Production of Formaldehyde during Metabolism of Dimethyl Sulfoxide by Hydroxyl Radical Generating Systems†

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ABSTRACT: Dimethyl sulfoxide is a potent hydroxyl radical scavenging agent. The production of methane from dimethyl sulfoxide has been used to detect the generation of hydroxyl radicals in biological systems. In the current report, evidence is presented that formaldehyde is produced during the interaction of dimethyl sulfoxide with hydroxyl radicals. Formaldehyde, rather than methane, represents a major product of this interaction. Three model hydroxyl radical generating systems were used in this investigation. They were (1) the oxidation of xanthine by xanthine oxidase, (2) the iron-catalyzed oxidation of ascorbic acid, and (3) NADPH-dependent electron transfer by rat liver microsomes. Formaldehyde was produced from dimethyl sulfoxide in a time-dependent manner by all three systems. Formaldehyde production was inhibited by competing hydroxyl radical scavenging agents such as benzoate, mannitol, and 2-keto-4-thiomethylbutyric acid. Catalase completely inhibited the production of formaldehyde in the xanthine oxidase and the ascorbate systems. Azide, an inhibitor of catalase (present as a contaminant in isolated microsomes), stimulated the production of formaldehyde during microsomal electron transfer. These results indicate that H_2O_2 serves as a precursor of hydroxyl radicals in the three systems. Superoxide dismutase inhibited formaldehyde production by the xanthine oxidase system but not by the ascorbate system. Diethylenetriaminepentaacetic acid inhibited the production of formaldehyde by the xanthine oxidase system to a much greater extent than it did in the ascorbate system. The addition of iron–EDTA stimulated formaldehyde production. It is suggested that hydroxyl radicals are generated by the xanthine oxidase system and by the ascorbate system via a Fenton reaction in which the reduction of iron is brought about either by superoxide radical or by ascorbate. Considerably greater amounts of formaldehyde than of methane were produced by all three hydroxyl radical generating systems. The production of formaldehyde from dimethyl sulfoxide may represent a convenient technique to detect and to evaluate the role of hydroxyl radicals in some biological systems. In view of the production of formaldehyde, dimethyl sulfoxide should not be considered to be an inert solvent in biological systems. The possibility that formaldehyde is a metabolite of the in vivo metabolism of dimethyl sulfoxide remains to be evaluated.

Dimethyl sulfoxide (Me_2SO)[1] is a potent hydroxyl radical (·OH) scavenging agent (Anbar & Neta, 1967; Dorfman & Adams, 1973). It interacts readily with ·OH ($K = 7 \times 10^9$ M^{-1} s^{-1}) (Anbar & Neta, 1967; Dorfman & Adams, 1973) to produce methyl radicals (·CH_3) (Dixon et al., 1964; Lagercrantz & Forshult, 1969; Ashwood-Smith, 1975) which can give rise to methane gas by hydrogen abstraction. The production of methane from Me_2SO has been used to detect a reactive species generated by several biological systems (Brownlee et al., 1977; Repine et al., 1979; Cohen & Cederbaum, 1979, 1980). However, it had been observed that during NADPH-dependent electron transfer by rat liver microsomes the production of methane from Me_2SO was at least 1 order of magnitude less than the generation of ethylene gas from either methional or 2-keto-4-thiomethylbutyric acid (KTBA) under the same experimental conditions (Cohen & Cederbaum, 1979, 1980). In this report, it is shown that formaldehyde is also produced and that the yield of formaldehyde is much greater than the yield of methane.

The initial observation of formaldehyde production from Me_2SO was made during studies of the microsomal oxidation of methanol (Cederbaum, 1981). It is known that alcohols can be oxidized to their respective aldehydes as a result of the interaction of alcohols with ·OH (Dorfman & Adams, 1973). It has been suggested that ·OH plays a role in the oxidation of alcohols by liver microsomes (Cederbaum et al., 1977, 1978, 1979, 1981; Cederbaum & Cohen, 1980). During a study of the effect of ·OH scavengers on methanol oxidation by microsomes, it was observed that Me_2SO produced formaldehyde even in the absence of methanol. The current study was carried out to characterize the ability of Me_2SO to be oxidized to formaldehyde by three different oxygen radical producing systems. In all three systems, the production of formaldehyde appears to be due to the interactions of Me_2SO with ·OH or a species with the oxidizing power of ·OH. Initial aspects of these results have been reported in a preliminary paper (Klein et al., 1980).

Experimental Procedures

Xanthine–Xanthine Oxidase Model ·OH-Generating System. The oxidation of xanthine by xanthine oxidase was used as a model ·OH-generating system (Beauchamp & Fridovich, 1970; Cohen, 1977a). The standard reaction mixture consisted of 50 mM potassium phosphate buffer, pH 7.4, 0.4 mM xanthine, 0.1 mM Na_4EDTA, 0.018 units of xanthine oxidase, and 33 mM Me_2SO in a final volume of 3.0 mL. The reactions were initiated at 37 °C by the addition of xanthine oxidase and were terminated by the addition of 1.0 mL of ice-cold 17.5% (w/v) trichloroacetic acid. A 1.5-mL aliquot was then assayed for formaldehyde. In view of the small amounts of formaldehyde which were made by this system, a more sen-

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[1] Abbreviations used: Me_2SO, dimethyl sulfoxide; ·OH, hydroxyl radical; KTBA, 2-keto-4-thiomethylbutyric acid; DETAPAC, diethylenetriaminepentaacetic acid; O_2^{-}·, superoxide anion radical.

