# Enzymatic N-Demethylation of cocaine and nutritional status

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SUMMARY

The parameters of the enzymatic system responsible for the N-demethylation of cocaine resulting in the formation or formaldehyde and nor-cocaine have been determined.

The microsomal enzyme system is found mainly in liver of various animals and requires NADPH, $Mg^{+2}$, and $O_2$ as cofactors. The Km of the reaction is approximately 1.09 x $10^{-6}$ M. The enzymatic activity is stimulated by feeding cocaine to well nourished animals but not to animals on a low protein diet. The amount of enzyme observed in diffrerent species does not correlate with that reported for the N-demethylation of other substances. These results suggest the possible involvement of different enzymes.

The use of coca leaf by the inhabitants of the highlands of the Andes has been associated with their poor nutritional status. As far as we know, no attempt has been made to elucidate the biochemical alterations that this type of addiction could induce in mammals.

Ramos-Aliaga and Arroyave (1) have reported that young rats maintained on a low protein diet (5% corn protein) for a period of 4 weeks developed fatty livers. However, when a group of rats were fed the same diet supplemented with co-

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caine, fatty livers were prevented. These results suggested that cocaine has a lipotropic activity.

Since there is an N-methyl group in cocaine, it is plausible that it acts as methyl donor and produces the observed lipotropic effect. Support for this hypothesis is found in the work of Brodie and Axelrod (2), Axelrod (3, 4), Butler and Busch (5, 6), and others (7) who have studied enzymatic systems that catalyze the demethylation of a variety of drugs containing a methyl-group attached to a tertiary nitrogen. Neubert and Hoffmeister (8) in a more specific study used cocaine as substrate for different N-demethylating enzymes and observed formation of formaldehyde although they could not conclusively prove the presence of nor-cocaine in the reaction products.

The observations and speculations mentioned in the previous paragraphs led us to conduc a study of the characteristics of N-demethylation of cocaine by the tissue of different animals.

## EXPERIMENTAL PROCEDURES

### *Preparation of the homogenate*

Livers, kidney, brain, heart, and intestinal mucosa from CF mice from our colony, PRNC, as well guinea pigs, rats and dogs in healthy condition and well nourished were homogenized in a Elvehjem-Potter homogenizer with 0.1 M phosphate buffer pH 7.4 (potasium salt) in a concentration of 1 part of tissue (wt.) to 3 parts of buffer (v.). Cellular fractions were separated in a Spinco centrifuge model L in 0.25 M sucrose following the Hogeboom technique (9), although with this method the resulting fractions are not 100% pure. As a standard procedure, the enzyme activity was measured in the supernatant obtained after centrifugation for 10 minutes at 9,000 x g of the homogenate in phosphate buffer. This supernatant contained the microsomes and soluble fraction.

### *Enzymatic Assay*

The procedure used was similar to the one of Neubert and Hoffmeister (8). The standard incubation media was prepared in 6 ml as follows: glucose 55 $\mu$ moles; $MgCl_2 . 3H_2O$ 40 $\mu$ moles; nicotinamide 98 $\mu$ moles; semicarbazide hydrochloride 50 $\mu$

moles; NADP 1 μ mole; phosphate buffer pH.4 200 μ moles; cocaine hydrochloride 30 μ moles; distilled water 0.75 ml and supernatant of 9,000 x g homogenate 1 ml.

The medium was incubated for one hour at 37°C in a Dubnoff-shaker with aeration. The reaction was stopped by adding 2 ml of barium hydroxide (saturated solution) and 2 ml of 20% zinc sulfate. The extent of the reaction was determined by measuring the formaldehyde formed. This was done by the method of Nash as modified by Cochin and Axelrod (11); the optical density was measured in a Beckman DB spectrophotometer at 420 mμ.

A total of 30 μ moles of cocaine was chosen as the standard assay quantity after preliminary trials to determine the saturation point of the enzymatic system.

The 9,000 x g supernatant was dialyzed in 0.01 M phosphate buffer pH 7.4 at 0-2°C to establish the role of different cofactors.

Microsomes were prepared and purified by the method of Hogeboom (9) using the 105,000 x g pellets obtained in the Spinco model L centrifuge.

*Identification of the reaction products*

In order to identify the reaction products, a batch of 10 ml of the supernatant was prepared and incubated in the standard system to which the constituents were added proportionatelly. When the reaction was completed, an aliquot was taken for formaldehyde determination. The remaining solution was alkalinized with $NH_4OH$ and extracted three times with chloroform. The chloroform was evaporated to 5 ml. Chromatographic studies were conducted with this chloroform extract to identifiy the possible cocaine metabolites such as benzoylecgonine, nor-cocaine, or ecgonine, using thin layer (cellulose) and paper chromatography, respectively.

