Table 2. Comparison of the inhibition constants for SAH analogs toward INMT, COMT, PNMT, HMT and HIOMT

Inhibitor	Inhibition constants*† ($K_i \pm$ S. E. M., μM)				
	INMT‡	COMT§	PNMT§	HMT§	HIOMT§
SAH	8·65 ± 0·71	36·3 ± 2·2	29·0 ± 2·8	18·1 ± 2·2	18·5 ± 1·9
3-Deaza-SAH	26·6 ± 1·2	80·6 ± 5·0	81·1 ± 14·9	59·2 ± 6·1	229 ± 11
N^6-methyl-3-deaza-SAH	70·2 ± 6·6		1243 ± 141		
3′-Deoxy-SAH	51·9 ± 7·8	138 ± 31	42·7 ± 2·5	2070 ± 864	
Arabinose derivative	85 ± 8·7		206 ± 27		

* When low inhibitory activity was observed from preliminary studies, no extensive studies were done to determine the inhibition constants.

† Each inhibitor showed linear competitive kinetics when plots of the reciprocal velocities vs reciprocal SAM concentrations were made. The inhibition constants were calculated as previously described [11–15].

‡ INMT was purified and assayed as described in the text and Table 1. SAM concentration = 24–210 μM; *N*-methyltryptamine concentration = 1·0 mM; inhibitor concentrations = 20–400 μM. In general, at least three concentrations of each inhibitor were used in determining the inhibition constants.

§ Data taken from Ref. 8 and from R. T. Borchardt and Y. S. Wu, *J. med. chem.* **18**, 300 (1975).

INMT. For the sake of comparison, the inhibition constants for these analogs toward PNMT, COMT, histamine-*N*-methyltransferase (HMT) and hydroxyindole-*O*-methyltransferase (HIOMT) are also listed in Table 2 [8,*]. The relatively strong inhibitory activity of N^6-methyl-3-deaza-SAH is of particular interest, because INMT appears to be the only methyltransferase investigated in our laboratory [8] which is strongly inhibited by this analog. A related compound, *N*-methyl-SAH was previously reported to inhibit tRNA methylase [16] and PNMT [8]. Another interesting difference in the inhibition profile for INMT is the strong inhibition produced by 3′-deoxy-SAH and the arabinose derivative. The only other enzyme showing a similar profile is PNMT [8].

From the inhibitory activities observed for the various analogs used in this study, it can be generally concluded that INMT shows a fairly strict specificity for the structural features of SAH, which appears to be common for most of the methyltransferases studied [9]. However, several unique characteristics of the binding site on INMT have been observed and several analogs of SAH have shown potent inhibitory activity toward INMT (3-deaza-SAH, N^6-methyl-3-deaza-SAH and 3′-deoxy-SAH). These observations suggest that analogs of SAH could be useful for the inhibition *in vivo* of this enzyme. This possibility is presently being explored in our laboratory.

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Repression of dimethylnitrosamine-demethylase by typical inducers of microsomal mixed-function oxidases

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Previous investigations in this laboratory have shown that the synthesis of the microsomal mixed-function oxidase, which *N*-demethylates the carcinogen, dimethylnitrosamine (DMN), is inhibited by pretreatment of the animals with the typical enzyme inducers, 3-methylcholanthrene (MC) [1–3] as well as other polycyclic hydrocarbons [4]

and phenobarbital [2]. It was ruled out that the direct action of hydrocarbon or hydrocarbon metabolite could be responsible for the decreased DMN-demethylase activity [1]. Decreased enzyme activity is due to decrease in the amount of enzyme, since pretreatment with MC brings about substantial decrease of the V_{max} but the K_m remains unchanged [2]. The repression of DMN-demethylase by MC pretreatment was confirmed by Somogyi *et al.* [5]. The case of DMN-demethylase is an example of the rare instances [6–14] where microsomal mixed-function oxidases are repressed by typical inducers of enzyme synthesis.

In a recent report, McLean and Day [15] took issue with the observations that compounds which induce the synthesis of many mixed-function oxidases and of cytochrome P-450 repress DMN-demethylase. Following our earlier studies [1–4], other investigations on DMN-demethylase have been carried out. Reports resulting from these investigations are being published [16] or have been submitted for publication; preliminary results of the latter have been presented [17]. The reports confirm and extend our previous findings that inducers of microsomal enzyme synthesis repress the *de novo* synthesis of DMN-demethylase which yet is a mixed-function oxidase supported by cytochrome-mediated electron transport. The present communication describes new data supporting the repression of DMN-demethylase by enzyme inducers and provides a critical analysis of the report and methodology of McLean and Day [15].