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sitive fluorometric modification (Steffen & Netter, 1979) of the method of Nash (1953) was used. A Perkin-Elmer fluorescence spectrophotometer (Model 650-10S) was used at an excitation wavelength of 415 nm and at an emission wavelength of 505 nm. It was necessary to construct a standard curve with known amounts of formaldehyde for each experimental condition in order to compensate for the effect of various constituents on the fluorescence emission intensity. For example, hydrogen peroxide quenched the fluorescence emission intensity. Since the samples are light sensitive, all fluorescent measurements were made under dim illumination to minimize decay of the fluorescent emission intensity. Control experiments verified identical scanning patterns with maximum excitation at 415 nm and maximum emission wavelength at 505 nm for both standards and experimental samples. Evidence that an aldehyde was produced from Me_2SO was provided by the observation that the contents of the experimental flasks promoted the oxidation of NADH in the presence of alcohol dehydrogenase. Iron–EDTA was added in some experiments as a 1:2 mixture of ferrous ammonium sulfate in aqueous Na_4EDTA.

Ascorbic Acid–Iron–EDTA Model ·OH-Generating System. The iron-catalyzed oxidation of ascorbic acid at 37 °C was used as a second model ·OH-generating system (Cohen, 1977a,b). The standard reaction mixture consisted of 100 mM potassium phosphate buffer, pH 7.4, 167 μM iron–EDTA (1:2 mixture), 0.1 mM EDTA, 2 mM ascorbic acid, and 33 mM Me_2SO in a final volume of 3.0 mL. The reactions were initiated by the addition of ascorbic acid. At time intervals, 1.0 mL of ice-cold 17.5% (w/v) trichloroacetic acid was added, and a 1.5-mL aliquot was assayed for formaldehyde by the spectrophotometric method of Nash (1953). An extinction coefficient of 8.0 cm^{-1} mM^{-1} was used to calculate the amount of formaldehyde which was produced.

Microsomal Experiments. Liver microsomes were prepared from male Sprague-Dawley rats as previously described (Cederbaum et al., 1976). The microsomes were washed once and were resuspended in 125 mM potassium chloride. The standard reaction mixture consisted of 83 mM potassium phosphate buffer, pH 7.4, 0.1 mM EDTA, 10 mM potassium pyrophosphate, 10 mM $MgCl_2$, 0.3 mM $NADP^+$, 10 mM glucose 6-phosphate, 7 units of glucose-6-phosphate dehydrogenase, 3–5 mg of microsomal protein, and 33 mM Me_2SO in a final volume of 3.0 mL. The reaction was initiated by the addition of the NADPH-generating system and was terminated by the addition of 1.0 mL of ice-cold 17.5% (w/v) trichloroacetic acid. A 1.5-mL aliquot was then assayed for formaldehyde according to the spectrophotometric method of Nash (1953).

Method for the Measurement of Hydrocarbon Gases. In some experiments, both formaldehyde and methane were measured in the same flasks in order to compare the yields of each product. The flasks were sealed with serum caps. Methane was measured by injecting a 0.5-mL aliquot of the head space directly into a Hewlett-Packard Model 5750 gas chromatograph. Operating conditions were as previously described (Cohen & Cederbaum, 1980). The flasks were then opened, and aliquots were removed for the measurement of formaldehyde. The production of ethylene from 10 mM KTBA was assayed by head-space gas chromatography (Cohen & Cederbaum 1979, 1980).

Materials. All materials were of the highest available grade from commercial sources. Materials were obtained from the following sources: DETAPAC and sodium 2-keto-4-thiomethylbutyrate, Sigma Chemical Co. (St. Louis, MO); xanthine, Eastman Kodak Co. (Rochester, NY); xanthine oxidase (0.45 unit/mg of protein) and catalase (65 000 units/mg of protein), Boehringer Mannheim (Indianapolis, IN); superoxide dismutase, Biotics Research Corp. (Houston, TX); Chelex-100 resin (200–400 mesh), Bio-Rad Laboratories (Richmond, CA). Water for the preparation of all solutions was purified by passage through a Millipore system. This water was subsequently distilled in an all-glass apparatus.

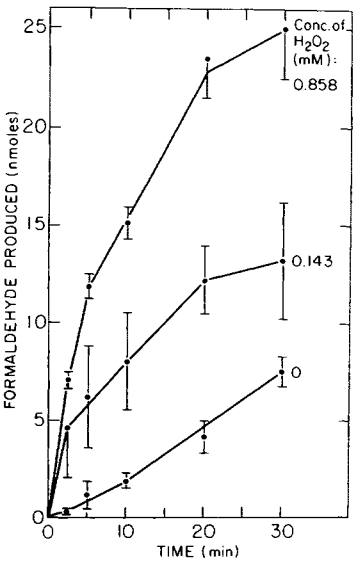

FIGURE 1: Time course of the generation of formaldehyde from dimethyl sulfoxide during the oxidation of xanthine by xanthine oxidase and the effect of the addition of hydrogen peroxide. The standard reaction mixture consisted of 50 mM potassium phosphate buffer, pH 7.4, 0.4 mM xanthine, 0.1 mM Na_4EDTA, 0.018 unit of xanthine oxidase, and 33 mM Me_2SO in a final volume of 3.0 mL. H_2O_2 was added to achieve the final concentrations which are indicated in the figure. The results represent the mean ± SEM from three experiments.

