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Enzymatic S-De-Ethylation and the Mechanism of S-Dealkylation[1] (38734)

THEODORE M. FARBER

Special Pharmacological Animal Laboratory, Division of Toxicology, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D.C. 20204

Enzymes capable of dealkylating many foreign compounds are present in the liver microsomes. These NADPH- and oxygen-requiring enzymes are capable of demethylating or de-ethylating many aromatic ethers and alkyl-substituted amines, forming the corresponding phenols, amines and aldehydes (1–3). It has been shown that an NADPH- and oxygen-requiring enzyme or enzymes localized in the liver microsomes appear capable of *S*-demethylating certain methylthioethers (4).

This paper describes a microsomal enzyme system which de-ethylates a variety of *S*-ethyl compounds and presents some anomalous characteristics of *S*-dealkylation which are inconsistent with the proposed mechanism for this reaction and proposes that the reaction is more complex than was originally postulated. An alternative mechanism is suggested.

Materials and Methods. Tissue preparation and incubation conditions. Male Sprague–Dawley rats, 150–200 g, were stunned and the liver, kidney, heart, lung and spleen were immediately removed, rinsed in ice-cold isotonic KCl and homogenized with two volumes of this solution. The homogenates were centrifuged at 9000 *g* for 30 min and the supernatant fraction obtained from 1 g of liver was used in most of the experiments. To determine the intracellular distribution of the enzyme, a differential centrifugation of a 33% homogenate in 0.25 *M* sucrose was performed to obtain nuclear, mitochondrial, microsomal and soluble fractions by the method of Schneider (5). The particulate fractions were washed twice with 0.25 *M* sucrose and resuspended in isotonic KCl. All procedures in preparing the homogenates and various fractions were carried out at 4°.

Tissue fractions from 1 g of liver were incubated with shaking for 2 hr at 37° in an air atmosphere in 6.1 ml containing varying amounts of substrate, 0.65 μmole of NADP, 1.0 μmole of glucose-6-phosphate, 50 μmoles of nicotinamide, 45 μmoles of semicarbazide hydrochloride, 25 μmoles of magnesium chloride and 3 ml of 0.5 *M* Na^+/K^+ phosphate buffer, pH 7.4.

Acetaldehyde and formaldehyde assays. The microsomal incubation reaction was terminated by adding 1 ml of cold 2.6 *M* perchloric acid. The incubation flasks were washed with 5 ml of cold 0.4 *M* perchloric acid and the washings combined with the reaction mixture. The precipitated protein was centrifuged at 5000 *g* for 5 min and the supernatant collected. After distillation of the supernatant, acetaldehyde in the distillate was assayed enzymatically by utilizing alcohol dehydrogenase and reduced NAD in the method of Klein and Korzis (6). All values were corrected for a small tissue blank. Substrates employed in these studies did not release acetaldehyde under the conditions used except in the presence of active preparations. All values are averages of two or more identical samples, and variation was less than 2% among samples. Demethylation of methylthio compounds was followed by determining the amount of formaldehyde formed during the reaction. For the analysis of an aliquot of the protein-free extract, the method of Nash (7) as modified by Cochin and Axelrod (8) was employed. Corrections were made for small tissue blanks. All values are averages of two or more identical samples and variation was less than 3% among samples.

Isolation and separation of 6-methylthiopurine (MTP), 6-ethylthiopurine (ETP), and

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6-mercaptopurine (MP).[2] The protein in the incubation mixture was precipitated with 1 ml of 2.6 *M* perchloric acid and washed twice with three volumes of 0.4 *M* perchloric acid. For the determination of the amount of MP formed, the washings and original supernatant were adjusted to pH 8 and placed on a 10 mm × 120 mm column of Dowex-1-formate × 8 (200–400 mesh). Any MP present was eluted with 30 ml of 1 *N* formic acid. This formic acid fraction was placed on a 10 mm × 120 mm column of Dowex-50W-H × 8 (200–400 mesh) to remove contaminating MTP or ETP. Any MP present was eluted by 3 *N* HCl and was found in the 15–40 ml fraction. MTP and ETP were eluted with 6 *N* HCl.