Weanling male Sprague-Dawley rats (weight range 50–70 g) maintained on a semisynthetic diet [1, 2] were pretreated with the compounds as follows: MC at 40 mg/kg of body weight, benzo[*a*]pyrene and β-naphthoflavone at levels equimolar to the above, and aminoacetonitrile at 200 mg/kg of body weight, all four administered by single i.p. injection 24 hr prior to sacrifice; pregnenolone-16α-carbonitrile administered in four i.p. injections at 60 mg/kg of body weight, at 43, 31, 19 and 6 hr prior to sacrifice; phenobarbital given daily for 4 consecutive days i.p. at 80 mg/kg of body weight, the last injection preceding sacrifice by 24 hr; Aroclor 1254 administered by single i.p. injection at 500 mg/kg of body weight, 4 days prior to sacrifice. Aminoacetonitrile and phenobarbital were in solution in 0·9% NaCl; all other compounds were in corn oil. Controls received the respective solvent following the same schedule. Standardized isolation of the microsomes, the demethylation reaction, determination by the Nash reaction of the formaldehyde produced, and determination of the microsomal protein by Lowry's Folin procedure were carried out as described previously [1–4, 16].

Table 1 shows the effects of MC, benzo[*a*]pyrene and several agents other than hydrocarbons on the liver weight, amount of microsomal protein per unit weight of tissue, and DMN-demethylase activity expressed as HCHO produced per mg of microsomal protein and per g of tissue. With the exception of benzo[*a*]pyrene and aminoacetonitrile, all compounds brought about a statistically signifi-

Table 1. Effect of typical enzyme inducers on hepatic tissue synthesis and dimethylnitrosamine-demethylase activity in the rat*

Compound	Liver wt (g/100 g body wt) Control	Exper.	Microsomal protein (mg/g liver) Control	Exper.	HCHO (nmoles/hr/mg microsomal protein) Control	Exper	HCHO (μmoles/hr/g liver) Control	Exper.
3-Methylcholanthrene	5·07 ±0·16	5·70 ±0·15	18·3 ±1·3	17·8 ±2·1	35·0 ±1·9	19·0 ±0·6	0·64 ±0·03	0·34 ±0·01
	12·4% increase		no change		45·7% decrease		46·9% decrease	
	0·02 > P > 0·01		0·90 > P > 0·80		P < 0·001		P < 0·001	
Benzo[*a*]pyrene	4·72 ±0·32	4·96 ±0·49	16·3 ±2·3	18·6 ±1·6	30·1 ±3·1	17·4 ±0·7	0·49 ±0·05	0·32 ±0·01
	no change		(14·1% increase)		42·2% decrease		34·7% decrease	
	0·70 > P > 0·60		0·50 > P > 0·40		0·02 > P > 0·01		0·01 > P > 0·001	
β-Naphthoflavone	4·34 ±0·17	5·30 ±0·11	17·9 ±1·2	20·3 ±0·8	30·3 ±1·5	15·5 ±1·4	0·54 ±0·03	0·31 ±0·03
	22·1% increase		(13·4% increase)		48·8% decrease		42·6% decrease	
	0·01 > P > 0·001		0·20 > P > 0·10		P < 0·001		P < 0·001	
Pregnenolone-16α-carbonitrile	4·22 ±0·03	6·28 ±0·09	14·8 ±0·9	16·5 ±0·9	44·1 ±3·6	17·8 ±1·9	0·65 ±0·05	0·29 ±0·03
	48·8% increase		(11·5% increase)		59·6% decrease		55·4% decrease	
	P < 0·001		0·30 > P > 0·20		P < 0·001		P < 0·001	
Phenobarbital	4·75 ±0·16	6·47 ±0·18	14·6 ±0·6	18·7 ±0·7	42·6 ±2·3	32·4 ±1·0	0·62 ±0·03	0·61 ±0·02
	36·2% increase		28·1% increase		23·9% decrease		no change	
	P < 0·001		P < 0·001		P < 0·001		0·40 > P > 0·30	
Aroclor 1254	4·70 ±0·21	6·80 ±0·60	12·8 ±0·7	7·8 ±0·7	33·8 ±1·1	12·8 ±0·9	0·43 ±0·01	0·10 ±0·01
	44·7% increase		39·1% decrease		62·1% decrease		76·7% decrease	
	0·02 > P > 0·01		0·01 > P > 0·001		P < 0·001		P < 0·001	
Aminoacetonitrile	4·45 ±0·14	4·63 ±0·11	14·1 ±1·0	13·5 ±1·6	27·3 ±1·4	6·8 ±1·2	0·38 ±0·02	0·09 ±0·02
	no change		no change		75·5% decrease		76·3% decrease	
	P ≈ 0·40		0·80 > P > 0·70		P < 0·001		P < 0·001	

