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Methanethiol and dimethylsulfide formation from 3-methylthiopropionate in human and rat hepatocytes

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This study was designed to investigate the metabolism of methanethiol, and the involvement of methanethiol and its metabolites in the transamination pathway of methionine. Gaseous methanethiol, methanethiol-mixed disulfides and dimethylsulfide were formed from 3-methylthiopropionate, a metabolite in the transamination pathway of methionine, during incubation with human and rat hepatocytes. An increase of the 3-methylthiopropionate concentration resulted in an increased formation of the products, up to a substrate concentration of 4.4 mM. Higher substrate levels resulted in a decreased methanethiol formation, probably due to poisoning of the system. However, in human hepatocytes the formation of dimethylsulfide increased up to a 3-methylthiopropionate concentration of 12.5 mM. The formation of methanethiol, dimethylsulfide and methanethiol-mixed disulfides from 3-methylthiopropionate in hepatocytes of both human and rat support the hypothesis that methanethiol can be formed from methionine via the transamination pathway.

Introduction

Methanethiol (MT) has often been suggested as one of the endogenous factors involved in the pathogenesis of hepatic encephalopathy. This association is based upon the work of Challenger and Walshe [1], who isolated MT from the urine of a patient with massive hepatic necrosis and strong fetor hepaticus. The authors suggested that MT was derived from methionine. Oral methionine is able to induce neurological deterioration in cirrhotic patients [2,3] and coma in rats [4] and dogs [5] with limited liver function. The toxicity of methionine has often been ascribed to formation of MT from methionine (Refs. 1, 2, 5; reviews Refs. 6–9).

Our interest in the formation of MT from methionine was stimulated by a recent study [10] concerning a man with a partial deficiency of hepatic methionine adenosyltransferase (EC 2.5.1.6), the first enzyme in the transsulfuration pathway of methionine (Fig. 1). In this patient we showed that, despite a 35-fold increase in methionine (serum), a 100-fold increase in MTP (urine) and MT-mixed disulfides (serum and urine) and a 15-fold increase in DMS (breath), the patient was clinically normal and healthy. An increase in MT and DMS levels without clinical side-effects has also been observed in normal subjects after oral methionine administration [11–13].

Abbreviations: MT, methanethiol; DMS, dimethylsulfide; MTP, 3-methylthiopropionate.

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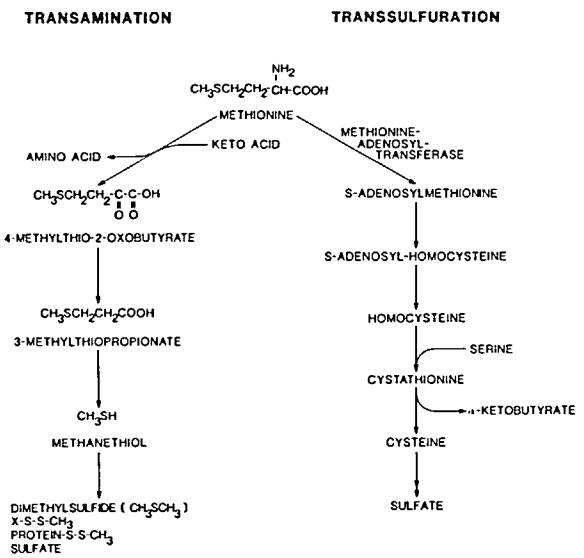


Fig. 1. Catabolism of methionine via the transamination and the transsulfuration pathways.

These observations seem to contradict the theory of methionine toxicity through MT formation.

In most texts, methionine degradation is depicted to occur only via the transsulfuration pathway (Fig. 1). However, MT is not formed via this pathway, because in this pathway the methylthio moiety is lost. 25 years ago, Canellakis and Tarver [14] reported the formation of MT from methionine in rat liver mitochondria, probably via 4-methylthio-2-oxobutyrate. The authors reported that MT became bound to protein, probably in a disulfide linkage. Recently, Benevenga and colleagues (reviewed in Ref. 6) have suggested that MT is formed via the methionine transamination pathway. The first step in this pathway is the formation of 4-methylthio-2-oxobutyrate by the transamination of methionine [6,8]. The oxidative decarboxylation of 4-methylthio-2-oxobutyrate to 3-methylthiopropionate (MTP) has been well characterized [6,15–18]. However, little is known about the next steps in the transamination pathway of methionine, viz., the conversion of MTP into MT and the catabolism of this compound (see for reviews Refs. 6,7–9,18,19). This is probably due to difficulties encountered in measuring volatile sulfur compounds. Recently, we have developed reliable methods for measuring volatile sulfur compounds [20,21], and MT-mixed disulfides (protein-S-S-CH_3 and X-S-S-CH_3) in body fluids [13]. In the present study we describe the formation of the volatile compounds MT and dimethylsulfide (DMS), and of MT-mixed disulfides by both human and rat hepatocytes from MTP.

