[CANCER RESEARCH 40, 4446–4450, December 1980]
0008-5472/80/0040-0000$02.00

Production of a Highly Reactive Alkylating Agent from the Organospecific Carcinogen Methylazoxymethanol by Alcohol Dehydrogenase[1]

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ABSTRACT

The carcinogen methylazoxymethanol acetate induces a high incidence of tumors in select tissues of the rat. It has been shown that methylazoxymethanol is a substrate for the enzyme horse liver alcohol dehydrogenase, and it has been suggested that the organotropic effects are related to the metabolism of methylazoxymethanol within the sensitive tissues by β-nicotinamide adenine dinucleotide (NAD^+)- or β-nicotinamide adenine dinucleotide phosphate-dependent dehydrogenase reactions. The fate of the product of the enzyme reaction, presumably the aldehydic form of methylazoxymethanol, and its mechanism of reaction with cellular macromolecules have heretofore not been investigated. We designed an assay to monitor the formation of carbonium ions using sodium-[^{14}C]acetate as the trapping agent. This nucleophile was added to an incubation mixture containing methylazoxymethanol, NAD^+, and alcohol dehydrogenase. The amount of methylazoxymethanol converted was determined by measuring the amount of reduced β-nicotinamide adenine dinucleotide formed. Significantly more methyl [^{14}C]acetate formed when methylazoxymethanol was incubated with enzyme and NAD^+ as compared to when it was incubated alone. Analysis of the data comparing the amounts of reduced β-nicotinamide adenine dinucleotide and of methyl[^{14}C]acetate formed indicates that the aldehydic form of methylazoxymethanol is a very unstable compound which rapidly releases carbonium ions. Experiments with *N*-methyl-*N*-nitrosourea, a methylating agent not requiring metabolic activation, showed no increase in the amount of methyl [^{14}C]acetate obtained when enzyme and NAD^+ were added as opposed to when enzyme was omitted. The significance of these findings is discussed in relation to the organotropism exhibited by methylazoxymethanol.

INTRODUCTION

MAM[2] acetate is a potent carcinogen that induces acute and chronic effects in a few tissues of the rat, mainly duodenum, colon, liver, and kidney (3, 8–10). Since the alkylating moiety derived from MAM (Chart 1) can occur spontaneously (4), it was unclear why only a few tissues are affected by this carcinogen. The selectivity exhibited by other carcinogens in different species, organs, and sexes is believed to be due, in part, to metabolism of the carcinogen by the tissue sensitive to its effects. Schoental (5) had postulated that MAM might be metabolized by ADH to MAMAL (Chart 1) which could then act as a bifunctional alkylating agent. Grab and Zedeck (2) have shown that MAM is a substrate for the enzyme horse liver ADH. Moreover, they found that the 169,000 × *g* supernatant fractions from those tissues which are sensitive to the effects of MAM could, unlike those tissues resistant to the carcinogen, also utilize MAM as a substrate in NAD^+- or $NADP^+$-dependent reactions. Pyrazole, an inhibitor of ADH (1), inhibited the toxic (2) and carcinogenic (11) effects of MAM, giving support to the role of this enzyme in the biological effects of this carcinogen. Zedeck *et al.* (7) reported that disulfiram, an inhibitor of aldehyde dehydrogenase, potentiates the biological effects of MAM. Thus, it appears that MAMAL is the activated form of MAM. It was now of interest to determine whether the product of the enzyme reaction, presumably MAMAL, can also act as an alkylating agent and, if so, to compare its rate of reactivity with the parent compound.

MATERIALS AND METHODS

Chemicals. Quaternary aminoethyl-Sephadex A25 was purchased from Pharmacia Fine Chemicals, Piscataway, N. J.; sodium [^{14}C]acetate (56.5 Ci/mmol) was from New England Nuclear, Boston, Mass.; BSA, horse liver ADH, and NAD^+ were from Sigma Chemical Co., St. Louis, Mo.; pyrazole was from Eastman Chemicals, Rochester, N. Y.; and MAM acetate was from Schwarz/Mann, Orangeburg, N. Y. Free MAM was prepared as described by Grab and Zedeck (2) and stored frozen in small aliquots.

