11. Petkovich M, Brand NJ, Krust A and Chambon P, A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature* **330**: 444–450, 1987.
12. Brand N, Petkovich M, Krust A, Chambon P, de The H, Marchio A, Tiollais P and Dejean A, Identification of a second human retinoic acid receptor. *Nature* **332**: 850–853, 1988.
13. Benbrook D, Lernhardt E and Pfahl M, A new retinoic acid receptor identified from a hepatocellular carcinoma. *Nature* **333**: 669–672, 1988.
14. Chytil F and Ong DE, Cellular retinol- and retinoic acid-binding proteins. In: *Advances in Nutritional Research* (Ed. Draper HH), pp. 13–29. Plenum Press, New York, 1983.
15. Rainier S, Herrera JM and McCormick AM. Rapid characterization of cellular retinoid binding proteins by high-performance size-exclusion chromatography. *Arch Biochem Biophys* **225**: 818–825, 1983.
16. Kwarta RF Jr, Kimmel CA, Kimmel GL and Slikker W Jr, Identification of the cellular retinoic acid binding protein (cRABP) within the embryonic mouse (CD-1) limb bud. *Teratology* **32**: 103–111, 1985.
17. Sani BP and Hill DL, Retinoic acid: A binding protein in chick embryo metatarsal skin. *Biochem Biophys Res Commun* **61**: 1276–1282, 1974.
18. Ong DE and Chytil F, Novel retinoic acid-binding proteins in the lumen of rat epididymis. *Arch Biochem Biophys* **267**: 474–478, 1988.
19. Maden M and Summerbell D, Retinoic acid-binding protein in the chick limb bud: Identification at developmental stages and binding affinities of various retinoids. *J Embryol Exp Morphol* **97**: 239–250, 1986.
20. Howard WB, Willhite CC and Sharma RP, Structure–toxicity relationships of tetramethylated tetralin and indane analogs of retinoic acid. *Teratology* **36**: 303–311, 1987.
21. Howard WB, Willhite CC, Dawson MI and Sharma RP, Structure–activity relationships of retinoids in developmental toxicology. III. Contribution of the vitamin A β-cyclogeranylidene ring. *Toxicol Appl Pharmacol* **95**: 122–138, 1988.
22. Scatchard T, The attraction of proteins for small molecules and ions. *Ann NY Acad Sci* **51**: 660–672, 1949.
23. Willhite CC and Shealy YF, Amelioration of embryotoxicity by structural modifications of the terminal group of cancer chemopreventive retinoids. *J Natl Cancer Inst* **72**: 689–695, 1984.
24. Williams KJ, Ferm VH and Willhite CC, Teratogenic dose–response relationships of etretinate in the Golden hamster. *Fundam Appl Toxicol* **4**: 977–982, 1984.
25. Flanagan JL, Willhite CC and Ferm VH, Comparative teratogenic activity of cancer chemopreventive retinoidal benzoic acid congeners (arotinoids). *J Natl Cancer Inst* **78**: 533–538, 1987.
26. Ong DE and Chytil F, Cellular retinoic acid-binding protein from rat testis. Purification and characterization. *J Biol Chem* **253**: 4551–4554, 1978.
27. Bonelli FC and DeLuca LM, A high-performance liquid chromatography technique that separates cellular retinol-binding protein from cellular retinoic acid-binding protein. *Anal Biochem* **147**: 251–257, 1985.
28. Siegenthaler G and Saurat JH, Cellular retinoid-binding proteins in normal and diseased human skin. In: *Retinoids: New Trends in Research and Therapy* (Ed. Saurat JH), pp. 168–174. Karger, Basel, 1985.
29. Brandes M, Gesell MS, Ueda H, Millan JC, Quinlan RM, Ernst C, Sindelar WF, Kurman LL, Harris LL and Brandes D, Retinoic acid binding protein in human and experimental pancreatic carcinomas in hamster. *Ann Clin Lab Sci* **13**: 400–406, 1983.
30. Sani BP, Titus BC and Banerjee CK, Determination of binding affinities of retinoids to retinoic acid-binding protein and serum albumin. *Biochem J* **171**: 711–717, 1978.
31. Frolik CA, *In vitro* and *in vivo* metabolism of all-*trans*- and 13-*cis*-retinoic acid in the hamster. *Ann NY Acad Sci* **359**: 37–44, 1981.
32. Chytil F and Ong DE, Cellular retinol and retinoic acid-binding proteins in vitamin A action. *Fed Proc* **38**: 2510–2518, 1979.
33. Takase S, Ong DE and Chytil F, Transfer of retinoic acid from its complex with cellular retinoic acid-binding protein to the nucleus. *Arch Biochem Biophys* **247**: 328–334, 1986.