Experimental

Materials

MT and DMS were obtained from Merck. Dithiothreitol, collagenase type I, bovine serum albumin, Tes and Hepes came from Sigma. Methyl(3-methylthio)propionate was from Aldrich. William's medium E and fetal calf serum were from Flow Laboratories. Dexamethasone disodium phosphate (Oradexon®) was from Organon, The Netherlands. Chemicals, unless otherwise specified, were of analytical purity.

Methods

Synthesis of MTP. Methyl(3-methylthio)propionate (1.0 g) was hydrolyzed to MTP by refluxing for 4 h in 100 ml 20% NaOH. The solution was then acidified (pH 1) and extracted three times with diethyl ether. The diethyl ether phases were pooled and re-extracted three times with 0.1 M NaOH. The 0.1 M NaOH phases were pooled, acidified (pH 1) and again extracted three times with diethyl ether. The diethyl ether phases were pooled and the ether was evaporated, resulting in a colourless oil (3-methylthiopropionic acid). The lithium salt of MTP was prepared by treating the acid with equimolar amounts of LiOH. The structure of MTP was confirmed by nuclear magnetic resonance (δ (ppm) [$CDCl_3$]: 2.1 [3H, s, CH_3-S], 2.7 [4H, m, S-CH_2-CH_2-COOH], 9.8 [1H, s, COO*H*]), mass spectrometry (*m/z* 120 [M^+], 103 [$M^+ - OH$], 75 [$M^+ - COOH$]) and gas chromatographic analysis. According to gas chromatographic analysis, the purity of MTP was > 98%.

Isolation of rat hepatocytes. Male Wistar rats (180–200 g) were used. Food and water were given ad libitum. The animals were anesthesized by an intraperitoneal injection of 0.2 ml sodium pentobarbital (60 mg/ml). Hepatocytes were isolated according to the method of Seglen [22] with the following modifications. The liver was perfused with a Ca^{2+}-free Hepes buffer (pH 7.6) for 20

min, followed by a Hepes buffer (pH 7.6) containing 0.05% (w/v) collagenase and 10 mM $CaCl_2$ for 10 min. The perfusion rate was 40 ml/min. The cells were pelleted and resuspended in William's medium E and incubated at 37°C for 30 min. After filtration and centrifugation, the hepatocytes were resuspended in William's medium E supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 20 mU/ml porcine insulin, 50 nM dexamethasone, 2.5 μg/ml fungizone, 50 μg/ml tetracycline and 100 μg/ml vancomycine, and diluted to a density of $10 \cdot 10^6$ viable cells/ml. The viability of hepatocytes was at least 95% before and 86% after the experiment, as estimated by the Trypan blue assay. Microscopic studies showed that the purity of the hepatocytes was greater than 98%.

Isolation of human hepatocytes. Post mortem human liver tissue was obtained from a kidney donor. Hepatocytes were isolated as described previously [23], with some modifications. EGTA was omitted in the Ca^{2+}- and Mg^{2+}free Hepes buffer. After the perfusion the cell suspension was pelleted, resuspended and incubated at 37°C for 30 min. After filtration and centrifugation, the hepatocytes were resuspended as described for rat hepatocytes. The viability of the hepatocytes was at least 68% before and 61% after the experiment, as determined by the Trypan blue exclusion test. The purity of the hepatocytes was greater than 98%, as was estimated by microscopic experiments.