Resin Preparation and Sodium [^{14}C]Acetate Purification. Fifty g of quaternary aminoethyl-Sephadex A25, a strong anion exchanger, were converted to a resin having low selectivity by washing it 6 times with 500 ml of 1 M sodium acetate to exchange the chloride counterion to acetate. The resin was stirred gently for 15 to 30 min during each washing and finally washed extensively with water to remove the salts.

The sodium [^{14}C]acetate was purified by loading approximately 20 μCi through a Chromaflex column (Kontes, Vineland, N. J.) filled with quaternary aminoethyl-Sephadex A25 resin (0.6 × 3.0 cm). The resin was washed with water, and the eluant was collected until no more radioactivity could be detected. The resin was next eluted with 1 M sodium acetate. Fractions of 0.2 ml were collected and assayed for radioactivity. The peak of radioactivity was collected and diluted with glass-distilled water to give a specific activity of 0.74 μCi/5 μmol/20 μl.

Alkylation Studies. All of the incubations were done at 37° in a total volume of 1 ml for 2 hr at pH 9.0 using 250 mM glycine buffer or at pH 7.0 using 250 mM phosphate buffer. The incubation vessels were capped, covered with parafilm, and immersed in a 37° water bath up to the level of liquid in the vessel. Rubber tubing was wrapped around the vessel

[1] Supported by USPHS Grants CA 08748 from the National Cancer Institute and CA 15637 from the National Cancer Institute through the National Large Bowel Cancer Project and by the Elsa U. Pardee Foundation.

[2] The abbreviations used are: MAM, methylazoxymethanol; ADH, alcohol dehydrogenase; MAMAL, methylazoxymethanal; MNU, *N*-nitroso-*N*-methylurea; BSA, bovine serum albumin.

Received March 14, 1980; accepted September 10, 1980.

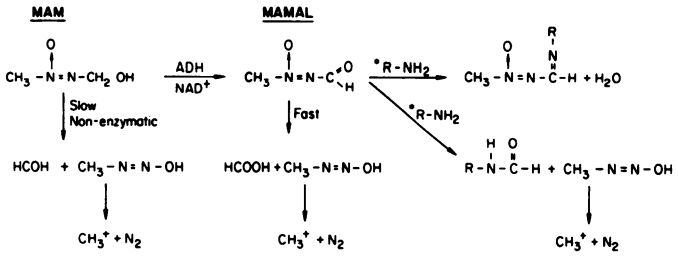

Chart 1. Pathways illustrating MAM decomposition, proposed metabolism, and subsequent interactions. *R—NH₂* represents either DNA, RNA, or protein.

above the level of liquid, and cold water was circulated through the tubing to condense any vapors.

For experiments at pH 9.0 and at pH 7.0, the amounts of NAD^+, pyrazole, MNU, BSA, and sodium [^{14}C]acetate were 50 μmol, 5 μmol, 10 μmol, 200 μg, and 0.74 μCi, respectively. The amounts of ADH used for the experiments at pH 9.0 and pH 7.0 were 200 μg and 1.6 mg, respectively. MAM was added to give varying concentrations. NAD^+ and pyrazole were dissolved in glass-distilled water, BSA, or ADH in 0.1 M phosphate buffer, pH 7.5 (containing 0.015% BSA to stabilize the enzyme), and MNU was added in 50 μl dried distilled acetone. NAD^+, ADH, and MNU solutions were prepared daily, and replicate experiments were performed on different days.

After the 2-hr incubation period, the tube was cooled in ice for 10 min. The sample was then loaded onto a 1.6-cm-diameter column containing 8 to 8.5 ml of swollen QAE Sephadex-A25 resin and eluted with 20 ml of distilled water to collect the unadsorbed methyl [^{14}C]acetate. This was followed by 1 M sodium formate to elute the bound [^{14}C]acetate. Sodium formate was chosen because, unlike other salts, it allowed the formation of one phase when mixed with the Hydrofluor scintillation fluid. The flow rate was 0.2 to 0.3 ml/min, and fractions of 1.4 to 1.6 ml were collected and then transferred by pipet into scintillation vials. Each collection tube was rinsed with two 200-μl portions of water which were also added to the vials. The fractions were mixed with 18 ml Hydrofluor, and the radioactivity was measured; the counting efficiency was 80%.