All values refer to the mean ± standard error of the mean (SEM). Statistical analyses were performed by Student's *t* test. The number of experiments is indicated in the table or in the figure legends.

Results

Generation of Formaldehyde from Me_2SO during the Oxidation of Xanthine by Xanthine Oxidase. During the oxidation of xanthine by xanthine oxidase, the superoxide anion radical ($O_2^{-}\cdot$), hydrogen peroxide, and ·OH are produced (Beauchamp & Fridovich, 1970). The production of hydroxyl radicals probably represents the interaction of $O_2^{-}\cdot$ and hydrogen peroxide in an iron-catalyzed Haber-Weiss type of reaction (McCord & Day, 1978).

$$O_2^{-}\cdot + H_2O_2 \xrightarrow{Fe} \cdot OH + OH^- + O_2 \qquad (1)$$

Other investigators observed the production of ethylene gas from methional (Beauchamp & Fridovich, 1970) and the production of acetaldehyde from ethanol (Cohen, 1977a) during the oxidation of xanthine by xanthine oxidase. When Me_2SO was substituted for methional or for ethanol, formaldehyde was produced (Figure 1). In control experiments, formaldehyde was not produced when either Me_2SO, xanthine, or xanthine oxidase was omitted from the reaction mixture, or when H_2O_2 (0.4 mM) was added in the absence of either xanthine or xanthine oxidase (data not shown). Although formaldehyde production does not occur as a result of a direct interaction between Me_2SO and H_2O_2, the addition of H_2O_2 to the complete reaction mixture stimulated the rate of formaldehyde production (Figure 1). Since H_2O_2 is a precursor of ·OH, the increased production of formaldehyde from

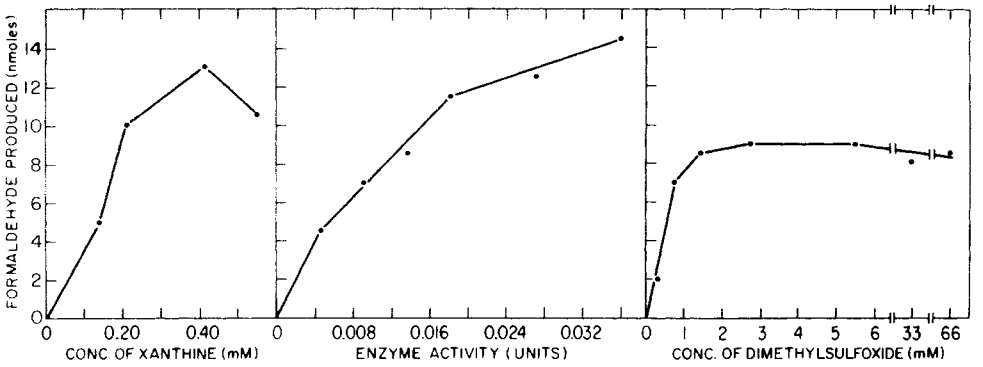

FIGURE 2: Titrations of formaldehyde production vs. the concentration of xanthine, xanthine oxidase, and dimethyl sulfoxide. The standard reaction mixture is described in the legend to Figure 1. The modification of the standard reaction mix are as follows: (Left panel) the concentration of xanthine was varied from 0 to 0.55 mM, while the concentrations of xanthine oxidase and Me_2SO were held constant at 0.018 unit and 33 mM respectively. (Center panel) the concentration of xanthine oxidase was varied from 0 to 0.036 unit while the concentrations of xanthine and Me_2SO were held constant at 0.4 and 33 mM, respectively. (Right panel) the concentration of Me_2SO was varied from 0 to 66 mM while the concentrations of xanthine oxidase and xanthine were held constant at 0.018 unit and 0.4 mM, respectively. Results are from a typical titration experiment.

Table I: Effect of Hydroxyl Radical Scavenging Agents on the Production of Formaldehyde from 3.3 and 33 mM Dimethyl Sulfoxide[a]

addition	concn of scavenger (mM)	rate of formaldehyde production (nmol/30 min)		effect of scavenger (%)	
		3.3 mM Me_2SO	33 mM Me_2SO	3.3 mM Me_2SO	33 mM Me_2SO
butanol (4)	0	9.12 ± 0.62	9.31 ± 0.61		
	10	4.31 ± 0.38	7.50 ± 0.54	−53[c]	−20[b]
	25	2.94 ± 0.22	6.38 ± 0.65	−68[c]	−32
	50	2.31 ± 0.19	5.19 ± 0.72	−75[c]	−44[b]
	100	3 06 ± 0.42	4.06 ± 0.16	−66[c]	−56[c]
ethanol (7)	0	10.78 ± 2.11	7.07 ± 0.54		
	10	5.75 ± 0.98	7.14 ± 1.07	−47[c]	+1
	25	4.61 ± 0.86	6.14 ± 0.53	−57[c]	−13
	50	3.43 ± 0.60	5.75 ± 0.99	−68[d]	−19
	100	3.00 ± 0.49	4.86 ± 1.60	−72[d]	−31
mannitol (3 or 4)	0	10.88 ± 2.12	12.12 ± 1.82		
	10	8.12 ± 1.52	11.35 ± 1.98	−25	−6
	25	5.98 ± 1.17	11.03 ± 1.77	−45[b]	−7
	50	4.50 ± 1.37	10.12 ± 1.38	−59[d]	−17
	100	2.45 ± 1.30	8.38 ± 1.59	−78[b]	−31
urea (3)	0	12.0 ± 0.5	10.64 ± 1.05		
	50	13.3 ± 1.2	11.06 ± 1.61	+10	+4
	100	15.0 ± 2.3	10.50 ± 0.70	+25	+1