For the determination of the amount of MTP or ETP remaining in the incubation mixture, the washings and original supernatant were placed directly on the Dowex-50 column described above and eluted with 6 *N* HCl. The UV absorption spectra of the various eluates were obtained with a Beckman DB recording spectrophotometer. The final eluates were evaporated *in vacuo*, and the residues taken up in a small amount of methanol and chromatographed on paper by using an isopropanol-sodium sulfate (5% isopropanol-5% aqueous sodium sulfate) or *n*–butanol–acetic acid-water solvent system (3.5:16.0: 9.6, v/v). Thin-layer chromatography (TLC) was also performed using silica gel G and a solvent system of *n*–butanol saturated with 2 *N* ammonium hydroxide. Final estimations of the amount of MP, MTP, and ETP were obtained by spectrophotometric analysis at 327 nm, 308 nm, and 296 nm, respectively of 0.1 *N* HCl eluates of spots having the same R_f values as authentic compounds developed in the same chromatogram. The UV absorption spectra of the eluates of the spots were also determined. Isotopic dilution assays for MP were performed by using the above column techniques and TLC.

Formation of intermediate metabolite by microsomes. The 9000 *g* supernatant fraction of rat liver was prepared as above and a microsomal pellet was prepared from an aliquot of the 9000 *g* supernatant in an International B-60 centrifuge (200,000 *g* for 1 hr). The soluble fraction was removed very carefully so as not to disturb the pellet and was stored at 4° for later use. The microsomal pellet was washed and resuspended with additional cold isotonic KCl and recentrifuged twice in order to free the pellet of any soluble fraction. The volume of the resuspended pellet was adjusted to the volume of the original 9000 *g* supernatant used to prepare the pellet.

The original 9000 *g* supernatant fraction was incubated for 1 hr in the complete reaction mixture with 10 μmoles of MTP as substrate, and the amount of formaldehyde formed was determined. A 3-ml aliquot of the washed microsomal preparation (equivalent to the microsomes present in 1 g of tissue) was incubated with 10 μmoles of MTP, but without the soluble fraction or NADP, for 1 hr. Four 10-mg portions of NADPH were added to these flasks at 10, 20, 30 and 40 min respectively after the start of the incubation. At the end of the incubation, one-half of the microsomal incubation mixtures were analyzed for the amount of formaldehyde formed and the other half were recentrifuged at 200,000 *g* for 1 hr. The supernatant was carefully removed and incubated for 1 hr with 3 ml of the soluble fraction that had been stored at 4°. These incubation mixtures were then analyzed for the formaldehyde formed.

Results. Preliminary experiments using the 9000 *g* supernatant fraction of liver homogenates indicated that *S*-ethyl compounds were converted to acetaldehyde. The requirements and characteristics of the de-ethylating process were studied in greater detail with ETP as the substrate, since Mazel *et al.* (4) used MTP to characterize the *S*-demethylation process. With incubates containing a complete cofactor system as

[2] 6-Mercaptopurine and 6-methylthiopurine were obtained from the Sigma Chemical Company; 2-ethylmercapto-4-hydroxy-6-methylpyrimidine, 2-ethylmercapto-4-hydroxypteridine and 6-ethylthiopurine from the Aldrich Chemical Company; the ethyl analog of thioridazine from the Sandoz Corporation; *S*-ethylisothiourea (sulfate) from K and K Laboratories; 8-ethylthiotheophylline from Dr. Raymond Burgison; 6-methylsulfinylpurine and 8-hydroxy-6-methylsulfinylpurine from Dr. Gertrude Elion; and 6-mercaptopurine-8-^{14}C from the Volk Radiochemical Company.

discussed above, 900 nmoles of acetaldehyde was formed from 10 μmoles of ETP. NADP, magnesium chloride, nicotinamide and oxygen were found to be necessary for maximal activity in the system. Lack of NADP, nicotinamide or oxygen caused an 85% or more reduction in acetaldehyde formation, while lack of magnesium chloride caused a 37% reduction in acetaldehyde formation. NAD could not replace NADP in this system. Nicotinamide was required to prevent the destruction of NADP by NADPases.

The rate of enzymatic de-ethylation of ETP is reflected by the formation of acetaldehyde which was linear during the first hour of incubation. After 2 hr, little if any additional acetaldehyde was formed. At substrate concentrations of approximately 1.8 m*M* (10 μmoles per flask) the rate of metabolism followed zero-order kinetics. For all subsequent incubations, 10 μmoles of any substrate used was added to each flask.

