Biochemical Pharmacology, Vol. 18, pp 2179–2186. Pergamon Press. 1969. Printed in Great Britain

N-DEMETHYLATION OF *p*-(N^1-METHYLHYDRAZINO METHYL)-*N*-ISOPROPYL BENZAMIDE (PROCARBAZINE),* A CYTOSTATICALLY ACTIVE METHYLHYDRAZINE DERIVATIVE, IN THE INTACT RAT AND IN THE ISOLATED PERFUSED RAT LIVER†

BEATRICE DEWALD,‡ MARCO BAGGIOLINI§ and HUGO AEBI

Medizinisch-Chemisches Institut, University of Berne, Switzerland‖

(*Received* 24 *December* 1968; *accepted* 17 *March* 1969)

Abstract—The oxidative *N*-demethylation of *p*-(N^1-methylhydrazino methyl)-*N*-isopropyl benzamide (procarbazine, Natulan) and of its postulated metabolite monomethyl hydrazine (MMH) has been studied in the intact rat, as well as in the isolated perfused rat liver, by measuring the formation of $^{14}CO_2$ from $^{14}CH_3$-labeled substrates. The CO_2 production rate from procarbazine was 1·83 and 1·18 μmoles/hr per 100 g body weight *in vivo* and in the isolated perfused rat liver respectively. In both systems, this rate was markedly lowered by SKF 525-A and enhanced 3·10 to 3·50-fold by the pretreatment with 3-methylcholanthrene and 2·5-fold by the pretreatment with phenobarbital. The demethylation rate of equimolar amounts of MMH was 0·7 μmoles/hr per 100 g body weight *in vivo* and 2·08 μmoles per hr per 100 g body weight in the isolated liver. In the isolated liver SKF 525-A and 3-methylcholanthrene treatment resulted in a 14 and 26 per cent decrease of the demethylation rate of MMH respectively. These results indicate that the main pathway of CO_2 production from procarbazine involves the primary cleavage of the N^1–C bond and not the intermediary formation of MMH. The response of the CO_2 production rate to SKF 525-A and 3-methylcholanthrene suggests that the N^1–C bond of procarbazine is split by a microsomal hydroxylase.

THE METHYL group of procarbazine (PCZ) appears to be associated with the mechanism of action of this compound, since only methylhydrazine derivatives display cytostatic activity.[1, 2] In previous experiments, $^{14}CO_2$ production from $^{14}CH_3$-labeled PCZ in mice and rats has been demonstrated. Furthermore, it was observed that pretreatment of the animals with phenobarbital or 3-methylcholanthrene resulted in a considerable increase in the $^{14}CO_2$ formation rate.[3, 4] The production of $^{14}CO_2$ after the administration of $^{14}CH_3$-labeled PCZ was also shown by Kreis *et al.*[5] and by Schwartz.[6] The inductive effect of phenobarbital on the $^{14}CO_2$ production was confirmed recently by Dost and Reed.[7] In the attempt to elucidate the pathway of $^{14}CO_2$ formation from $^{14}CH_3$-PCZ, three possibilities have been considered: (1) the cleavage of the N^1–CH_3 bond, i.e. direct demethylation of PCZ; (2) primary cleavage of the *N*–*N* bond,

*Natulan (Roche).

†Part of this work has been presented at the Third Conference of the German Society for Biological Chemistry on Metabolism of the isolated perfused liver, held in Oestrich/Rheingau on the 27th to 29th of April, 1967.

‡Present address: Department of Pharmacology, New York University, New York, N.Y.

§Present address: The Rockefeller University, New York, N.Y.

‖Address for reprint request.

formation of methylamine and secondary methylamine demethylation; (3) primary cleavage of the N^2–C bond, formation of monomethylhydrazine (MMH) and secondary demethylation of the latter. Evidence has been presented that methylamine is not an intermediate in the $^{14}CO_2$ production and that the cleavage of the *N–N* bond does not occur to a measurable extent in the isolated perfused rat liver.[4] Although no MMH could be detected by radiochromatography in the deep-freeze liver biopsy after administration of PCZ,[4] the question whether MMH might be an intermediate remained unsolved. By means of a kinetic analysis of the $^{14}CO_2$ production from $^{14}CH_3$-PCZ and ^{14}C-MMH, two different mechanisms of demethylation could be demonstrated for the two substrates.

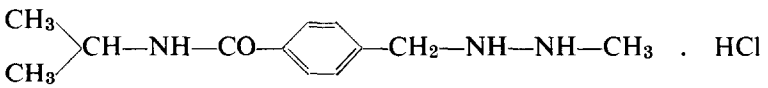

FIG. 1. Procarbazine HCl, Natulan (Roche).

MATERIALS AND METHODS

Radioactive materials. N^1-$^{14}CH_3$-procarbazine. HCl and ^{14}C-methylhydrazine. H_2SO_4 were synthesized by Dr. R. Barner and Dr. J. Würsch from the chemical laboratories of Hoffmann-La Roche, Basle.

Experiments in vivo. Male Wistar rats (strain "Füllinsdorf" Hoffmann-La Roche) weighing 100–120 g kept on a standard diet were injected i.p. with the labeled compound (0·4 m-mole per kg body weight) and immediately placed in a 750-ml glass metabolic cage. During the experiment, air was drawn by vacuum through a 10% NaOH trap into the animal cage, subsequently dried by a trap containing concentrated H_2SO_4, and methanol-saturated by a methanol gas washing bottle before entering the CO_2-trapping mixture. The air-flow rate was kept constant at 200 ml/min and controlled by a rotameter placed between the methanol gas washing bottle and the CO_2 trap.

