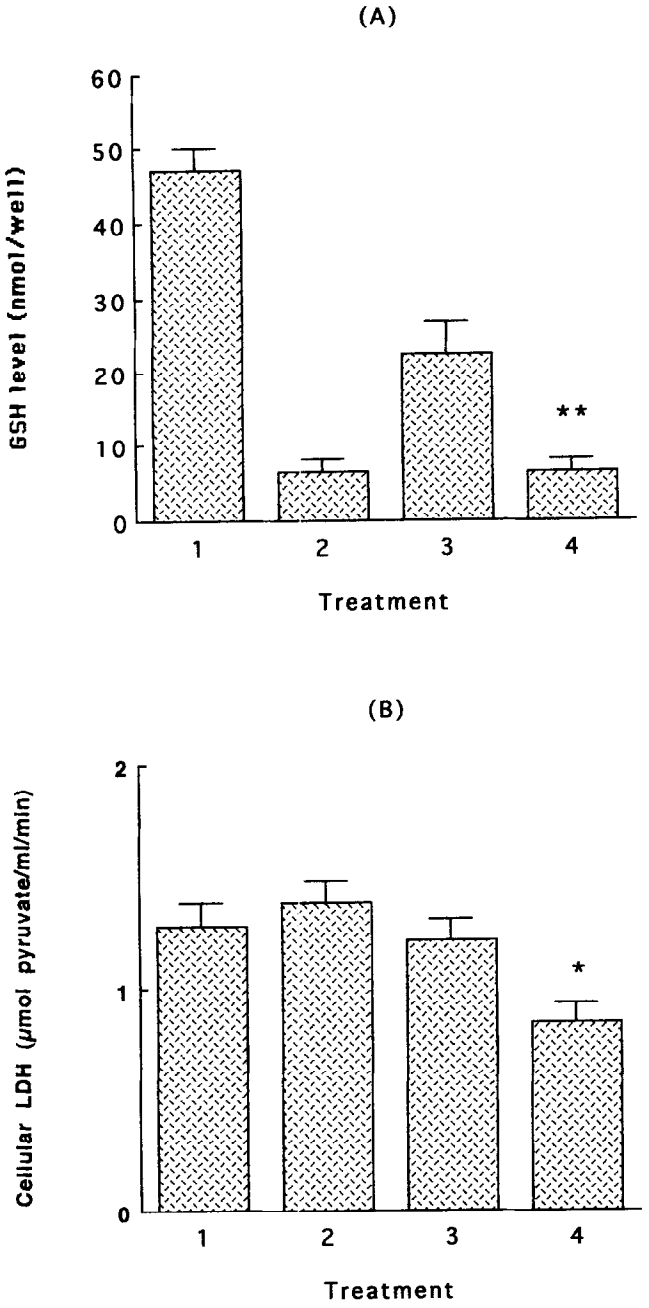

FIG. 5. Effect of menadione without or with adenosine on (A) the GSH level and (B) the LDH level of rat hepatocytes. Treatment conditions were: 1, incubation in salt solution for 30 min; 2, incubation with menadione (1 mM) for 30 min; 3, incubation in methionine-containing solution for 2 hr following menadione treatment; and 4, incubation in methionine-containing solution with 1 mM adenosine for 2 hr following menadione treatment. Results are mean ± SEM of four experiments. Where indicated, values for treatment condition 4 are significantly different from those for treatment condition 3 at *P < 0.05, **P < 0.01.

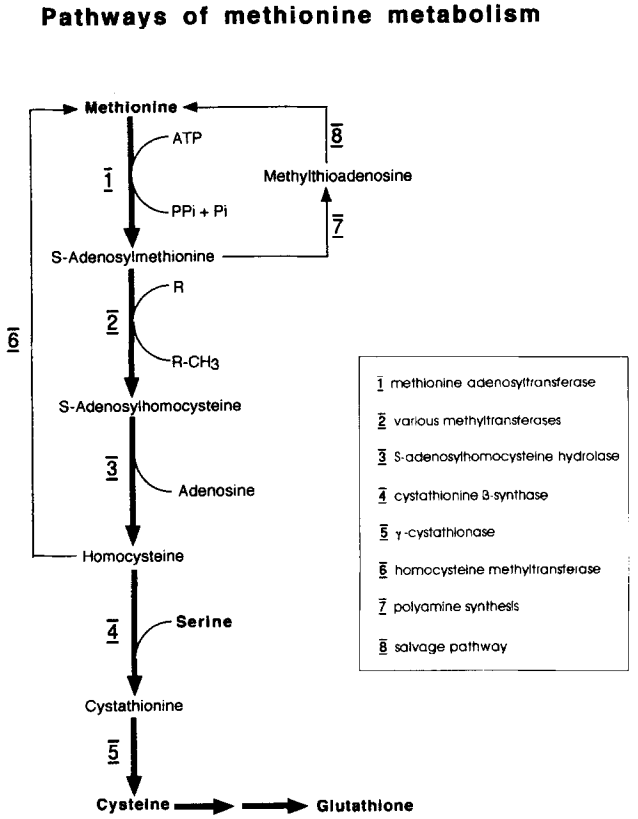

FIG. 6. Pathways of methionine metabolism in mammalian liver. R, methyl acceptor.

The recently described Ado-mediated inhibition of protein synthesis in rat hepatocytes [12, 14, 15] bears many properties similar to those described in this report on inhibition of GSH synthesis. Thus, Ado and 2CIA in the concentration range 10^{-5}–10^{-3} M inhibited protein synthesis by a mechanism unrelated to Ado receptor activation [12, 15], which has subsequently been localised to inhibition of SAH hydrolase [14]. Therefore, Ado-mediated inhibition of SAH hydrolase may lead to two possible deleterious outcomes for hepatocytes: inhibition of protein synthesis and of GSH synthesis.

GSH plays a central role as a cytoprotective agent, and the possibility that Ado may modulate xenobiotic-mediated toxicity to hepatocytes was demonstrated by the finding that menadione, which causes a loss of GSH by conjugation and oxidation [16], became toxic only when GSH resynthesis was blocked by Ado.

Adrenaline has also been reported to inhibit GSH synthesis in rat hepatocytes, an effect that occurs through α-receptor-mediated inhibition of γ-glutamylcysteinyl synthetase [17], the first enzyme in the production of GSH from cysteine. This inhibition of GSH synthesis was confirmed in the present study; furthermore, co-incubation of adrenaline with Ado produced an additive inhibition of GSH synthesis. Increased levels of adrenaline together with Ado would probably be produced under different pathophysiological situations, and the findings of an additive inhibition of GSH synthesis points to the possibility that submaximal concentrations of these and possibly other agents may combine to produce a substantial inhibition of hepatic GSH synthesis.

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