A study of the intracellular localization of the enzyme was conducted on the various fractions obtained from a differential centrifugation of a sucrose homogenate of liver. Little or no enzymatic activity was found in any of the separate fractions. Only when the microsomal pellet was combined with the soluble fraction was the activity equivalent or somewhat higher than that of the whole homogenate.

Kidney, heart, brain, spleen and lung tissues were examined for de-ethylating activity with ETP as a substrate. Activity was found in only the 9000 *g* supernatant of kidney tissue and was approximately 20% of that in liver.

Rats were given intraperitoneal injections of phenobarbital (in saline, 50 mg/kg), methylcholanthrene (in corn oil, 10 mg/kg) or benzpyrene (in corn oil, 20 mg/kg) daily for 3 days and sacrificed on the fourth day. Control rats were given only the vehicles. De-ethylating activity of the 9000 *g* supernatant of liver homogenates was increased 280% by phenobarbital, 310% by methylcholanthrene and 335% by benzpyrene. Additionally, phenobarbital pretreatment caused an increase in the demethylation of MTP by the 9000 × *g* supernatant of 112% over that seen with controls.

TABLE I. SUBSTRATE SPECIFICITY OF THE DE-ETHYLATING ENZYME[a].

Compound	Acetaldehyde formed (nmoles)
6-Ethylthiopurine	1500
2-Ethylthio-4-hydroxymethyl-pyrimidine	275
2-Ethylthio-4-hydroxypteridine	160
S-Ethylthiourea	500
S-Ethylcysteine	150
Ethionine	143
Ethylmercaptan	1520
Ethyl sulfide	120
Diethyl disulfide	5550
Ethyl thiocyanate	0
Ethyl analog of thioridazine	95
8-Ethylthiotheophylline	0

[a] The 9000 *g* supernatant fraction obtained from 1 g of liver was incubated 2 hr at 37° with 10 μmoles of 6-ethylthiopurine, 0.65 μmole NADP or NAD, 1.0 μmoles of glucose-6-phosphate, 50 μmoles of nicotinamide, 45 μmoles of semicarbazide hydrochloride, 25 μmoles of $MgCl_2$, 3.0 ml of 0.5 *M* phosphate buffer, pH 7.4, in a final volume of 6.1 ml.

As seen in Table I, the 9000 *g* supernatant fraction of rat liver appeared to de-ethylate many diverse sulfur-containing compounds. Simple compounds like diethyl disulfide and ethyl mercaptan were converted to acetaldehyde, but it is difficult to determine the true extent of conversion of ethyl mercaptan because of its extreme volatility.

Up to this point, the microsomal *S*-de-ethylation seems to be quite similar or perhaps identical to the system described by Mazel *et al.* (4) for microsomal demethylation. However, further study of the de-ethylation process raised the following points which were not consistent with a microsomal oxidative dealkylation process:

1. Attempts were made to establish the NADPH requirements of the system by replacing the soluble fraction with buffers containing NADPH or a known NADPH-generating system. When suspensions of washed microsomal pellets were fortified with up to 20 mg of NADPH per flask or a combination of 2 μmoles of NADP, 20 μmoles of glucose-6-phosphate, 50 units of

glucose-6-phosphate dehydrogenase and 50 mg of bovine albumin per flask and were incubated with 10 μmoles of MTP or ETP, no formaldehyde or acetaldehyde was formed. Apparently the soluble fraction of the hepatic cell must be present in the preparation before acetaldehyde or formaldehyde is formed. A similar finding has been observed by Mazel (personal communication).

2. A balance study of the reaction products resulting from the incubation of 10 μmoles of ETP with the 9000 *g* supernatant was performed; 1.5 μmoles of acetaldehyde were formed and 8.4 μmoles of ETP were recovered. However, no evidence for the formation of MP could be found. Spectral scans of the final Dowex-50-H^+ eluates revealed no peak at 327 nm characteristic of MP, although 650 nmoles of authentic MP added to the incubation mixture at zero time could easily be detected in the column eluates. No MP spot was found by paper chromatography or TLC in several solvent systems with which 65 nmoles could easily be detected. Similarly, when MTP was incubated with the 9000 *g* supernatant fraction and cofactors, formaldehyde was formed but MP could not be found.