Biochemical Pharmacology, Vol. 40, No. 3, pp. 648–651, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00
Pergamon Press plc

Effects of *S*-adenosyl-L-methionine on phospholipid methyltransferase activity changes induced by thioacetamide

(*Received* 25 *October* 1989; *accepted* 11 *February* 1990)

Acute administration of thioacetamide (TAA*) induces a maximum degree of hepatic necrosis [1]. Schriewer and Lohman [2] have reported an increase in the microsomal PE/PC ratio by acute effect of thioacetamide. In a more detailed study of microsomal phospholipid composition we have confirmed those results as an acute response of microsomal membranes to the toxic action of thioacetamide [3]. Microsomal PE/PC ratio is the substrate/product ratio of the reaction catalysed by *S*-adenosyl-L-methionine; phospholipid methyltransferase (E.C. 2.1.1.17).

Several pharmacological effects have been reported regarding *S*-adenosyl-L-methionine [4–7], although its biochemical mechanism of action is unknown. *S*-Adenosyl-L-methionine partly recovered the TAA-induced liver necrosis [8]. This observation suggests that *S*-adenosyl-L-methionine could prevent the TAA-induced alteration in PE/PC ratio. SAM:phospholipid methyltransferase as a potential responsible for these microsomal changes induced by TAA is an enzyme whose activity can be modulated by exogenous administration of *S*-adenosyl-L-methionine [9]. In this paper we have studied the role of phospholipid methyltransferase in TAA-induced liver necrosis and the effect of exogenous administration of Ado-met on this activity.

* Abbreviations: Ado-hcy: *S*-adenosyl-L-homocysteine; Ado-met, *S*-adenosyl-L-methionine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TAA, thioacetamide.

Materials and Methods

Materials. *S*-Adenosyl-L-[^{3}H-*methyl*]-methionine was purchased from The Radiochemical Centre (Amersham, U.K.). Thioacetamide, silicagel G and organic solvents were obtained from Merck (Darmstadt, F.R.G.). Ado-met was obtained from Europharma (Madrid, Spain). Phospholipid standards were supplied by the Sigma Chemical Co. (St Louis, MO).

Animals. Hepatic injury was induced in inbred male Wistar rats (200–250 g, aged 2 months) by intraperitoneal injection of thioacetamide at a dose of 50 mg/kg body weight every 24 hr. Control rats were injected with an equal volume of sterile 0.15 mol/L NaCl every 24 hr during the experimental period. To test the effect of Ado-met on TAA-induced liver damage the animals received an additional (i.p.) daily injection of Ado-met (2 mg/kg body weight) in 0.5 mL of sterile saline as well as TAA. These groups of animals were kept on a standard laboratory diet and allowed free access to food and water.

Preparation of microsomal membranes. Before killing, animals were fasted for 18 hr. For each experimental group, six animals were used, which were handled and killed always observing criteria from the European Community for care and use of laboratory animals in research. Microsomal membranes were obtained and characterized as we have previously published [10]. Basically, postmitochondrial supernatants were collected and centrifuged at 100,000 *g* for 55 min. The resulting pellet was resuspended in 0.25 M sucrose and again centrifuged at the same conditions. The resulting pellet was resuspended in 0.25 M sucrose.

Lipid composition studies. Lipids were extracted from the microsomal membranes by the method of Folch *et al.* [11]. Phospholipids were separated by two-dimensional thin layer chromatography on silica gell G-60 plates [12] and quantified by the method of Rouser *et al.* [13].