Different solvent systems were used; that of Castañeda and Chiriboga (11) for separation of cocaine from its metabolites (acetic acid, n-butanol, water, 4:100:50) and that of Schmidt and Werner (12) for separation of cocaine from nor-cocaine (n-butil acetate, acetic acid, water, n-butanol, 47:28:16:9).

Ascending and descending paper chromatography were also run, using the previously mentioned systems as well as that described by Maglat and Bayer (13), consisting of ethylmethyl-ketone, dimethyl-formamide, water (20:10:10) for the separation of ecgonine and benzoyl-ecgonine. Dragendorff solution was used to develop the color of the alkaloids following the procedures described by Block (14).

Quantitation of the cocaine metabolites was done by eluting the compounds from paper chromatography strips with methyl-alcohol and determining the concentration by spectrophotometry at 360 mμ.

The standards used were commercial grade cocaine; benzoyl-ecgonine, ecgonine and nor-cocaine were prepared in this laboratory. Benzoyl-ecgonine was prepared by hydrolyzing cocaine by refluxing it for 4 hours at pH 8. Ecgonine was prepared by oxidizing benzoyl-ecgonine at pH 9 with $KMnO_4$ and re-esterifying the nor-benzoyl-ecgonine obtained to nor-cocaine with HCl-methanol. All these compounds were purified by recrystallization. Melting points and infrared spectrometry were used to characterize the compounds and to establish their purities.

*Biological Experiments: Cocaine effect in vivo on enzymatic activity*.

Four groups of 8 Wistar rats each were used; as soon as they were removed from the mothers (21 days old) two groups were fed Purina chow, containing 23% of high quality protein and the two others received the experimental diet, containing 5% corn protein, such as was described previously (1).

The diet of one group of each dietary treatment was supplemented with 15 mg of cocaine hydrochloride per 10 g of diet. The other two groups did not receive cocaine and served as controls. The experimental treatment with these diets was for a period of 4 weeks. At the end of this experimental period the rats were killed, the livers homogenized and centrifuged at 9,000 x g and the supernatant tested for ability to produce formaldehyde after incubation with cocaine hydrochloride.

## RESULTS

The studies reported here confirm the characteristics and distribution of the demethylase system. The enzymatic system is localized in the microsomal fraction (Table 1) and requires

NADPH, $Mg^{2+}$ and $O_2$ (Table 2). A direct relation exists between the reaction rate and the concentration of NADPH (Table 3). Neither mitochondria, nucleus, and microsomes nor the soluble fraction alone catalyze the reaction (Table 1).

When the supernatant was dialyzed, it lost its ability to catalyze the reaction (Table 2). The addition of glucose-6-phosphate regenerated the activity.

*Stoichiometry of the reaction:*

The amount of formaldehyde produced by the enzymatic systems is proportional to the nor-cocaine formed.

In 1 hour at 37°C in the standard medium 1.44 μ mole of formaldehyde and 2.07 μ moles of nor-cocaine were produced per gram of tissue. Errors intrinsic in the methodology probably accounts for the difference observed. The N-demethylation of cocaine was difficult to determine exactly because the system simultaneously yielded some benzoyl-ecgonine.

TABLE 1

COCAINE N-DEMETHYLATION ACTIVITY OF DIFFERENT CELLULAR FRACTIONS FROM MOUSE LIVER*

| Cellular Component | μ moles of HCHO per g of wet tissue |
|---|---|
| Total homogenate | 0.910 |
| Supernatant (9,000 x g) | 2.761 |
| Nuclear fraction | 0.031 |
| Mitochondrial fraction | 0.0 |
| Microsomal fraction | 0.063 |
| Soluble fraction | 0.094 |
| Microsomes plus soluble fractions | 2.573 |
| Mitochondries plus soluble fractions | 0.766 |
| Nucleus plus soluble fractions | 0.876 |

* Standard incubation medium was used. In a volume of 6 ml: glucose 55 μ moles; $MgCl_2 3H_{20}$ 40 μ moles; nicotinamide 98 μ moles; semicarbazide hydrochloride 50 μ moles; NADP 1 μ mole; phosphate buffer 200 μ moles; cocaine hydrochloride 30 μ moles; distilled water 0.75 ml and sample (equivalent to 333 mg from liver) 1 ml.