[a] The complete reaction system is described in the legend to Figure 1. The various hydroxyl radical scavenging agents were added to achieve the final concentrations which are indicated in the table. The number of experiments is indicated in parentheses. [b] $p < 0.05$. [c] $p < 0.01$. [d] $p < 0.005$.

Me_2SO may reflect increased production of ·OH in the presence of H_2O_2.

The concentrations of xanthine, xanthine oxidase, and Me_2SO were varied in order to determine optimal conditions for the production of formaldehyde. A reaction period of 30 min was used in these experiments. The production of formaldehyde reached a maximum rate at approximately 0.4 mM xanthine and above 1.25 mM Me_2SO and was dependent upon the concentration of xanthine oxidase (Figure 2).

Effect of ·OH Scavenging Agents. Previous results (Klein et al., 1980) indicated that the production of formaldehyde from Me_2SO was completely inhibited by either catalase or superoxide dismutase, which indicates that neither H_2O_2 nor $O_2^{-}\cdot$ radicals alone have the ability to produce formaldehyde from Me_2SO. The effect of ·OH scavenging agents on the production of formaldehyde was evaluated. The ·OH scavenging agents and their rate constants ($\times 10^9$ M^{-1} s^{-1}) for interaction with ·OH are as follows: ethanol, $K = 1.8$; butanol, $K = 3.7$; mannitol, $K > 1$ (Dorfman & Adams, 1973). Two concentrations of Me_2SO, 33 and 3.3 mM, were used. All three ·OH scavenging agents suppressed the production of formaldehyde in a dose-dependent manner (Table I). The extent of inhibition was greater with 3.3 mM Me_2SO than with 33 mM Me_2SO as the substrate (Table I), consistent with a competition between the scavengers and Me_2SO for the generated ·OH. Urea, a relatively weak scavenger of ·OH ($K = 7 \times 10^5$ M^{-1} s^{-1}) (Anbar & Neta, 1967), was used as a negative control in these experiments. Urea did not inhibit formaldehyde production (Table I).

Effect of Iron–EDTA. Iron–EDTA is known to stimulate the generation of ·OH during the oxidation of xanthine by xanthine oxidase (McCord & Day, 1978; Halliwell, 1978a,b; Cederbaum et al., 1980). Under our conditions, the addition of iron–EDTA stimulated the production of formaldehyde from Me_2SO (Figure 3). In control experiments, formaldehyde was not produced in the presence of iron–EDTA when either xanthine or xanthine oxidase was omitted from the reaction mixture. The addition of 50 μM ammonium sulfate in 100 μM EDTA, instead of 50 μM ferrous ammonium sulfate, had no effect on the production of formaldehyde. The further addition of 10, 30, 50, or 100 μM EDTA alone, beyond the normal 100 μM which was usually present in the standard reaction mixture, had no effect on the oxidation of Me_2SO to formaldehyde. The addition of either catalase, superoxide dismutase, or benzoate inhibited the production of formaldehyde in the presence of 50 μM iron–EDTA.

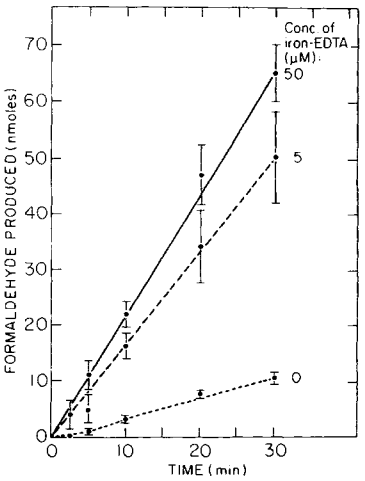

FIGURE 3: Effect of iron–EDTA on the production of formaldehyde from dimethyl sulfoxide during the oxidation of xanthine by xanthine oxidase. The standard reaction mixture is described in the legend to Figure 1. Iron–EDTA was added as a 1:2 mixture to achieve the final concentrations of iron which are indicated in the figure. The results represent the mean ± SEM of six or seven experiments.

Table II: Effect of DETAPAC on the Production of Formaldehyde from Dimethyl Sulfoxide[a]

reaction system	concn of DETAPAC (mM)	rate of formaldehyde production (nmol/30 min)	effect of DETAPAC (%)
xanthine oxidase (4)	0	9.00 ± 0.50	
	0.007	10.50 ± 0.57	+17
	0.067	7.77 ± 0.44	−14
	0.33	4.17 ± 0.73	−54[c]
	0.67	3.42 ± 0.12	−62[d]
	1.0	3.83 ± 0.33	−57[d]
	2.0	2.33 ± 0.15	−74[c]
ascorbate plus iron-EDTA (3)	0	782 ± 56	
	1.0	658 ± 23	−16
	2.0	587 ± 32	−25[b]

[a] Formaldehyde production from 33 mM dimethyl sulfoxide by the xanthine oxidase system or by the ascorbate system was carried out as described in the legends to Figures 1 or 5, respectively. Results are from three or four experiments. [b] $p < 0.05$. [c] $p < 0.005$. [d] $p < 0.001$.