To determine the molarity of the free MAM following its preparation from MAM acetate, an aliquot of the solution was diluted with 5 ml water to give a concentration of 1 A_{215}/ml. MAM acetate, unlike free MAM, is readily extractable from water by ether. This solution was then extracted 15 times with 5 ml of ether saturated with water to extract any remaining MAM acetate, and the A_{215} of the water layer containing the free MAM was measured after each 3 extractions. By extrapolating, we found that, in preparation of the free MAM, 86% of the MAM acetate had been hydrolyzed, resulting in a final concentration of 36 mM free MAM.

Spectrophotometric Studies. The amount of MAM, alcohol, and MNU metabolized by ADH was determined by measuring the production of NADH spectrophotometrically at 340 nm. The reaction mixtures and conditions used were the same as described above except that sodium [^{14}C]acetate was not added. At various intervals of incubation, 20 μl were withdrawn from the incubation mixture and diluted to a total volume of 1 ml for determination of the amount of NADH. The half-lives of MAM at different pH's were determined by monitoring the absorbance at 215 nm at 15-min intervals using an automatic cell programmer.

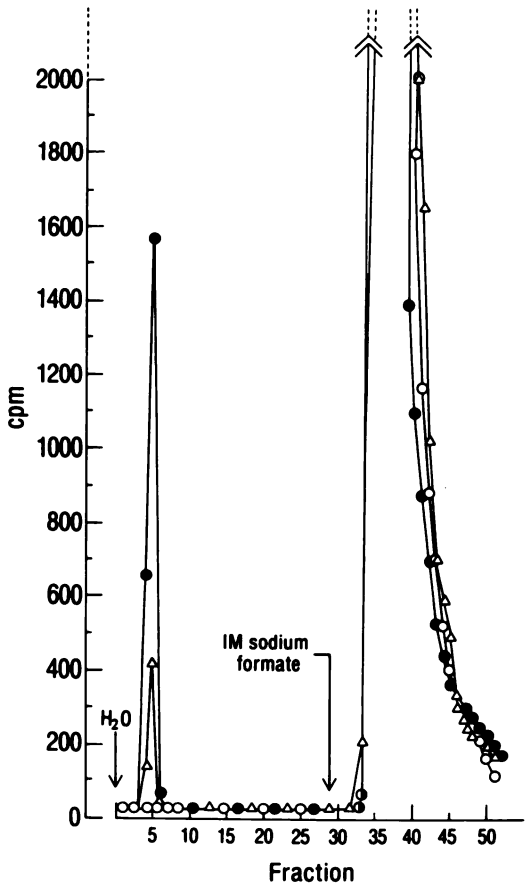

Chart 2. Formation of methyl [^{14}C]acetate and its separation from sodium [^{14}C]acetate. Sodium [^{14}C]acetate (○), sodium [^{14}C]acetate and MAM (△), or sodium [^{14}C]acetate with MAM and ADH (●) was incubated, and the mixtures were analyzed as described under "Materials and Methods"; the total cpm eluted in Fractions 34 to 39 were 1,292,303 (○); 1,255,093 (△); and 1,262,256 (●). *Arrows*, time when either H_2O or 1 M sodium formate was used as eluant.

Identification of Alkylated Product. A 4-ml reaction mixture containing NAD^+ and enzyme in concentrations described above with 17.2 mM MAM was incubated for 2 hr at 37°. The mixture was extracted 6 times with 4-ml portions of cyclohexane. To the combined fractions were added 25 ml of distilled methyl acetate, and the mixture was distilled. The distillate was collected in 5-ml portions directly into counting vials kept cool with dry ice, and the radioactivity of each fraction was determined.