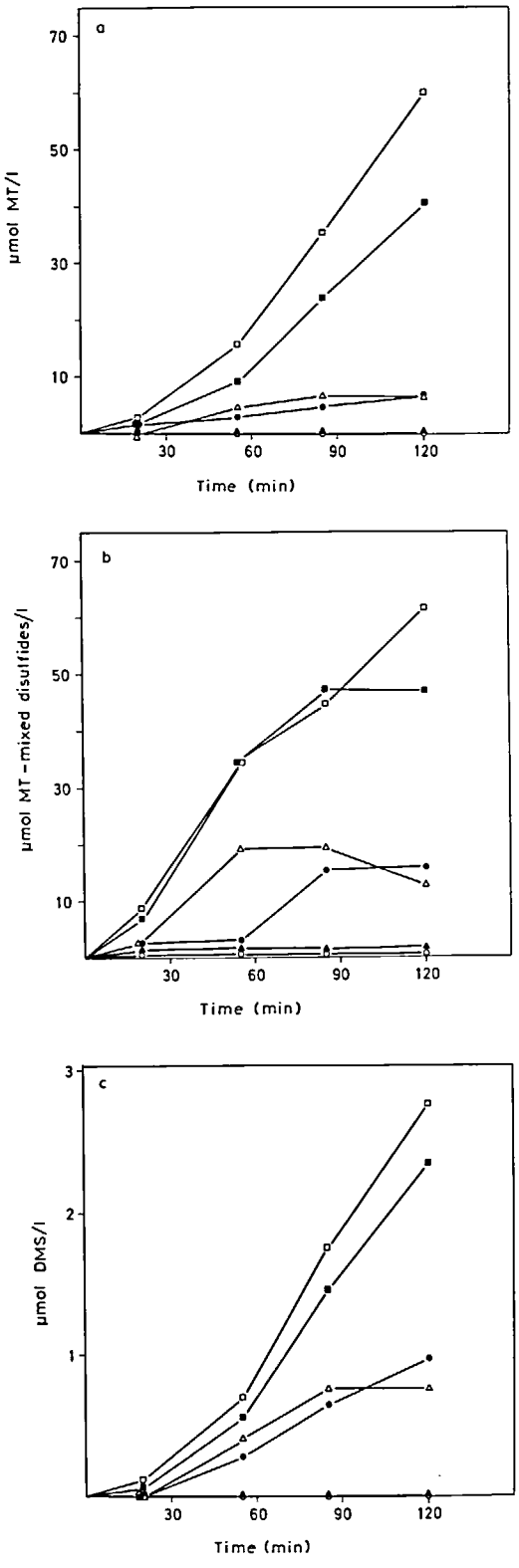
Incubation procedure. All incubation experiments were performed in duplicate. After addition of 100 μl of a neutralized aqueous MTP solution in a 15 ml glass vial containing 1 ml of the hepatocyte suspension ($10 \cdot 10^6$ viable cells) and 2 ml William's medium E (pH 7.8), the glass vial was sealed by means of a septum and gently shaken at 37°C. A sample of the head space of the vial was withdrawn at regular intervals with gas-tight syringes, concentrated onto Tenax trap tubes and analyzed for volatile sulfur compounds by means of gas chromatography. The volume of the sample of the head space varied from 100 μl to 12 ml, depending on the concentrations of the volatile sulfur compounds. The concentrations of MT and DMS, as depicted in Figs. 2a, 2c, 3a and 3c, represent the sum of concentrations as determined in the separate head space samples. After sampling of the head space, the vial was flushed with air for a few seconds and a liquid sample (200 μl) was taken out of the incubation mixture. The vial was sealed again by means of a septum and the incubation was continued. The liquid sample was cooled on ice and the hepatocytes were pelleted by centrifugation. The supernatant was frozen and stored at −80°C until analysis. The MT-mixed disulfides, which were measured in this supernatant, were shown to be stable at −80°C for at least 2 years.

Gas chromatographic analysis. Gaseous MT and DMS were measured as described previously [20,21]. The MT-mixed disulfides were measured by conversion to MT by means of dithiothreitol, as described recently [10,13]. MTP was measured by a modified method of Kaji et al. [13,24].

Results

Up to a concentration of 0.125 mM MTP gaseous MT and DMS were not formed in detectable amounts (less than 5 nM) by rat hepatocytes (Fig. 2). However, MT-mixed disulfides were detected after 20 min when the incubation was carried out with 0.125 mM MTP. Increasing amounts of MT, MT-mixed disulfides and DMS were recovered with increasing substrate concentrations up to 4.4 mM MTP. A higher substrate concentration (12.5 mM) resulted in a decrease in the formation of all three products.