S-Adenosyl-L-methionine: phospholipid N-methyltransferase assay. The assay was performed according to Sastry *et al.* [14] with modified Ado-met concentration according to its K_m value [15]. Incubation mixture contained: 4 mmol/L magnesium chloride, 0.04 mol/L EDTA, 10 mmol/L Tris/glycilglycine buffer, pH 10, 1 mmol/L *S*-adenosyl-L-[^{3}H-*methyl*]methionine (sp. act. 11.8 GBq/mmol) and 40 μg microsomal protein in a total volume of 50 μL. The assay was performed at 37° for 40 min. Reactions were stopped and their products were analysed according to Sastry *et al.* [14]. The radioactivity of the gel from the TLC plate at the localization of PC spots was measured in a Kontron liquid scintillation spectrometer. Quenching was corrected by external standardization. Radioactivity incorporated into PC spots from tubes containing the heat-inactivated enzyme was discounted from that found in PC spots coming from tubes containing the unheated microsomal preparations.

Statistical analysis. The Shapiro–Wilk test was applied to establish the behaviour of distributions. Whenever the Shapiro–Wilk test rejected the hypothesis of normal distribution, or when the Bartlett test for homogeneity of variances was significantly different, the overall significance of differences was calculated with the Kruskal–Wallis (one-way analysis of variance test; if the differences were significant ($P < 0.05$), we also tested the differences between the groups pair-wise with the Mann–Whitney U-test [16].

Results and Discussion

Ado-met administered exogenously recovers liver from the TAA-induced necrosis [8]. The mechanism involved in this fact is unknown, even more so if we consider that liver TAA-induced necrosis is a multifactorial phenomenon which involves some mechanism such as: hepatic lysophosphatidylcholine content [1], alterations of microsomal phospholipids [2, 3], changes in ionic content [17] etc. In this paper we check whether Ado-met prevents microsomal phospholipid alterations induced by effect of TAA. After 3 days of TAA administration, a decrease in PC and an increase in PE was observed (Table 1). A similar finding was published by Schriewer and Lohman [2] with a single dose of TAA six-fold higher than that used in our research.

The slight difference between control and TAA-treated rats (Table 1), regarding the PE/PC molar ratio in liver microsomal membranes has to be of physiological relevance, bearing in mind the relatively constant molar percentages of PC and PE in control rats. Even though in this experimental approach interanimal variations have not been considered, the invariable phospholipid composition of microsomal membranes coming from the liver of 13 control groups, each composed of six adult rats underlines the stable composition of these membranes. A metabolic failure that causes striking imbalances between PE and PC as membrane components has never been described but a slight alteration of their balance in erythrocytes has been associated to hemolytic disease [18]. The statistical significant decrease in the PC molar percentage by effect of TAA (Table 2) is recovered by Ado-met administration.

Phosphatidylethanolamine is the substrate in the methylation pathway of phosphatidylcholine biosynthesis and this step is catalysed by a single enzyme in liver [15, 19]. The observed changes in PE/PC ratio induced by TAA treatment after 3 days (Table 1) tentatively show that this enzyme activity is involved. This hypothesis has been confirmed by assaying this enzyme activity under TAA treatment (Table 3). Changes in activity of this enzyme in

Table 1. Time course of main phospholipids during thiocetamide treatment

	Phospholipid/protein*		PC†		PE†	
Days	Control	TAA	Control	TAA	Control	TAA
1	330 ± 20	311 ± 22	58.5 ± 1	56 ± 1‡	22.4 ± 1.2	23.0 ± 1
3	352 ± 50	360 ± 33	59.0 ± 1	54 ± 2‡	23.3 ± 1.3	27.8 ± 2‡
30	340 ± 20	350 ± 30	60.0 ± 1	55 ± 2‡	22.5 ± 1	22.0 ± 0.5
90	290 ± 30	261 ± 10	59.3 ± 1	51 ± 1‡	23.4 ± 1	21.0 ± 2

* Values in nmol/mg protein.

† % molar of total phospholipids.

Data are means ± SD of three preparations assayed by duplicate (six rats each preparation) except for control 3 days (N = 10). TAA animals received an i.p. injection of 50 mg/kg body weight/day (‡$P < 0.01$ vs control).