Therefore, both H_2O_2 and $O_2^{-}\cdot$ radicals are involved in the increase of ·OH generation which occurs as a result of the addition of iron–EDTA.

Effect of DETAPAC and Treatment with Chelex-100. The production of ·OH in the xanthine oxidase system appears to occur as a result of an iron-catalyzed Haber-Weiss reaction (McCord & Day, 1978; Halliwell, 1978a,b). DETAPAC, an iron chelator, prevents the iron-catalyzed Haber-Weiss reaction (Halliwell, 1978a,b; Buettner & Oberley, 1978; Buettner et al., 1978). DETAPAC inhibited formaldehyde generation from Me_2SO during the oxidation of xanthine by xanthine oxidase (Table II). EDTA was present at a final concentration of 100 μM. In the absence of EDTA, formaldehyde was not detectable regardless of whether or not DETAPAC was added (data not shown). Therefore, DETAPAC cannot substitute for EDTA in this regard.

Iron may be present as a contaminant of the water or of the phosphate buffer in the reaction mixture. Chelex-100 resin was used to remove extraneous iron. A single passage of the solutions through a column of Chelex-100 resulted in a 50% decrease in the rate of formaldehyde production at all time points tested (data not shown).

Generation of Formaldehyde from Me_2SO during the Iron-Catalyzed Oxidation of Ascorbic Acid. Figure 4 illustrates the time course for the production of formaldehyde from Me_2SO during the iron–EDTA-catalyzed oxidation of ascorbic acid. The addition of either KTBA or mannitol inhibited the production of formaldehyde (Figure 4). This inhibition suggests that ·OH is responsible for the production of formaldehyde by this system. The complete inhibition produced by catalase (Figure 4) indicates that H_2O_2 is a precursor of ·OH in this system. In contrast, superoxide dismutase did not inhibit the production of formaldehyde (Figure 4). In fact, the addition of superoxide dismutase resulted in a slight stimulation of formaldehyde production. DETAPAC was a much less effective inhibitor of formaldehyde production from Me_2SO during the iron-catalyzed oxidation of ascorbic acid than during the xanthine oxidase reaction (Table II). This relative lack of inhibition may be related to the lack of inhibition by superoxide dismutase (see Discussion).

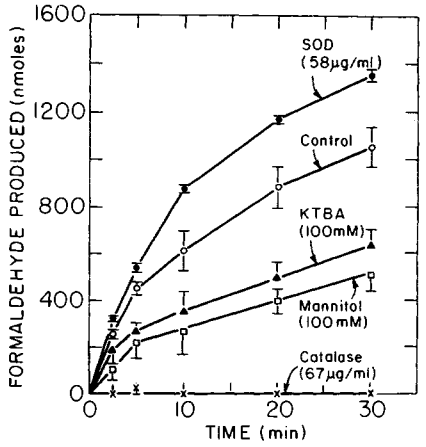

FIGURE 4: Generation of formaldehyde from dimethyl sulfoxide during the iron-catalyzed oxidation of ascorbic acid. The standard reaction mixture consisted of 100 mM potassium phosphate, pH 7.4, 0.1 mM EDTA, 0.167 mM iron–EDTA, 2 mM ascorbic acid, and 33 mM Me_2SO in a final volume of 3.0 mL. The final concentrations of the various additions to the basic reaction mixture are indicated in the figure. The results represent the mean ± SEM of three experiments.

Table III: Comparison between the Production of Formaldehyde and Methane from Dimethyl Sulfoxide[a]

reaction system	reaction time (min)	production of formaldehyde (nmol)	production of methane (nmol)
ascorbate–iron-EDTA	2.5	292 ± 33	1.60 ± 0.8
	5	380 ± 67	3.01 ± 1.2
	20	849 ± 117	3.87 ± 0.64
	30	1037 ± 140	3.93 ± 0.3
	40	1119 ± 119	3.50 ± 0.8
xanthine oxidase	20	5.2 ± 0.5	0.33 ± 0.3
	30	8.2 ± 0.5	1.09 ± 0.6
	40	10.2 ± 0.2	1.2 ± 0.7

[a] The oxidation of 33 mM dimethyl sulfoxide to either formaldehyde or methane by the xanthine oxidase system or by the ascorbate system was carried out as described under Experimental Procedures. Results are from three experiments.