RESULTS

Methylation of Sodium [^{14}C]Acetate. Sodium acetate was chosen as the nucleophile for these studies because it can be easily separated from both the methylated compound (Chart 2) and the MAM, and it is available double labeled with ^{14}C in high specific activity, allowing for the detection of minute quantities of the methylated derivative.[3] Since MAM eluted with the void volume while the sodium acetate was retained, no further

[3] The sensitivity of this assay is illustrated by the fact that the 763 dpm methyl [^{14}C]acetate obtained 2 hr after spontaneous decomposition of 8.6 mM MAM represents 2.3 nmol methyl acetate. This is equivalent to 0.2% of the carbonium ion released, the remainder probably interacting with water and other nucleophiles present in the reaction mixture.

alkylation of sodium acetate by unchanged MAM could occur on the column itself.

Experiments at pH 9.0. To determine whether the rate of carbonium ion released from MAMAL was equal to or greater than that released from MAM, it was desired to obtain as much MAMAL as possible within a reasonable time and under conditions where there was not much decomposition of MAM. The data in Table 1 show the half-life data of MAM at various pH's. At pH 9, used routinely for assay of ADH activity, MAM was rather stable, and, in a 2-hr period, only 13% of MAM spontaneously decomposed. For enzymatic studies, large amounts of enzyme (200 μg/ml) were used with a saturating amount of NAD^+ (50 mM); these amounts did not interfere with the chromatography. Increasing amounts of MAM were used to obtain as much NADH as possible and yet not saturate the enzyme. It was determined that the V_{max} was 72 nmol NADH per min, and the K_m was 2.5 mM. Under similar conditions, the V_{max} and K_m for alcohol was 313 nmol NADH per min and 0.21 mM, respectively. We used 1.7-, 3.4-, and 6.8-fold the K_m which theoretically represents 63, 78, and 87% of the V_{max}. Using 8.6 mM MAM, it was found that about 30% of the MAM was metabolized as measured by NADH formation. The difference between the spontaneous and enzymatic conversion appeared large enough to detect a difference in any methyl acetate formation that might occur as a result of enzymatic metabolism.

The data in Table 2, Groups 1 to 3, indicate that the amount of methyl [^{14}C]acetate formed following the spontaneous decomposition of MAM was proportional to the concentration of MAM used. The data also indicate that the experimental findings were very reproducible. The presence of NAD^+ did not influence the amount of methyl [^{14}C]acetate formed; similar results were obtained without NAD^+ (Group 4) as with NAD^+ (Group 2). Also, enzyme alone (Group 5) gave results similar to those obtained in Groups 2 and 4. Incubation of MAM in the presence of NAD^+ and enzyme (Groups 6 to 8) resulted in the formation of amounts of methyl [^{14}C]acetate which were significantly greater than those obtained without enzyme. The addition of pyrazole (5 mM, Group 9) to the enzyme mixture resulted in alkylation of sodium [^{14}C]acetate similar to that occurring spontaneously (Group 2).

To further verify that the additional alkylation obtained in the presence of enzyme in Groups 6 to 8 was indeed due to the metabolism of MAM, we reacted MNU with sodium [^{14}C]acetate under the same conditions used for MAM. Prior spectrophotometric analysis had indicated that no NADH was formed when MNU was incubated with enzyme and NAD^+. The data are shown in Table 3. MNU, unlike MAM, decomposes rapidly under the conditions utilized, and the 10-fold higher alkylating activity observed reflects this difference. Unlike what was observed with MAM, the addition of enzyme did not increase the amount of methyl [^{14}C]acetate formed. In fact, there was a decrease. Since it was possible that the enzyme was an effective competitor for the carbonium ion, we incubated MNU with and without BSA, a nonenzymatic protein. The data indicated that the addition of BSA also caused a decrease in methyl [^{14}C]acetate, suggesting that nonspecific alkylation of protein does occur.