Although the amount of semicarbazide present in the mixture would have significantly inhibited the xanthine oxidase in the 9000 *g* supernatant (10), ETP and MTP were incubated in the presence of a xanthine inhibitor, 4-hydroxy-pyrazolo(3,4-d)pyrimidine (9), 120 μg per flask. Even this treatment did not result in the formation of any MP. An isotopic dilution assay for MP was performed using 6-mercaptopurine-8-^{14}C of a known specific activity. The radioactive MP was added to the fortified 9000 *g* supernatant preparation along with 10 μmoles of MTP at zero time and the mixture incubated for 1 hr. The specific activity of the radioactive MP, isolated and purified by Dowex-1 and Dowex-50 column chromatography and TLC, was the same as the specific activity of the radioactive MP added to the incubation mixture, confirming that no MP had been formed.

3. SKF-525A a compound known to significantly inhibit *N*- and *O*-dealkylation, did not significantly inhibit *S*-demethylation. SKF-525A, at a dose of 50 mg/kg, was administered intraperitoneally to rats and the animals were sacrificed 1 hr later. At most, only a 10% reduction in *S*-dealkylation was seen in the reaction mixtures after incubation with the 9000 *g* supernatant of liver homogenates. A similar finding has been seen by Mazel (personal communication).

TABLE II. DEMONSTRATION OF PRESENCE OF INTERMEDIATE METABOLITE FORMED IN MICROSOMAL PREPARATION[a].

Type of preparation	No. of incubates	Formaldehyde formed (nmoles) ±S.D.
9000 *g* supernatant	4	650 ± 70
Microsomal suspension	4	112 ± 25
Supernatant from microsomal incubate + soluble fraction	4	700 ± 71

[a] Intracellular fractions obtained from 1 g of rat liver were incubated with 10 μmoles of 6-methylthiopurine as described in Table I, except that the microsomal suspension was incubated with four 10-mg portions of NADPH. Some of the incubated microsomal suspensions were recentrifuged at 200,000 *g* for 1 hr and the supernatants were subsequently incubated with soluble fraction obtained during the preparation of the original microsomal pellet.

As stated earlier, no formaldehyde was formed when a microsomal preparation, free of soluble fraction, was incubated with MTP and NADPH. However, the results shown in Table II indicate that an intermediate metabolite was formed in the presence of a microsomal pellet and NADPH, which in the presence of the soluble fraction, yielded formaldehyde as a product of its metabolism. The possibility was considered that the MTP or ETP was converted to alkylsulfoxides by the microsomal preparation and that the alkylsulfoxide was subsequently metabolized to aldehydes by an enzyme in the soluble fraction. Schuster (11) and McMahon (12) have proposed such a mechanism for the formation of aldehydes from alkylthioethers.

TABLE III. THE DE-METHYLATION OF 6-METHYLTHIOPURINE, 6-METHYLSULFINYLPURINE AND METHYLMERCAPTAN[a].

Fraction	Formaldehyde formed (nmoles)		
	Methylthiopurine	Methylsulfinylpurine	Methylmercaptan
9000 *g* supernatant	610+[b]	1250+	1970
Soluble fraction	50	1168+	920
Microsomal suspension	10	10	—
Soluble fraction without added cofactors	0	1190+	1826
0.5 *M* PO_4 buffer, pH 7.4	0	0	—

[a] Fractions obtained from 1 g of rat liver were incubated with 10 μmoles of 6-methylthiopurine, 6-methylsulfinylpurine or methylmercaptan as described in Table I except that the microsomal suspension also contained 2 units of glucose-6-phosphate dehydrogenase.

[b] Plus sign indicates the development of a mercaptan odor during incubation.

Table III indicates that methylsulfinylpurine, the sulfoxide of MTP, is indeed metabolized to formaldehyde and is a better substrate than MTP. A strong odor of methylmercaptan was detected during the incubation. The formation of formaldehyde and the development of a mercaptan odor did not require the presence of microsomes but did require the presence of the soluble fraction. Formaldehyde formation occurred without the addition of exogenous cofactors, but did not occur when boiled soluble fraction was incubated with methylsulfinylpurine. As seen in Table III, conversion of methylmercaptan to formaldehyde did not require the presence of microsomes. The conversion can occur in the soluble fraction alone and without the addition of exogenous cofactors. Preliminary evidence indicates that the reaction may be inhibited by the addition of a cofactor. A boiled soluble fraction preparation was inactive in the metabolism of methylmercaptan to formaldehyde. Additionally, when 6-methylsulfonyl-purine, the sulfone of MTP, 8-hydroxy-6-methyl sulfinylpurine and 6-methyl-8-hydroxy-thiopurine were used as substrates, no formaldehyde formation was seen in the 9000 *g* supernatant incubates, indicating that the compound undergoing dealkylation is not an 8-hydroxy derivative formed by the action of aldehyde oxidase (13).