Comparison between the Production of Formaldehyde and the Production of Methane from Me_2SO. Experiments were carried out to compare the amounts of formaldehyde and methane, two products of the interaction of Me_2SO with ·OH, produced under identical reaction conditions. Either the xanthine oxidase system or the ascorbate system was used to generate ·OH. The rate of formaldehyde production was considerably greater than the rate of methane production from Me_2SO by both model ·OH-generating systems (Table III). In fact, the measurements of methane production were carried

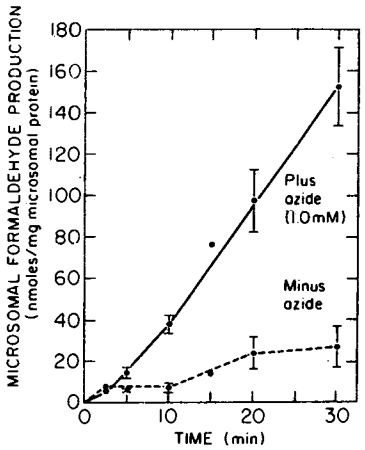

FIGURE 5: Generation of formaldehyde from dimethyl sulfoxide during NADPH-dependent electron transfer by rat liver microsomes. The standard reaction mixture consisted of 83 mM potassium phosphate buffer, pH 7.4, 10 mM potassium pyrophosphate, 10 mM $MgCl_2$, 0.3 mM $NADP^+$, 10 mM glucose 6-phosphate, 7 units of glucose-6-phosphate dehydrogenase, 3–5 mg of microsomal protein, and 33 mM Me_2SO in a final volume of 3.0 mL. Experiments were carried out in the absence or presence of 1 mM sodium azide. Results are from four experiments except for the 2.5- and 15-min time points where the results are from two experiments.

out near the limits of sensitivity of the gas chromatograph. These data indicate that formaldehyde, rather than methane, represents the predominant product of the interaction of Me_2SO with ·OH generated by these two model systems.

Production of Formaldehyde from Me_2SO by Rat Liver Microsomes. NADPH-dependent electron transfer by rat liver microsomes was used as a third ·OH-generating system (Cohen & Cederbaum, 1979, 1980). Rat liver microsomes are invariably contaminated with catalase during the preparation of the microsomal fraction. Therefore, formaldehyde production was measured in both the absence and presence of azide, an inhibitor of catalase activity. The microsomes produced formaldehyde from Me_2SO in a time-dependent manner (Figure 5). Azide, which prevents the decomposition of H_2O_2 by catalase, stimulated the production of formaldehyde. This stimulation is consistent with the presumption that H_2O_2 serves as a precursor of ·OH in this system in much the same manner as it did in the other ·OH-generating systems. Previously, the microsomal oxidations of other ·OH scavenging agents such as methional, KTBA, and 1-butanol were also shown to be enhanced in the presence of azide (Cohen & Cederbaum, 1980; Cederbaum et al., 1978).

Formaldehyde was not produced in the absence of either microsomes, the NADPH-generating system, or Me_2SO. Heat-denatured microsomal protein was inactive with respect to formaldehyde production. Hydrogen peroxide could not substitute for the NADPH-generating system. This suggests that NADPH-dependent electron transfer was required for the production of ·OH and therefore for the production of formaldehyde from Me_2SO by rat liver microsomes.

The effect of a competing ·OH scavenging agent on the production of formaldehyde from Me_2SO by the microsomal system was tested. Benzoate produced a dose-dependent inhibition of formaldehyde production from Me_2SO (Table IV). Benzoate had no effect on other microsomal functions such as the activities of aniline hydroxylase and aminopyrine demethylase (Cederbaum et al., 1978).

The rate of formaldehyde production from Me_2SO [about 4 nmol min^{-1} (mg of microsomal protein)$^{-1}$ (Figure 5, Table IV)] was of the same order of magnitude as the rate of ethylene generation from KTBA [about 1.5–2 nmol min^{-1} mg of microsomal protein)$^{-1}$ (Cohen & Cederbaum, 1980)]. These rates should be contrasted with the rate of methane production from Me_2SO [about 0.16 nmol min^{-1} (mg of microsomal protein)$^{-1}$ (Cohen & Cederbaum, 1980)]. These results indicate that formaldehyde, rather than methane, represents a main product of the metabolism of Me_2SO by the microsomal system.

Table IV: Effect of Benzoate on the Production of Formaldehyde from Dimethyl Sulfoxide by Rat Liver Microsomes[a]

concn of benzoate (mM)	rate of formaldehyde production [nmol (30 min) (mg of microsomal protein)$^{-1}$]	effect of benzoate (%)
0	146.8	
10	121.4	−17
25	87.6	−40
50	65.5	−55
100	55.5	−62

[a] NADPH-dependent oxidation of 33 mM dimethyl sulfoxide was carried out as described in the legend to Figure 5. Results are from two experiments carried out in duplicate.

Discussion

The ability of Me_2SO to be oxidized to formaldehyde was characterized in three different systems which are known to produce oxygen radicals. These systems are (1) the coupled oxidation of xanthine by xanthine oxidase, (2) the iron-catalyzed oxidation of ascorbate, and (3) NADPH-dependent microsomal electron transfer. Previously, the oxidation of Me_2SO to methane gas was utilized to detect a reactive oxygen species which was generated by several biological systems (Brownlee et al., 1977; Repine et al., 1979; Cohen & Cederbaum, 1979, 1980). In all three reaction systems which were tested, the oxidation of Me_2SO was found to yield formaldehyde, rather than methane, as a predominant product. The oxidation of Me_2SO to formaldehyde appears to be mediated by ·OH or by a species with the oxidizing power of ·OH since (a) formaldehyde production is inhibited by competing ·OH scavengers, (b) formaldehyde production is augmented by the addition of iron–EDTA, and (c) conditions known to alter the rate of ·OH production also alter the rate of formaldehyde production. For example, in the xanthine oxidase system, the production of ·OH is prevented by the addition of either superoxide dismutase or catalase (Beauchamp & Fridovich, 1970), or by DETAPAC (Buettner & Oberley, 1978; Buettner et al., 1978). These additions also inhibit formaldehyde production by the xanthine oxidase system. On the basis of the effects of catalase (xanthine oxidase and ascorbate systems) and azide (microsomal system) on the production of formaldehyde from Me_2SO, it appears that H_2O_2 serves as a precursor of ·OH in all three systems. Taken as a whole, these observations indicate that the production of formaldehyde from Me_2SO is due to the interaction of Me_2SO with a "·OH-like" species. In view of these considerations, the production of formaldehyde from Me_2SO may represent a convenient tool by which to detect the presence of ·OH in biological systems.