Table 1

Half-lives of MAM at different pH at 37°

pH	Buffers used	Half-life (hr)
10	0.1 M sodium borate	2.8
9.5	0.1 M sodium borate	5.9
9	0.1 M sodium borate	9.8
8	0.1 M sodium phosphate	11.4
7	0.1 M sodium phosphate	11.6
6	0.1 M sodium phosphate	11.9
4	0.1 M sodium phosphate	12.3

Table 2

Formation of methyl [^{14}C]acetate from sodium [^{14}C]acetate and the alkylating moiety released from MAM following either its spontaneous or enzyme-mediated decomposition at pH 9.0

Group	MAM (mM)	NAD^+	ADH	Pyrazole	dpm	Av. dpm
1	4.3	+	−	−	414 428	421
2	8.6	+	−	−	716 753 759 825	763
3	17.2	+	−	−	1431 1479 1518	1476
4	8.6	−	−	−	864 909	886
5	8.6	−	+	−	825 829	827
6	4.3	+	+	−	1193 1339	1266
7	8.6	+	+	−	2390 2680 2849	2640
8	17.2	+	+	−	3561 3771	3666
9	8.6	+	+	+	598 664	631

Table 3

Formation of methyl [^{14}C]acetate from sodium [^{14}C]acetate and the alkylating moiety released from MNU at pH 9.0

Group	NAD^+	ADH	BSA	dpm	Av. dpm
1	+	+	−	6009 6193 6194 6221	6154
2	+	−	−	7205 7826 7931 7948 8076 8413	7899
3	+	−	+	7040 7350	7195

Experiments at pH 7.0. Studies at pH 7.0 allow for the study of the stability of MAMAL at physiological pH. Because at pH 7.0 ADH is not very active, different conditions had to be set up that would allow for increasing amounts of NADH to be formed and measured. Thus, it was necessary to use 1.6 mg of enzyme per reaction vessel and concentrations of MAM lower than those used at pH 9.0.

The data obtained at pH 7.0 were similar qualitatively to those at pH 9.0 and indicate that MAMAL is very unstable. The analysis of the alkylation activity of MAM at both pH's in the absence and presence of ADH is shown in Table 4. The lower specific activity of the methyl [^{14}C]acetate per μmol carbonium ion obtained in the experiments performed at pH 7.0 might be due to the presence of the phosphate ion which probably is a better nucleophile than glycine and thus competes more effectively with acetate for the carbonium ion.

Nature of Alkylation Product. Though unlikely, it was pos-

Table 4
Comparison of the alkylating activity of MAM following spontaneous and enzymatic reactions

		Spontaneous decomposition			Enzyme reaction + spontaneous decomposition			
							dpm methyl [^{14}C]acetate formed	
pH	MAM (μM)	μmol carbonium ion (based on half-life)	dpm methyl [^{14}C]acetate	dpm/μmol carbonium	μmol NADH produced	Total μmol carbonium ion[a]	Theoretical[b]	Actual
9.0	4.3	0.56	421	752	2.1	2.66	1854	1266
	8.6	1.12	763	681	2.6	3.72	2593	2640
	17.2	2.24	1476	659	3.2	5.44	3792	3666
7.0	3.6	0.59	348	589	0.53	1.12	630	940
	5.4	0.89	473	531	0.81	1.70	956	1213
	12.9	1.41	799	566	0.98	2.39	1345	1803

[a] Assuming that 1 μmol NADH is equivalent to the generation of 1 μmol carbonium ion.
[b] Based on an average dpm methyl [^{14}C]acetate per μmol carbonium ion of 697 and of 563 at pH 9.0 and pH 7.0, respectively.

sible that the material appearing in the void volume was not methyl [^{14}C]acetate but rather a hemiacetal formed from the addition of acetate ion to MAMAL. To more precisely determine the nature of the product formed, 4 ml of 17.2 mM MAM were incubated with enzyme and NAD^+ at pH 9.0 for 2 hr. The incubation mixture was then chromatographed, and the activity eluting in Fractions 4 to 6 (Chart 2) was extracted with cyclohexane. After 6 extractions, the cyclohexane had removed 94% of the radioactivity. To the cyclohexane fractions, unlabeled methyl acetate was added, and the solution was fractionally distilled. Chart 3 shows the distillation of this mixture. All the counts distilled with the fractions that boiled at 55°. Though the boiling point of methyl acetate is 56°, in our apparatus, pure methyl acetate distilled over at 55°. Since the boiling point of MAM acetate is 191°, it is highly unlikely that the hemiacetal derivative would have a boiling point at 55°. The data indicated that the radioactivity measured in the void volume peak was due to methyl [^{14}C]acetate.