Table 2. Effect of Ado-met on major phospholipid of microsomal membranes

	Phospholipid/protein (nmol/mg protein)	PC (% molar total phospholipids)	PE (% molar)
Control (10)	352 ± 50	59 ± 1	23.3 ± 1.3
TAA	360 ± 33	54 ± 2*†	27.8 ± 2*†
TAA + Ado-met	365 ± 20	58 ± 1	24.5 ± 1.3

Data are means ± SD of three preparations assayed by duplicate (six rats each preparation) unless another number is specified in brackets. TAA animals received an i.p. injection of 50 mg/kg body weight/day thioacetamide for 3 days. TAA + Ado-met received an additional (i.p.) daily injection of Ado-met (2 mg/kg body weight) for 3 days, as well as TAA.

Statistical analysis was performed using the Mann–Whitney U-test. (When $P > 0.05$, not significant; *$P < 0.01$ TAA vs control; and †$P < 0.01$ TAA vs TAA + Ado-met).

Table 3. Specific activity of Ado-met: phospholipid *N*-methyltransferase

	pmol [^{3}H-methyl]/min/protein mg
Control (10)	258 ± 50
TAA	108 ± 20*†
TAA + Ado-met	290 ± 30

Incubation mixture contained 50 nmol *S*-adenosyl-L-[^{3}H-*methyl*]methionine (sp. act. 0.32 Ci/mmol) and 40 μg protein in a total volume of 50 μL. Results are expressed as pmoles [^{3}H-*methyl*] incorporated in phosphatidylcholine/min/mg protein at 37° and are means ± SD of three preparations assayed by duplicate (six rats each preparation) unless another number is specified in brackets. TAA animals received an i.p. injection of 50 mg/kg body weight/day thioacetamide for 3 days. TAA + Ado-met received an additional (i.p.) daily injection of Ado-met (2 mg/kg body weight) for 3 days, as well as TAA. Statistical analysis was performed using the Mann–Whitney U-test. (When $P > 0.05$, not significant; *$P < 0.01$ TAA vs control; and †$P < 0.01$ TAA vs TAA + Ado-met).

hepatocytes have been shown in different experimental situations [19]. Ado-met reverses the diminished activity induced by TAA (Table 3). Ado-met does not fully reverse TAA-induced necrosis [8]; however, it fully reverses microsomal lipid alterations induced by TAA (Table 2), which suggest that these alterations are not the primary event in TAA-induced necrosis. In this way, the observed microsomal lipid alterations can contribute to the progression of hepatic necrosis by several mechanisms. One could be by failure in sequestering Ca^{2+} by endoplasmic reticulum. Intracellular increase in the concentration of this cation is one of the most early events found in TAA-induced necrosis [17]. In some cells, ionic changes control the Ado-met/Ado-hcy ratio [20] and this ratio controls Ado-met:phospholipid methyltransferase activity [15, 19]. The failure of this enzyme as a consequence of ionic changes would block the transformation of PE into PC. The metabolites involved in this reaction seem to have a role in ionic transport and their failure would complicate the intracellular management of Ca^{2+} and would facilitate the progression of hepatic necrosis. To prove that these events are involved in TAA-induced necrosis is a speculative hypothesis which we plan to investigate in the future.

In conclusion, Ado-met reverses the decrease in microsomal molar percentage of phosphatidylcholine and the increase in phosphatidylethanolamine and Ado-met also reverses the diminished specific activity of *S*-adenosyl-L-methionine:phospholipid methyltransferase found in thioacetamide-treated rats after 3 days of thioacetamide treatment, suggesting that the alteration of this enzyme could be implicated in the changes of microsomal phospholipids.

Acknowledgements—The authors thank Dr Isabel Millan for her advice and statistical evaluation of data and Dr Ordovas for his critical reading. We are also grateful to Miss Sandra Kennelly and Erik Lundin for their help in preparing the manuscript.

This investigation was supported by grant 87030 awarded by the CAYCIT (M.E.C.), by grant 87/1336 awarded by FISS (INSALUD) and by grant provided by EUROPHARMA.

JESÚS OSADA*
HORTENSIA AYLAGAS
EVANGELINA PALACIOS-ALAIZ†

*Human Nutrition Research Center on Aging 711 Washington Street, Boston MA 02111, U.S.A.;
†Instituto de Bioquímica (Centro mixto UCM-CSIC) Facultad de Farmacia Universidad Complutense Ciudad Universitaria 28040-Madrid, Spain

† To whom correspondence should be addressed.