TABLE 2

**NADP, $Mg^{2+}$ and $O_2$ REQUIREMENTS BY COCAINE N-DEMETHYLATION FROM MOUSE LIVER***

| Supernatant condition | μ moles of HCHO per g of wet tissue |
|---|---|
| Supernatant in standard incubation medium (NADP ≠ $Mg^{2≠}$ with aeration)** | 3.828 |
| Dyalized supernatant in standard incubation medium (DS) | 0.125 |
| DS plus glucose-6-phosphate (DSG-6-P) | 2.592 |
| DSG-6-P but without NADP | 0.0 |
| DSG-6-P but without $Mg^{2≠}$ | 1.544 |
| DSG-6-P but without aeration ($N^2$ atmosphere) | 0.0 |

* Dialyzed mouse liver supernatant (9,000 x g) from 333 mg mouse liver was used with standard incubation medium.

** Reference system.

TABLE 3

**COCAINE-N-DEMETHYLATION BY MOUSE LIVER MICROSOMES AND NADPH REQUIREMENTS**

| Microsomes condition | | | μ moles of HCHO per g of wet tissue |
|---|---|---|---|
| Supernatant in standard incubation medium with aeration* | | | 3.326 |
| Microsomes plus soluble fraction in standard incubation medium with aeration | | | 2.950 |
| Microsomes plus reduced coenzyme in same condition above: | | | |
| with: | NADPH | 1.2 μ mole | 0.502 |
| | NADPH | 3.6 μ mole | 0.847 |
| | NADPH | 7.2 μ mole | 1.381 |
| | NADPH | 10.8 μ mole | 1.663 |
| with: | NADH | 1.4 μ mole | 0.157 |
| | NADH | 4.2 μ mole | 0.169 |

* Reference system.

TABLE 4
DIFFERENT RF OF COCAINE METABOLITES

| | Solvents | | | | |
|---|---|---|---|---|---|
| | Thin Layer | | Ascendent | | Decendent |
| | 1 | 2 | 1 | 2 | 3 |
| Standards | | | | | |
| Cocaine | 0.83 | 0.87 | 0.66 | 0.71 | 0.89 |
| Nor-cocaine | 0.34 | 0.64 | 0.20 | 0.34 | 0.72 |
| Benzoyl-ecgonine | 0.81 | 0.89 | 0.70 | 0.74 | 0.79 |
| Ecgonine | 0.14 | 0.52 | 0.12 | 0.26 | 0.56 |
| Sample | 0.83* | 0.86* | 0.67* | 0.67* | 0.88* |
| | 0.36** | 0.64** | 0.20** | 0.35** | 0.73** |
| | | | | | 0.78*** |

Cocaine*, nor-cocaine**, and benzoyl-ecgonine***, produced after the N-demethylation reaction for mouse liver.

(1) Solvent: acetic acid, n-butanol, water: 4:100.50;

(2) n-buthyl acetate, acetic acid, water: 47:28:16:9;

(3) ethylmethyl ketone, dimethyl formamide, water:

*Km of reaction*

The Km for the N-demethylation of cocaine by normal mouse liver N-demethylase was estimated by Lineweaver-Burke plot. This value was 1.09 x $10^{-6}$ M (Fig. 1).

*Identification of the reaction products*

The chromatographic test showed the identity of nor-cocaine obtained enzymatically. Different Rf values were obtained indifferent solvents (Table 4) but always the same Rf for the same solvent. Also, the synthetic and natural nor-cocaine had the same ultraviolet absorption spectrum and gave the maximum absorption in the visible spectrum with Dragendorff reagent.