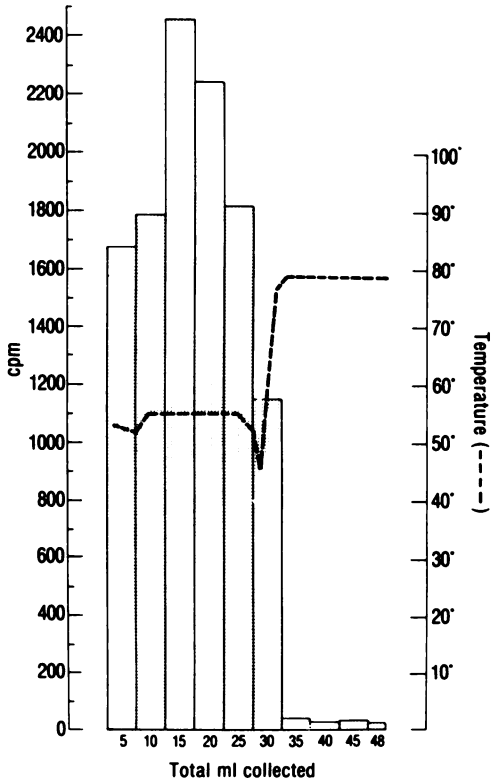

Chart 3. Distillation of the cyclohexane extract. Following incubation of MAM with ADH, NAD^+, and sodium [^{14}C]acetate, the mixture was loaded onto a quaternary aminoethyl-Sephadex column and eluted with water. Fractions 4 to 6 were pooled and extracted with cyclohexane. Unlabeled methyl acetate was added to the extract, and the mixture, containing 11,100 cpm, was distilled as described under "Materials and Methods."

DISCUSSION

MAM selectively induces a high incidence of tumors in rat liver and colon after a single treatment (8, 9). It has been shown that the 169,000 × *g* supernatant fraction isolated from different tissues varied in its ability to metabolize MAM in a NAD^+- or $NADP^+$-dependent reaction, and a correlation was found between those organs able to metabolize MAM and those organs in which MAM caused biochemical and pathological effects (2). It was then suggested that, although MAM spontaneously produced methyl carbonium ion, this process by itself was not sufficient to initiate carcinogenesis and that prior metabolic conversion of MAM to MAMAL was required (2). It was of interest to determine whether MAMAL is reactive in its natural state or decomposes to an alkylating moiety.

The calculations and data given in Tables 2 and 4 indicate that the half-life of MAMAL is exceptionally short. The fact that the addition of enzyme results in an increase in methyl [^{14}C]-acetate relative to that obtained with MAM only (Table 2) indicates that the product of the enzyme reaction decomposes to an alkylating agent faster than does the parent compound. If the half-life of the new product was the same as that for MAM, then the amount of methyl [^{14}C]acetate would have been equal to that obtained from MAM alone. The data in Table 4 show the amount of NADH produced during the enzyme reaction. Assuming that an equivalent amount of carbonium ions arises instantly from the new product, the total μmol of carbonium ion formed would then be equal to those arising spontaneously and those produced by enzyme reaction. Based on the finding that, at pH 9.0, on the average, 697 dpm methyl [^{14}C]acetate are produced per μmol carbonium ion (Table 4), the total dpm that could be expected from the spontaneous and enzyme-derived carbonium ion were calculated. Since the amount of methyl [^{14}C]acetate obtained approximates that amount expected from both these processes, it is reasonable

to conclude that MAMAL does decompose rapidly to carbonium ions.[4]

MNU is a compound the biological activity of which does not appear to be dependent on enzymatic activation, and it decomposes spontaneously to liberate carbonium ions. The addition of ADH to MNU does not result in any more methyl [^{14}C]acetate being produced than without enzyme. In fact, there is a decrease, possibly due to the carbonium ion interacting with the enzyme protein. The addition of BSA also results in lesser amounts of methyl [^{14}C]acetate as compared to incubation of MNU alone.