As demonstrated in Fig. 3, incubation of MTP with human hepatocytes also results in the formation of gaseous MT, MT-mixed disulfides and DMS. MT and DMS were not formed in detectable amounts up to a concentration of 0.125 mM MTP, while the formation of MT-mixed disulfides was already measurable at a concentration of 0.044 mM MTP after 15 min. An increase in substrate concentration resulted in an elevated formation of MT, MT-mixed disulfides and DMS. Maximum production of MT and MT-mixed disulfides was observed at 4.4 mM MTP. A higher concentration of MTP (12.5 mM) resulted in decreased MT formation. In contrast to MT, DMS formation still increased, when the substrate concentration was increased from 4.4 mM to 12.5 mM MTP.



Blanks contained no hepatocytes or hepatocytes boiled for 2–4 min. MT, MT-mixed disulfides or DMS were not formed in detectable amounts under these conditions. However, when the incubation was performed without MTP, small amounts of MT-mixed disulfides were detected (maximal 1.07 μM in human hepatocytes and 0.80 μM in rat hepatocytes). Corrections were made for the endogenous formation of MT-mixed disulfides. This formation of small amounts of MT is probably due to the degradation of methionine present in the incubation mixture.

Discussion

The results clearly demonstrate that MT, MT-mixed disulfides and DMS are products of MTP catabolism in human and rat hepatocytes. To our knowledge, the formation of MT-mixed disulfides and DMS from MTP has not been described before. The formation of MT from MTP has been reported once before in rat liver homogenates [25]. Although our gas chromatographic system is quite capable of measuring H_2S [20,21], this compound was not detected during incubation of MTP with human or rat hepatocytes. Steele and Benevenga [25] reported formation of H_2S from MTP on incubation of MTP with rat liver homogenates at low pH (6.3). However, when they used the same pH as we used in our hepatocyte studies (pH 7.8), they did not detect H_2S.

Increasing amounts of MT and DMS were formed when the incubation was carried out with increasing substrate concentrations. A maximal MT formation was obtained at 4.4 mM MTP. The conversion of MTP to MT did not show saturation kinetics. On the contrary, MT formation was markedly decreased, when the substrate con-

Fig. 2. Formation of methanethiol (MT), MT-mixed disulfides and dimethylsulfide (DMS) from 3-methylthiopropionate in rat hepatocytes. The formation of gaseous MT (a), MT-mixed disulfides (b) and gaseous DMS (c) from 3-methylthiopropionate in rat hepatocytes was measured as described in the Experimental section. When necessary, corrections were made for endogenous formation of MT-mixed disulfides. Each point represents the mean of duplicate experiments. 3-Methylthiopropionate concentrations used (mM): ○, 0.044; ▲, 0.125; △, 0.44; ■, 1.25; □, 4.4; and ●, 12.5 mM.