The production of ·OH by the xanthine oxidase system appears to be mediated by iron that is present in the solutions, especially the phosphate buffer. The inhibition of formaldehyde production by DETAPAC or by Chelex treatment is consistent with this possibility. The stimulation of formaldehyde production by iron–EDTA involves ·OH since catalase, superoxide dismutase, and benzoate all prevent the stimulation. Iron–EDTA has been shown to stimulate the production of ·OH by the xanthine oxidase reaction (McCord

& Day, 1978; Halliwell, 1978a,b; Cederbaum et al., 1980). Iron also catalyzes the production of formaldehyde during the metabolism of Me_2SO by the ascorbate system since less formaldehyde is produced from Me_2SO when iron–EDTA is omitted from the reaction mixture (data not shown).

Iron is rapidly oxidized by oxygen to the ferric state (half-life less than 15 s) in the presence of EDTA and phosphate buffer (Cohen & Sinet, 1980). Therefore, reduction of Fe^{3+} back to Fe^{2+} is required when iron acts to catalyze the production of ·OH. In the xanthine oxidase system, $O_2^{-}\cdot$ reduces the ferric iron to the ferrous state.

$$Fe^{3+}\text{–EDTA} + O_2^{-}\cdot \rightarrow Fe^{2+}\text{–EDTA} + O_2 \quad (2)$$

$$Fe^{2+}\text{–EDTA} + H_2O_2 \rightarrow Fe^{3+}\text{–EDTA} + \cdot OH + OH^- \quad (3)$$

$$\text{net:} \quad O_2^{-}\cdot + H_2O_2 \rightarrow \cdot OH + OH^{-}\cdot + O_2 \quad (4)$$

In the ascorbate system, ferric iron is reduced by ascorbate to the ferrous state (Winterbourn, 1979, Fee, 1980), analogous to the role of $O_2^{-}\cdot$ in the xanthine oxidase system. H_2O_2 is produced during the autoxidation of Fe^{2+} or ascorbate (ascorbate system) or during the xanthine oxidase reaction. A Fenton reaction between the H_2O_2 and Fe^{2+} results in the production of ·OH.

In light of the above discussion, superoxide dismutase should inhibit formaldehyde production in the xanthine oxidase system but need not inhibit in the ascorbate system. The different sensitivities of the xanthine oxidase and the ascorbate systems to inhibition by DETAPAC (Table II) may reflect the greater reactivity of the iron–DETAPAC complex with ascorbate than with $O_2^{-}\cdot$. It has been suggested that DETAPAC inhibits the iron-catalyzed Haber-Weiss reaction rather than the Fenton-type reaction (eq 3) (Cohen et al., 1981) as a result of its ability to interfere with superoxide-dependent reduction of iron (eq 2). It is also possible that the different rates of formaldehye production by the ascorbate system as compared to the xanthine oxidase system may contribute to the different sensitivities of the two systems to superoxide dismutase and DETAPAC. The inability of H_2O_2 alone, in the absence of either xanthine or xanthine oxidase, to promote formaldehyde production (or oxidation of methional; Beauchamp & Fridovich, 1970) is probably due to the lack of an agent to reduce Fe^{3+} to Fe^{2+}. If superoxide dismutase was added to the xanthine oxidase system after 10 min of incubation, then further production of formaldehyde from Me_2SO was prevented (data not shown). It appears that continuous generation of $O_2^{-}\cdot$ is required for the continuous generation of H_2O_2 and for the continuous reduction of ferric to ferrous iron.

Previous studies have demonstrated that in the presence of NADPH, rat liver microsomes oxidize several ·OH scavengers to their respective products (Cohen & Cederbaum, 1979, 1980; Cederbaum et al., 1977, 1978, 1979, 1981). When Me_2SO was utilized, a relatively small yield of methane was obtained (Cohen & Cederbaum, 1979, 1980). It is now clear that formaldehyde, rather than methane, is the major product generated from Me_2SO by microsomes during electron transfer. Stimulation by azide, which permits H_2O_2 to accumulate, and inhibition by benzoate, a competitive scavenger, suggest a role for a ·OH-like species in the production of formaldehyde from Me_2SO. The ability of the microsomes to metabolize Me_2SO thus appears to be similar to the ability of the microsomes to metabolize other ·OH scavengers such as KTBA, methional, and alcohols. Further studies with microsomes are in progress.