* Dosage levels and i.p. administration schedules of compounds are given in third paragraph of text. Each determination on pretreated animals was carried out together with a control determination, using rats with the same birthdate for both. The means ± standard errors represent the results of 4 to 6 individual determinations, except for phenobarbital where 9 controls and 18 experimental determinations were run; each determination was carried out in duplicate. Changes between control and experimental values greater than 10 per cent, but with P > 0·05, are given in parentheses.

cant increase of liver weight. The increase of liver weight with MC, after 24 hr, is comparable to the value found earlier [18]. The previous study showed, however, that after one MC administration liver hypertrophy continues to progress well beyond 24 hr and reaches an increase of 42 per cent after 4 days [18]. In earlier experiments benzo[*a*]-pyrene was found generally less potent than MC to induce azo dye *N*-demethylase [18] and to repress DMN-demethylase [1]; thus, it is probable that, allowing a longer time, benzo[*a*]pyrene would have shown a significant effect on liver hypertrophy. The high potency of pregnenolone-16α-carbonitrile to produce liver hypertrophy confirms the observation of Tuchweber *et al.* [19].

Phenobarbital and the polychlorinated biphenyl mixture, Aroclor 1254, were the only two agents which brought about liver hypertrophy coincident with a statistically significant change in the amount of microsomes. Increase of liver weight together with substantial proliferation of the endoplasmic reticulum after pretreatment with phenobarbital has been described [20]. Aroclor 1254 produced a diametrically opposite effect in that liver hypertrophy was accompanied by a decrease in microsomal protein; this is in accord with our previous result [16].

The fourth column in Table 1 presents the effects of the agents on DMN-demethylase activity in terms of nmoles HCHO produced per hr per mg of microsomal protein. All agents brought about substantial and statistically significant decreases in enzyme activity, aminoacetonitrile being the most and phenobarbital the least potent. The repression of DMN-demethylase by pregnenolone-16α-carbonitrile confirms the finding of Somogyi and Selye [21].

The enzyme activities expressed as μmoles HCHO produced per hr per g of liver are given in column 5 of Table 1. Since this expression is the product of the microsomal yield (in terms of mg microsomal protein/g of liver) and of the enzyme activity (expressed per mg microsomal protein), a decrease of the yield magnifies the decrease of enzyme activity, while an increase of microsomal yield has a compensatory effect and tends to lessen the decrease. Table 1 shows that, with the exception of phenobarbital, all compounds substantially repress DMN-demethylase activity when expressed per g of liver; in the case of phenobarbital, there is no change because the decrease of enzyme activity per mg microsomal protein is compensated for by an increase of microsomal yield of similar magnitude.

With respect to the analysis of the report of McLean and Day [15] below, it should be pointed out that there is no convention in the literature preferentially prescribing expression of a mixed-function oxidase activity (determined on an isolated microsomal fraction) as per g of tissue rather than per mg microsomal protein. Both ways of expressing enzyme activity are currently used. We hold the view that, in analogy with the expression of activity of isolated enzyme preparations in general, activity on a per mg microsomal protein basis is more correct, in particular, when a selective effect on the synthesis of a specific mixed-function oxidase is studied as distinct from the proliferation of the endoplasmic reticulum. Regarding a possibly greater biological relevance *in vivo* of one way over the other way of expressing enzyme activity, it should be remembered that the three DMN-demethylase repressors—MC [22], phenobarbital [23] and aminoacetonitrile [24]—are inhibitors of nitrosamine hepatocarcinogenesis consistent with the requirement of dealkylation for nitrosamine carcinogenesis. Hence, the enzyme concentration per unit weight of endoplasmic reticulum appears to be of greater biological relevance than total enzyme activity present per g of liver.

McLean and Day [15] argued that the repression of DMN-demethylase activity (expressed per mg microsomal protein) observed by us "...could have been brought about by one of two mechanisms. Either, the amount of enzyme activity per g of liver could remain constant while the amount of protein recovered in the microsomal fraction increased, or else there was a true fall in enzyme activity; per cell, per animal, or per g of liver." In so stating, the authors disregarded the evident fact that enzyme activity on a per mg microsomal protein basis is independent of the microsomal yield, since the determination of microsomal protein is carried out on the microsomal fraction already isolated.