REFERENCES

1. Osada J, Aylagas H, Miró-Obradors MJ and Palacios-Alaiz E, Lyso-phosphatidylcholine is implicated in thioacetamide- induced necrosis. *Biochem Biophys Res Commun* **154**: 803–809, 1988.
2. Schriewer H and Lohman J, Phospholipid metabolism of whole liver, mitochondria and microsomes disturbed by acute thioacetamide intoxication and action of silymarin. *Arzn Fors* **26**: 65–69, 1976.
3. Palacios-Alaiz E, Osada J, Aylagas H and Cascales M, Modification of membrane lipid composition of subcellular fractions from rat liver by effect of thioacetamide. In: *Proceedings of 16th Meeting of FEBS, Moscow, 1984 June*. pp. 362, 1984.
4. Stramentinoli G, Gualano M and Ideo G, Protective role of *S*-adenosyl-L-methionine on liver injury induced by D-galactosamine in rats. *Biochem Pharmacol* **27**: 1431–1433, 1978.
5. Di Padova C, Tritapepe R, Cammareri G, Humpel M and Stramentinoli G, *S*-Adenosyl-L-methionine antagonizes ethynylestradiol-induced bile cholesterol supersaturation in humans without modifying the estrogen plasma kinetics. *Gastroenterology* **82**: 223–227, 1982.

6. Frezza M, Pozzato G, Chiesa L, Stramentinoli G and Di Padova C, Reversal in intrahepatic cholestasis of pregnancy in women after high dose *S*-adenosyl-L-methionine administration. *Hepatology* **4**: 274–278, 1984.
7. Stramentinoli G, *S*-Adenosyl-L-methionine as a drug: pharmacokinetic and pharmacological aspects. In: *Biological Methylation and Drugs Design* (Eds. Borchardt RT and Creveling CR), pp. 315–326. Humana Press, Clifton, 1986.
8. Osada J, Aylagas H, Sanchez-Vegazo I, Gea T, Millán I and Palacios-Alaiz E, Effect of *S*-adenosyl-L-methionine on thiocetamide-induced liver damage in rats. *Toxicol Lett* **32**: 97–106, 1986.
9. Traver J, Varela I and Mato JM, Effect of exogenous *S*-adenosyl-L-methionine on phosphatidylcholine synthesis by isolated hepatocytes. *Biochem Pharmacol* **33**: 1562–1564, 1984.
10. Osada J, Aylagas H, Sanchez-Vegazo I, Sanchez-Prieto J and Palacios-Alaiz E. Isolation of lysosomes by phase partition in Dextran/Poly(ethylene)glycol. *Anal Biochem* **185**: 249–253, 1990.
11. Folch J, Lees M and Sloane-Stanley GH, A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* **226**: 497–509, 1957.
12. Alsasua MT and Palacios-Alaiz E, Changes in phospholipid levels during cold stratification and germination of *Pinus pinnea* seeds In: *Advances in the Biochemistry and Physiology of Plant Lipids* (Eds. Appelqvist LA and Liljenberg C), pp. 251–256. Elsevier, Amsterdam, 1979.
13. Rouser G, Siakotos AN and Fleischer S, Quantitative analysis of phospholipids by thin layer chromatography and phosphorus analysis of spots. *Lipids* **1**: 85–86, 1966.
14. Sastry BUR, Stathan CN, Axelrod J and Hirata F, Evidence of two methyltransferases involved in the conversion of phosphatidylethanolamine to phosphatidylcholine in rat liver. *Arch Biochem Biophys* **211**: 762–773, 1981.
15. Vance DE and Ridgway ND, The methylation of phosphatidylethanolamine. *Progr Lipid Res* **27**: 61–79, 1988.
16. Siegel S. *Non Parametric Statistics for Behavioral Sciences*. McGraw-Hill. New York, 1970.
17. Anghileri LJ, Crone-Escanye MC, Martin JA and Robert J, Calcium and ATPase activity during hepatic intoxication with thioacetamide. *Int J Clin Pharmacol Ther Toxicol* **24**: 270–273, 1986.
18. Jaffe ER and Gottfried EL, Hereditary nonspherocytic hemolytic disease associated with an altered phospholipid composition of the erythrocytes. *J Clin Invest* **47**: 1375–1388, 1968.
19. Mato JM, Regulation of phospholipid methylation and its effect on membrane proteins. In: *Progress in Protein-Lipid Interactions* (Eds. Watts A and De Pout JJH), pp. 267–302. Elsevier, Amsterdam, 1986.
20. Hirata F, Hatanaka M, Wano Y and Matsuda K, Phospholipid methylation and cellular differentiation. In: *Biological Methylation and Drugs Design* (Eds. Borchardt RT and Creveling CR), pp. 67–74. Humana Press, Clifton, 1986.