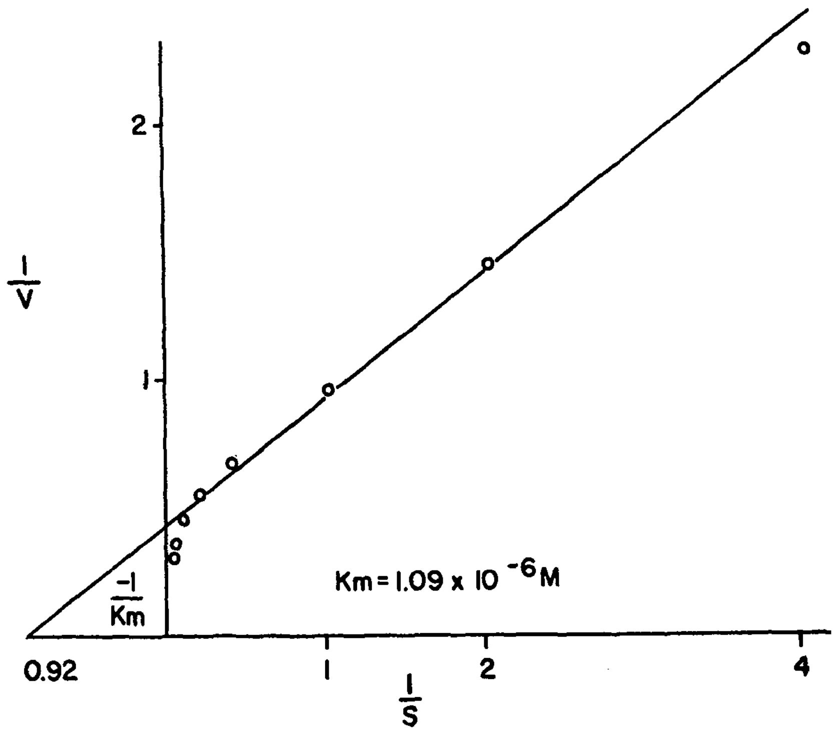


Fig. 1.—Lineweaver-Burk plot of cocaine N-demethylation. The experimental conditions are described in Table 1.

*Stimulation of liver N-demethylation activity by cocaine in vivo*

After 4 weeks feeding the stock diet supplemented with cocaine rats exhibited increased liver levels of N-demethylase when compared with the controls that did not receive cocaine. This difference was found to be statistically significant at P>0.05 level when the data was expressed either per gram of wet tissue, miligram of DNA or per gram of total body weight of the animals (Fig. 2).

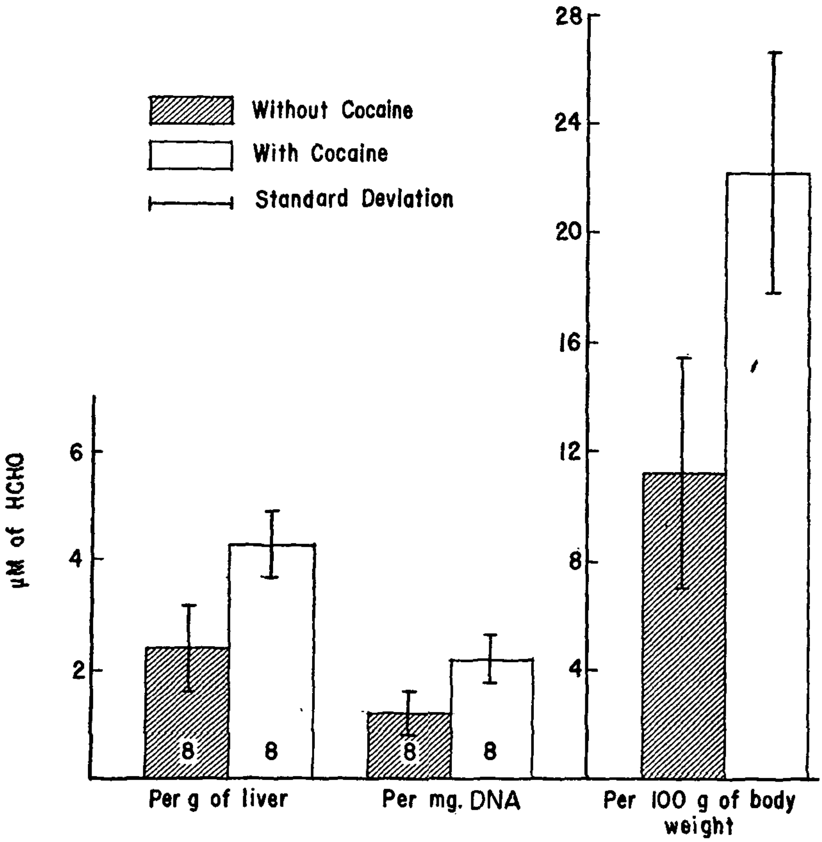


Fig. 2.—Cocaine-N-demethylation activity in the livers of rats fed with purine chow (23% of protein) with and without added cocaine chlorhidrate (15 mg/10 g). Each value is an average of eight determination.

On the other hand, no difference in enzymatic activity was observed between the animals receiving the 5% corn protein diet with or without the cocaine supplement.

*Distribution of the cocaine N-demethylation enzyme in different tissues*

The livers of different animal shown the highest activity. Brain, heart and intestinal mucosa show practically no activity (Table 5).

## DISCUSSION

The study of the N-demethylation of cocaine by different animals was undertaken keeping in mind the possible physiological role this alkaloid could play as a methyl-donor in the economy of individuals who routinely chew coca leaf and are usually poorly nourished.