Me_2SO interacts with ·OH radicals to give rise to methyl radicals ($\cdot CH_3$) (Dixon et al., 1964; Ashwood-Smith, 1975). The $\cdot CH_3$ radical may abstract a hydrogen to form methane, or it may dimerize to ethane (Cohen & Cederbaum, 1980). Alternatively, the methyl radical may react with molecular oxygen to produce the methylperoxy radical ($CH_3OO\cdot$). The decomposition of the methylperoxy radical via a Russell-type mechanism (Russell, 1957) can result in the production of formaldehyde.

$$2CH_3\text{–}O\text{–}O\cdot \rightarrow CH_2O + CH_3OH + O_2 \quad (5)$$

The hydroxyl radical is a powerful oxidizing agent, and by virtue of this characteristic, it is considered to be highly toxic in biological systems [e.g., see Myers (1973), Fong et al. (1973), Salin & McCord (1975), Cohen & Heikkila (1974), and Cohen (1978)]. It has been suggested that ·OH may play a role in the microsomal ethanol oxidizing system (Cohen & Cederbaum, 1979, 1980; Cederbaum et al., 1977–1979). Therefore, the ability to detect ·OH is important. The production of formaldehyde from Me_2SO appears to represent a new, sensitive, and convenient technique for the detection of ·OH.

There is currently much interest in the therapeutic properties of Me_2SO. The antiinflammatory activity of Me_2SO has been ascribed to the ability of this compound to scavenge ·OH (Ashwood-Smith, 1975). Can formaldehyde be a product of the in vivo metabolism of Me_2SO? If this is the case, then the production of formaldehyde in vivo as a result of therapeutic administration of Me_2SO to human subjects may have biological consequences. Therefore, Me_2SO should not be considered to be an unreactive component in biological systems.

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Distribution of Cytokinin-Active Nucleosides in Isoaccepting Transfer Ribonucleic Acids from *Agrobacterium tumefaciens*†

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ABSTRACT: The cytokinin-active isoprenoid nucleosides of *Agrobacterium tumefaciens* transfer ribonucleic acid were identified by high-pressure liquid chromatography, permethylation, and mass spectroscopy. Besides the expected 6-[(3-methylbut-2-enyl)amino]-9-(β-D-ribofuranosyl)purine (i^6A) and its 2-methylthio derivative (ms^2i^6A), substantial amounts of *cis*- and *trans*-ribosylzeatin (io^6A) and *cis*-2-(methylthio)ribosylzeatin ($c\text{-}ms^2io^6A$) were present. These hydroxylated side chain derivatives are normally characteristic of plant tRNA. Fractionation of the total bacterial tRNA on BD-cellulose and RPC-5 allowed isolation of purified isoaccepting species whose cytokinin nucleoside contents were then determined. Distribution of the isoprenoid nucleosides among the U-group tRNA species was not uniform. *cis*-Ribosylzeatin was found almost exclusively in one $tRNA^{Ser}$ while ms^2io^6A was found predominantly in $tRNA^{Phe}$, $tRNA^{Ser}$, and $tRNA^{Tyr}$. Not all cytokinin-active species were found in every member of the U-group tRNAs. The only species present in $tRNA^{Trp}$ was i^6A; it contained no zeatin derivatives. The hydroxylation and methylthiolation processes appear to be highly specific and dependent upon tRNA structure or sequence.

Four chemically distinct cytokinin-active nucleosides have been found to occupy the position adjacent to the 3′ side of the anticodon triplet in those transfer ribonucleic acid (tRNA)[1] species which recognize codons whose initial base is uracil. They differ in the extent to which they are chemically modified. Broadly speaking, the nature and extent of modification (Skoog & Armstrong, 1970; Leonard, 1974) depend upon whether the organism is an animal (in which case its tRNA contains i^6A), a bacterium (ms^2i^6A and i^6A), or a plant (io^6A and ms^2io^6A). An exception exists to this generalization, however, in that some plant pathogenic bacteria, notably *Agrobacterium tumefaciens* (Chapman et al., 1976; Cherayil & Lipsett, 1977) and *Corynebacterium fascians* (Einset & Skoog, 1977; Cherayil & Lipsett, 1977), have been shown to contain ribosylzeatin and ms^2io^6A in their tRNA. In light of the possible involvement of cytokinins in tumorigenesis initiated by *A. tumefaciens* it was of interest to determine the relative proportions of cytokinins present in bacterial tRNA and to determine the cytokinin distribution among the U-group species. We report here the identification of io^6A, ms^2io^6A, i^6A, and ms^2i^6A in tRNA from *A. tumefaciens* strain C58 and their distribution among the isoaccepting tRNA subspecies. The distribution of ribosylzeatin (io^6A) and the other species

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[1] Abbreviations used: i^6A, 6-[(3-methylbut-2-enyl)amino]-9-(β-D-ribofuranosyl)purine; ms^2i^6A, 2-(methylthio)-6-[(3-methylbut-2-enyl)-amino)]-9-(β-D-ribofuranosyl)purine; *c*-io^6A and *t*-io^6A, *cis*- and *trans*-ribosylzeatin; ms^2io^6A, 2-(methylthio)ribosylzeatin; DEAE, diethylaminoethyl; BD-cellulose, benzoylated DEAE-cellulose; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; GLC–MS, gas–liquid chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; tRNA, transfer ribonucleic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; ATP, adenosine 5′-triphosphate; TLC, thin-layer chromatography.

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