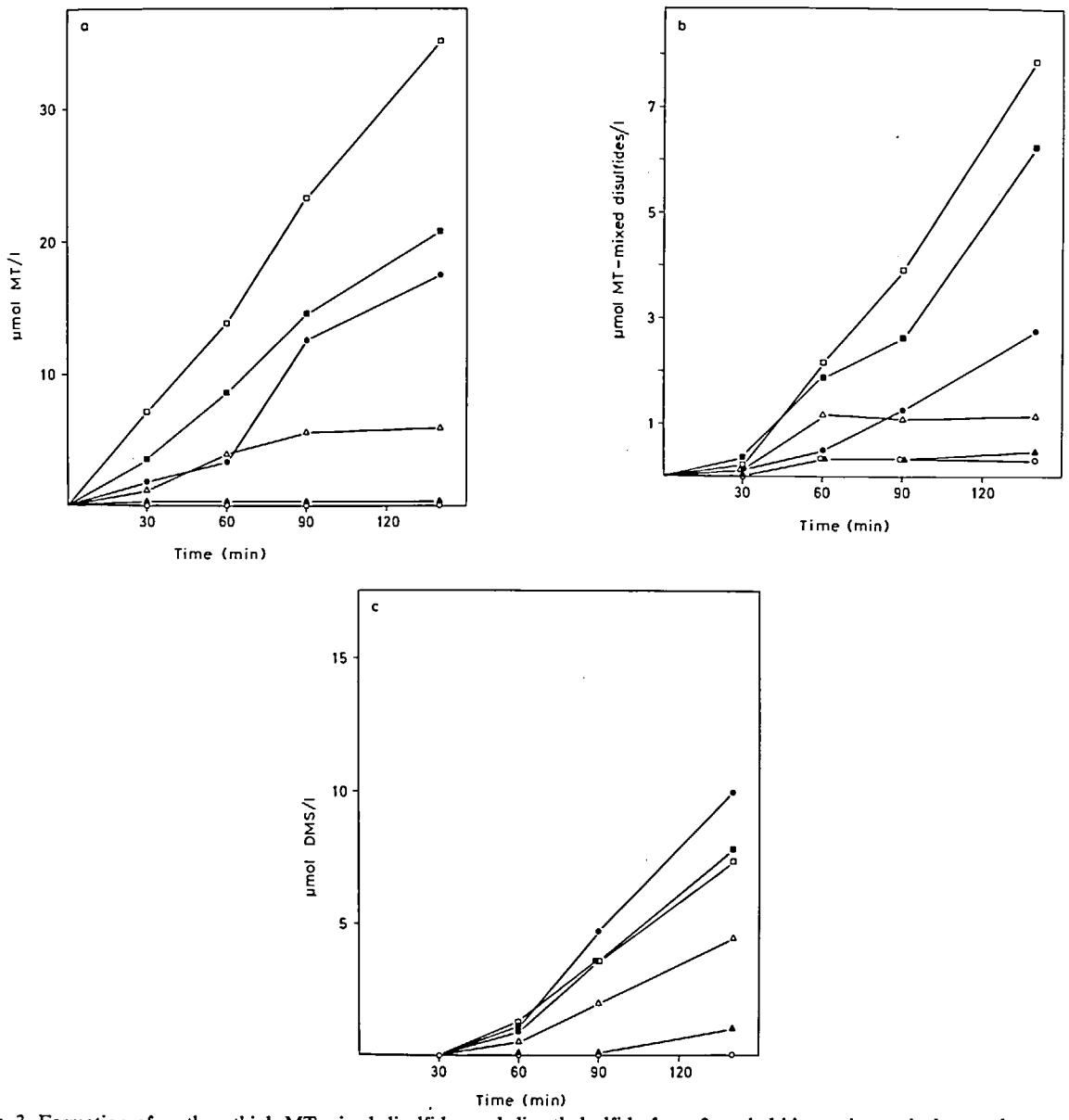


Fig. 3. Formation of methanethiol, MT-mixed disulfides and dimethylsulfide from 3-methylthiopropionate in human hepatocytes. The formation of gaseous MT (a), MT-mixed disulfides (b) and gaseous DMS (c) from 3-methylthiopropionate in human hepatocytes was measured as described in the Experimental section. When necessary, corrections were made for endogenous formation of MT-mixed disulfides. Each point represents the mean of duplicate experiments. 3-Methylthiopropionate concentrations used (mM): ○, 0.044; ▲, 0.125; △, 0.44; ■, 1.25; □, 4.4 and ●, 12.5 mM.

centration was increased from 4.4 to 12.5 mM, both in the human and in the rat hepatocyte system. This phenomenon can be explained by assuming that at high substrate concentrations the incubation system was inactivated by MTP or one of its metabolites. Although MT is a well-known toxin, MT itself is probably not responsible for the inactivation, because MT formation was markedly decreased at 12.5 mM MTP. The observed decrease in MT formation is in agreement with results of Steele and Benevenga [25], who reported a decreased product formation at levels

of MTP > 10 mM.

Theoretically, DMS can be formed from MTP via two different pathways: an MT-dependent or an MT-independent route. In the MT-dependent route MT is methylated to DMS by thiol-*S*-methyltransferase, an enzyme present in liver and many other organs [26]. The existence of an MT-independent route in humans has been suggested by Kaji et al. [11]. They observed that after administration of methionine to human subjects, the increase of DMS in breath was not parallelled by an increase of MT. An MT-independent route can proceed via methylation of MTP on the S-atom. This reaction results in the formation of dimethyl-β-propiothetin and DMS can easily be formed from this compound [27]. This pathway is well known in algae and various species of plankton [27]. The existence of this MT-independent DMS-producing pathway in humans could explain our observation that in the human hepatocyte system at a substrate concentration of 12.5 mM the formation of DMS increased while MT formation was markedly decreased.

In this paper we demonstrate that, in human hepatocytes, the formation of DMS is not totally dependent on MT and might proceed additionally via dimethyl-β-propiothetin. Evidence is provided that the volatile sulfur compounds, MT and DMS, and the MT-mixed disulfides are formed at least in part via the degradation of MTP in both human and rat hepatocytes. This supports the hypothesis that MT is formed from methionine via the transamination pathway. In future studies the measurement of MT, DMS, and MT-mixed disulfides will be used to investigate the methionine transamination pathway more extensively.

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