A number of methodological aspects of the report of McLean and Day [15] raise doubts regarding the validity of several of their results. The use of anesthetic agents in the sacrificing of animals is well known to affect different enzyme activities; hence, it is not impossible that the use of ether by McLean and Day for sacrificing could have differently affected control, fasted, and benzopyrene-pretreated rats. The unusually high protein yield in the microsomal fractions together with the unnecessarily long (8 sec) ultrahigh speed homogenization (Ultra Turrax blender) used for the liver strongly suggest that both the postmitochondrial and microsomal fractions may have been contaminated with co-sedimenting fragments of broken up mitochondria which are notoriously sensitive to shear stress. Mitochondrial contamination of the two fractions is also suggested by the low speed ("7000 *g* maximum centrifugation for 10 min") used to obtain the postmitochondrial supernatant fraction. Speeds ranging from 6000 to 13,000 *g* for 10–12 min have been used by various workers to sediment mitochondria; however, the low speeds have been generally abandoned in recent years because of the low yield due to slow sedimentation as well as the loose pellets obtained.

McLean and Day [15] state that by using 1·4 μmoles DMN/ml of incubation medium in the demethylation reaction, "There are difficulties in measuring metabolism of DMN because the reaction proceeds slowly." Comparison of this low DMN level to our kinetic data [2, 3] indicates that the amount of substrate was notably limiting in their experiments. This explains why the reaction proceeded slowly. The "large amounts of tissue needed" because of the low rate of DMN demethylation led McLean and Day to a minor modification of the Nash reaction, regarded by the authors to be a new method, involving amyl-alcohol extraction of the color before the measurement of absorbance; no data are given on the extent of recovery of the extraction.

Contrary to the views of McLean and Day [15], the demethylation of DMN, though of great biological importance for leading to the proximate toxicant and carcinogen, is a minor pathway for the clearance of DMN from the plasma. This is shown by the observations that the potent repressor of DMN-demethylase, pregnenolone-16α-carbonitrile, does not influence the disappearance of DMN from the plasma [5], while the similarly potent repressor, aminoacetonitrile, powerfully inhibits clearance from the plasma [5, 25]. This clearly indicates that plasma clearance must overwhelmingly depend on the activity of as yet unidentified enzymes which metabolize DMN to various non-carcinogenic degradation products [*rev.* 26].

In an unexpected contradiction to their strong claim that DMN-demethylase activity is increased by typical enzyme inducers, McLean and Day [15] state that after benzo[*a*]-pyrene pretreatment there is "...an apparent fall in DMN demethylating activity if this were calculated per mg of protein." Surprisingly, the authors overlooked that the report of Somogyi *et al.* [5], quoted by them in another context, actually confirms the repression of DMN-demethylase by the typical polycyclic hydrocarbon, MC. McLean and Day [15] incorrectly quoted the DMN-demethylase K_m values obtained in two different investigations; the actually reported values were 0·22 mM [2] and 0·35 mM [3], rather than 0·22 and 0·53. Finally, it should be pointed

out that the very large induction of DMN-demethylase by fasting and by feeding a carbohydrate-devoid protein diet was first reported from our laboratory [3]. In describing their own finding with fasting, which confirms our result, McLean and Day [15] failed to note the priority of our report [3], yet it was cited by them in another context.

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Effect of ATP upon dopamine-β-hydroxylase

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A stimulating effect of ATP upon the conversion of dopamine to noradrenaline was first reported when crude adrenal extracts were used [1, 2]. This effect, however, was much less apparent when the dopamine-β-hydroxylase (DBH) was purified; in this case the ATP caused only a 1·5-fold stimulation instead of the 5–10-fold stimulation noted when crude adrenal extracts were used [3]. As even this slight effect disappeared when initial rates of DBH were measured, it was concluded that ATP was not intimately involved in the hydroxylation reaction [4]. The fortuitous observation that a nucleotide preparation from a different origin was ineffective, even with a crude extract, prompted us to investigate a possible interaction between the apparent stimulation of DBH by ATP and the presence of endogenous inhibitors.

Fresh bovine adrenal glands were obtained from the local slaughterhouse and immediately chilled in ice-cold 0·25 M sucrose. The medullae were dissected from the total gland, minced and then homogenized in 10 vol 0·25 M sucrose with a Duall homogenizer. The total homogenate was centrifuged at low speed (1000 *g* for 10 min) and the pellet was discarded. The supernatant was further centrifuged at 27,000 *g* for 10 min and the pellet, corresponding to the M + L fraction described in a previous paper [5], was washed twice and resuspended in 20 vol. The final supernatant fraction was obtained by centrifugation at 120,000 *g* for 60 min.

Another fractionation procedure—that described by Kuzuya and Nagatsu [6]—was also followed. In this case the medullae were homogenized with an Ultraturrax in 6 vol of a 0·25 M sucrose solution containing 0·02 M potassium phosphate buffer (pH 7·0). After a low speed run, a particulate fraction was obtained by centrifugation at 20,000 *g* for 30 min.