Discussion. A possible mechanism for the formation of aldehydes from 6-alkylthiopurines is presented in Fig. 1. This scheme offers an explanation for the failure to detect MP as a metabolite of the alkylthiopurines and any aldehyde formation in microsomal suspensions fortified with only NADPH or an NADPH-generating system; and for the lack of inhibitory effect of SKF-525A on the production of aldehyde formation, and for the development of mercaptan odor in incubation mixtures containing the alkylthiopurines. Sulfoxidation is mediated by liver microsomes which require NADPH and oxygen and is unaffected by SKF-525A (14), as seen in the dealkylation of ETP and MTP. The alkylsulfinylpurine may then be metabolized to a mercaptan via enzymes present in the soluble fraction. The alkyl mercaptans can be converted to an aldehyde directly or transthiolated to an alcohol and inorganic sulfate (15). The alcohol can then be metabolized to an aldehyde.

Some support for this scheme was obtained from the literature which indicates that the major excretory products of MP are unchanged drug and 6-thiouric acid, whereas these two compounds represent only minor metabolites in the case of MTP. The latter compound, which would be expected to yield an excretion pattern identical to that of MP if it were largely converted to the latter *in vivo*, in fact has quite a different pattern of excretion products, in which inorganic sulfate and 6-methylsulfinyl-8-hydroxypurine predominate (10, 16, 17). *In vivo* observations such as these are not incompatible with the possibility that a significant fraction of administered MTP is

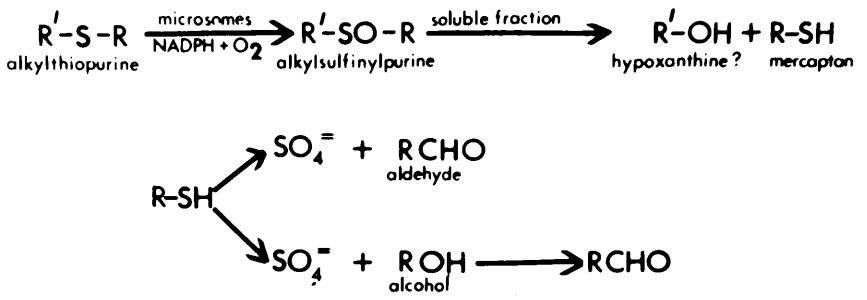

FIG. 1. Proposed mechanism for the formation of aliphatic aldehydes from alkylthiopurines.

converted to free MP, but they do suggest that this cannot be its major metabolic pathway.

To explain the extensive formation of inorganic sulfate seen during the *in vivo* metabolism of alkylthiopurines in man, rat and mouse, some investigators have postulated that mercaptans are formed (10, 16). Additionally, propylmercaptan has been detected on the breath of patients receiving 6-propylmercaptopurine (18) and, after administration of the ^{35}S-labeled compound, large amounts of labeled inorganic sulfate were found in the urine of both humans and mice (19). This scheme should not be considered as an explanation of aldehyde formation from all alkylthioethers but it should be given consideration when examining the metabolism of 6-alkylthiopurines. It is possible that *S*-dealkylation is truly a microsomal reaction for other substrates.

Summary. An NADPH- and oxygen-dependent enzymatic system responsible for the formation of acetaldehyde from ethylthioethers is present in the 9000 *g* supernatant fraction of rat liver. This system appears to be quite similar to the one responsible for the formation of formaldehyde from methylthioethers. Although both of these systems appear to be microsomal mixed-oxidase systems, several anomalous findings regarding the mechanism of microsomal *S*-dealkylation have been observed and have led us to the conclusion that the mechanism of this reaction is more complex than originally believed. A scheme is postulated by which an enzyme in the soluble fraction causes microsomal sulfoxidation of alkylthioethers to alkylsulfoxides, followed by metabolism of the alkylsulfoxides to aldehydes.

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