While the theoretical and the actual amounts of methyl [^{14}C]acetate obtained from MAM agree closely, they are not exact. The discrepancy might be due to several factors. (*a*) Since a significant amount of MAM is removed from the incubation mixture by enzymatic oxidation, especially in the experiment at pH 9.0 using only 4.3 μmol MAM (49% MAM converted based on NADH production), the amount of carbonium ion that forms from the spontaneous decomposition of MAM, as calculated from the half-life data, should be less than the amount obtained when no enzyme was present. (*b*) Since the data obtained with MNU indicate that a fraction of the carbonium ions generated interact with the enzyme protein, it can be assumed that, in the reaction of MAM, the ADH also results in lesser amounts of measurable methyl [^{14}C]acetate. (*c*) We have measured the stability of a constant concentration of NADH in the presence of NAD^+ and enzyme as in the experimental condition, but without substrate, at 37° at pH 7.0 and pH 9.0 at 340 nm. NADH slowly decomposes at pH 7.0, and the absorbance decreases about 30% over the 2-hr period. At pH 9.0, the absorbance increases about 25%. Since in the actual experiments NADH is constantly being formed while some is being altered, it is not possible to determine accurately the exact percentage of change and to correct the data accordingly. Hence, we have not made any corrections. Regardless of all these factors, the data clearly indicate that MAMAL is unstable, even at physiological pH.

The schema in Chart 1 shows the possible reactions leading to production of carbonium ions from MAM. Methylcarbonium ions may arise not only spontaneously from MAM but also following the interaction of MAMAL with nucleophiles, such as water or the amino group of macromolecules. This results in cleavage of the C—N bond and transfer of a formyl group to the nucleophile. The present studies could not determine such reactions with macromolecules or whether MAMAL acts as a cross-linking agent as proposed by Schoental (5), and these reactions are now being investigated to determine which of the pathways is responsible for the biological effects of MAM. While it is not probable that MAMAL will react with macromolecules as depicted because of its instability, the reactions are chemically possible. We cannot rule out the possibility of these reactions occurring under biological conditions in the presence of many macromolecules.

Previously, we reported (12) that pyrazole could prevent the MAM acetate-induced colon tumors and inhibition of RNA and protein synthesis but could not prevent inhibition of DNA synthesis. We suggested that induction of tumors and depression of RNA and protein synthesis were due to MAMAL while the carbonium ions inhibited DNA synthesis. In view of the current findings, we must now consider whether tumorigenesis and inhibition of all macromolecule synthesis are induced by carbonium ions with DNA synthesis being significantly more sensitive than the other biological processes. The data suggest that the sensitivity to MAM exhibited by only certain organs is related to their ability to metabolize MAM to MAMAL by NAD^+- or $NADP^+$-dependent dehydrogenase reactions resulting in the production of carbonium ions at levels significantly greater than would be expected to be found in those organs in which MAM decomposes only spontaneously. In preliminary experiments, we observed[5] that the content of 7-methylguanine in DNA of liver from rats treated with MAM, 70 mg/kg, (6) was decreased from 5.3/1000 guanine to 1.7/1000 guanine when rats were treated 2 hr earlier with pyrazole, 90 mg/kg; thus, it appears that *in vivo* conversion of MAM by dehydrogenase(s) also results in increased production of methylcarbonium ions.

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[4] Dr. N. Frank and Dr. M. Wiessler of the German Cancer Center, Heidelberg, attempted to synthesize MAMAL from MAM. They used standard chemical procedures for converting alcohols to aldehydes and included hydrazine to trap the aldehyde. Even though MAM disappeared from the reaction mixture, no MAMAL or hydrazone derivative of MAMAL could be isolated. These data support the suggestion that MAMAL is very unstable (personal communication).

[5] M. S. Zedeck and G. B. Brown, unpublished observations.