Biochemical Pharmacology, Vol. 40, No. 3, pp. 651–654, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00
Pergamon Press plc

Failure of adenosine analogues to affect N-type voltage sensitive Ca^{2+} channels in chicken brain synaptosomes

(*Received* 13 *July* 1989; *accepted* 7 *September* 1989)

Adenosine (or ATP) is released, along with neurotransmitters, from nerves in the central nervous system [1]. These nucleotides (and their analogues) inhibit further neurotransmitter release by negative feedback on presynaptic adenosine A_1 receptors [2, 3]. Several mechanisms have been proposed to explain this activity. One hypothesis suggests that adenosine analogues interfere with Ca^{2+} influx [4–6] or promote Ca^{2+} sequestration in the presynaptic terminal [7]. However, reports of the effects of these compounds on Ca^{2+} influx have been inconsistent [4–6, 8–10], while the direct measurement of the effect of these compounds on intraterminal free Ca^{2+} concentrations ($[Ca^{2+}]_{int}$) has not, to our knowledge, been performed in neural tissue. Another possibility has been suggested [11, 12]: that adenosine analogues may preferentially inhibit Ca^{2+} flux through N-type voltage sensitive Ca^{2+} channels (VSCCs*), an alternative that also has not been directly explored.

We first studied the effects of the A_1 agonists 2-chloroadenosine (2-CADO) and N^6-cyclohexyladenosine (CHA) on Ca^{2+} movements and ^{125}I-labeled ω-conotoxin (^{125}I-ω-CgTx) binding to the N channel in rat brain preparations. These analogues were found to have no effect on any aspects of Ca^{2+} influx studied, confirming the results of Barr *et al.* [8] and Garritson *et al.* [9]. Further, extending the previous investigations, these analogues had no effect on the rise in $[Ca^{2+}]_{int}$ evoked by K^+ or on ω-CgTx binding in rat brain preparations. Since the N channel inhibitor ω-CgTx tends to have relatively minor effects on Ca^{2+} influx in rat brain [13], we deemed it necessary to repeat the experiments in chicken brain for the following reasons: chicken brain is rich in adenosine A_1 receptors [14], and, unlike the situation in rat brain, inhibition of the N-type Ca^{2+} channel in chicken brain with ω-CgTx blocks virtually all of the parameters of Ca^{2+} mobility investigated in this species [15–17]. The present results deal only with the effects of A_1 agonists in chicken brain preparations, but are qualitatively similar to results we obtained in rat brain.

The $^{45}Ca^{2+}$ influx studies were carried out essentially by the method of Blaustein [18]. The effect of ω-conotoxin, 2-CADO or CHA on Ca^{2+} influx was studied under resting (5 mM K^+) and depolarizing conditions (40 mM K^+), and the K^+-stimulated component (40 mM minus 5 mM) was calculated.

$[Ca^{2+}]_{int}$ was estimated in chicken brain preparations essentially as described by Komulainen and Bondy [19]. Briefly, the synaptosomal F_4 pellet, isolated using the Percoll gradient technique of Robinson and Lovenberg [20],

* Abbreviations: VSCCs, voltage sensitive Ca^{2+} channels; CHA, N^6-cyclohexyladenosine; 2-CADO, 2-chloroadenosine; ω-CgTx, ω-conotoxin GVIA; and EGTA, ethyleneglycol-bis-(β-aminomethyl ether)tetra-acetate.