The results show that liver, mainly the microsomal fraction, has an enzymatic system that N-demethylates cocaine *in vitro*. We do not have data to verify the specificity of this enzymatic system; is is very possible that it is not specific. The system described may demethylate other drugs that have a methyl group on a tertiary nitrogen; however, the Km of the system for cocaine is very low, implying a high affinity of the enzyme for the substrate.

The finding of Neubert and Hoffmeister (8) that rat hepatoma tissue, in contrast to normal liver tissue does not N-demethylate any other drug but cocaine suggest that there is more than one enzymatic system within the microsome which catalyzes oxidative N-demethylation.

The probability that there is more than one enzyme system is also supported when our data on the distribution and rate of enzymatic N-demethylation of cocaine by different animals is compared with Axelrod's (3, 4) data on N-demethylation of ephedrine.

Nor-cocaine, the product of the N-demethylation reaction, has never been demonstrated to be a metabolite of cocaine in *in vivo* experiments. Ecgonine has been reported as the only metabolite of cocaine excreted in urine by rabbits injected with the drug (15). Ecgonine has also been found and its presence in the urine of human cocaine addicts has been discussed (16, 17).

TABLE 5

ACTIVITY IN μ MOLES OF HCHO PER g OF WET TISSUE

Average values of the cocaine N-demethylation enzymatic activity of different tissues in different animals

| | Liver | Kidney | Brain | Heart | Intestinal mucosa |
|---|---|---|---|---|---|
| Guinea Pig | 5.96 ± 1.23 | 0.125 | 0.026 | 0.025 | 0.0 |
| Rats | 3.29 ± 0.44 | 0.235 | 0.044 | 0.025 | 0.075 |
| Mouse | 2.77 ± 0.06 | 0.079 | 0.022 | 0.031 | 0.0 |
| Dog | 1.43 ± 0.14 | 0.37 | 0.005 | 0.026 | 0.063 |

Note: Supernatant (9,000 x g) of each tissue were used.

Other investigators (18), however, report that benzoyl-ecgonine is the only product detected when liver is incubated with cocaine *in vitro*. Serum of rabbits injected with cocaine has been shown to contain only benzoyl-ecgonine (19). Benzoyl-ecgonine was also the only product obtained when intestinal mucosa was incubated with cocaine (20).

Under our experimental conditions, intestinal mucosa *in vitro* produced benzoyl-ecgonine only, but liver produced benzoyl-ecgonine and nor-cocaine. It is possible that this disagreement may be due to different experimental conditions.

The stimulation of a N-demethylase system by cocaine in rats fed an adequate protein diet requires some comment. It will be necessary to determine wheter the action of cocaine is direct, or if it is mediated through the release of hormones or other substance that could affect this stimulation.

Our data lead us to suppose that the observed increase in enzymatic activity was due to an inductor action as it has been postulated in the case of other N-demethylases studied by Conney, et al. (21). Further work must confirm this hypothesis and check the synthesis rate of enzymatic protein.

Our data also showed that microsomal activity is greatly limited in the liver of animals receiving a low protein diet (5% corn). In these animals *in vivo* enzymatic system does not respond to cocaine as a simulator. The sensitivity of this microsomal system to different diets contrasts with the data reported by Chiriboga (22) o nthe influence of starvation on the *in vivo* conjugation with glucuronic acid of naphthalene and anthranilic acid.

Many questions arise with regard to the physiological role played by this enzyme in the metabolism and detoxification of cocaine either experimentally administered or ingested as coca leaf by some people in the South Andean region. Perhaps the formaldehyde produced enters the general one-carbon-pool. Recent studies in this laboratory (23) show that when methionine labeled in the methyl group with carbon 14 was injected into animal with cocaine in their diet the specific activity of the choline is less than that observed in the controls that did not receive cocaine. These results imply that cocaine could be operating as a one-carbon-source.

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## RESUMEN

### N-demetilación enzimática de la cocaína y estado nutricional

Se presenta un estudio sobre los diferentes parámetros responsables por la N-demetilación de la cocaína, con producción de formaldehido y norcocaína.

Este sistema enzimático microsomal ha sido encontrado solamente en el hígado de los diferentes animales estudiados (ratas, ratones, cobayos y perros). El Km de la reacción es aproximadamente 1.09 x $10^{-6}$ M. La actividad de la enzima es estimulada por la cocaína en ratas que poseen buen estado nutricional (dieta de Purina con 23% de proteína), pero no en las malnutridas (dita con 5% de proteína de maíz). La actividad enzimática encontrada en las diferentes especies de animales estudiados no guarda relación con lo observado para la N-demetilación de otras drogas. Esto sugiere que son diferentes enzimas las que participan en el proceso de N-demetilación.


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