Apparatus for the perfusion of the isolated liver. The perfusion apparatus shown in Fig. 2 is a modification of that used in earlier experiments.[3, 4] It allows an accurate and rapid measurement of the $^{14}CO_2$ formed. The temperature of the liver and the circulating medium is kept constant by two thermostated water baths; one for the multibulb glass oxygenator, the other for the liver chamber and the constant temperature coil for the inflowing medium. All parts of the apparatus, designed for two simultaneous perfusions or for two-circuit experiments, are easily accessible from three sides. The medium is pumped from the collecting vessel by way of a roller pump through a disposable plastic mesh transfusion filter. It then flows through a 1-m long constant temperature coil into the overflow vessel which is placed 20 cm above the liver. From the overflow vessel the medium moves to the liver portal cannula through a 2-mm wide plastic tubing with an adjustable microlamp which regulates the flow rate. The excess medium bypasses the liver and joins the liver outflow before entering the hydrostatic valve. A calibrated vessel in the liver outflow line is used to measure the flow rate, the outflow tubing is clamped off and the time needed to collect a 5-ml sample is recorded. The medium moves by gravity through the oxygenator back into the collecting vessel equipped with a magnetic stirrer. If CO_2 is collected, pure oxygen is used for the oxygenation of the medium. From an overflow tank humidified oxygen is drawn by vacuum through a rotameter into the oxygenator. The outgoing gas is dried

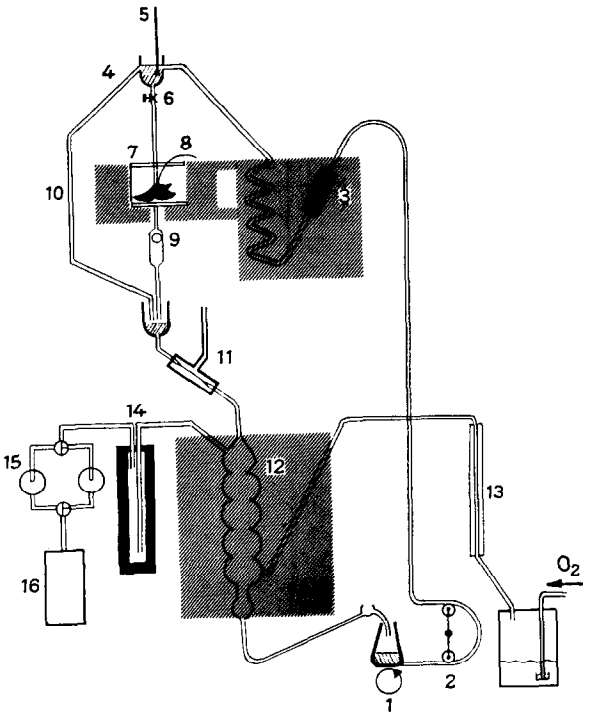

FIG. 2. Liver perfusion apparatus. 1, Collecting vessel; 2, roller pump; 3, filter; 4, overflow vessel; 5, thermometer; 6, microlamp for the regulation of the flow rate; 7, thermostated liver chamber; 8, bile duct cannula; 9, calibrated vessel for the measurement of the liver outflow rate; 10, bypass of excess medium; 11, hydrostatic valve; 12, oxygenator; 13, rotameter measuring the O_2 flow rate; 14, ice trap; 15, CO_2 absorption vessels; 16, vacuum pump; hatched areas correspond to thermostatized water baths.

in an ice trap and then passes through the CO_2 absorption vessel. Transparent polyethylene tubing (i.d. 3·5 mm) is used throughout the apparatus except for the overflow tubing and the pump tubing which are made of silicon rubber (i.d. 6·0 mm). The isolated liver is placed on a round lucite plate with the portal vein upward and the outflow cannula going through a center hole. The plate is placed at the bottom of the liver chamber and the portal cannula is connected to the inflow tube. The bile duct cannula is fixed to the top of the chamber and the liver is covered with moist lens paper to avoid desiccation. The chamber is then closed by a lucite thermostatic jacket. The temperature on the liver plate in the closed chamber is maintained at 36–37°. The bile is collected in a small vessel on top of the thermostated jacket.

Perfusion experiments. The liver was isolated from male rats weighing 230–250 g as described by Schimassek.[8] The perfusion medium was prepared by diluting 40 vol. of fresh bovine erythrocytes (washed three times with Ringer-phosphate-bicarbonate solution. The medium (100 ml) contained 9 to 9·5 g hemoglobin, 1·5 g dried bovine albumin, 0·4 g glucose and 7·5 mg Aureomycin. For each experiment, 200 ml of this medium was used. The initial pH of the medium was 7·45 and dropped to 7·2 to 7·3 after 3 hr of perfusion if pure oxygen was used for the oxygenation. The oxygen flow rate was kept constant at 180 ml per min and the temperature at 37 ± 0·5°. During

the first 10–15 min, the perfusion rate was adjusted to 2·5 ml/min per g of liver and then reduced to 1·5 ml/min per g of liver. The labeled substrate, dissolved in 2 ml of isotonic NaCl solution, was added to the collection vessel 45 min after the perfusion was started. In all experiments, the initial concentration of the substrate in the perfusion medium was 5×10^{-4} M.

Measurement of radioactive CO_2. In vivo as well as in the perfusion experiments, CO_2 was trapped directly in Packard scintillation counting vials containing 11 ml of a mixture of 20 vol. of ethanolamine and 80 vol. of methanol.[9] After addition of 10 ml of scintillation fluid (4 g of BBOT (Ciba) per l. of reagent grade toluene) the radioactivity was counted in a Packard scintillation spectrometer model 314 EX. The formation of radioactive CO_2 was measured in 15-min fractions during the first 3 hr

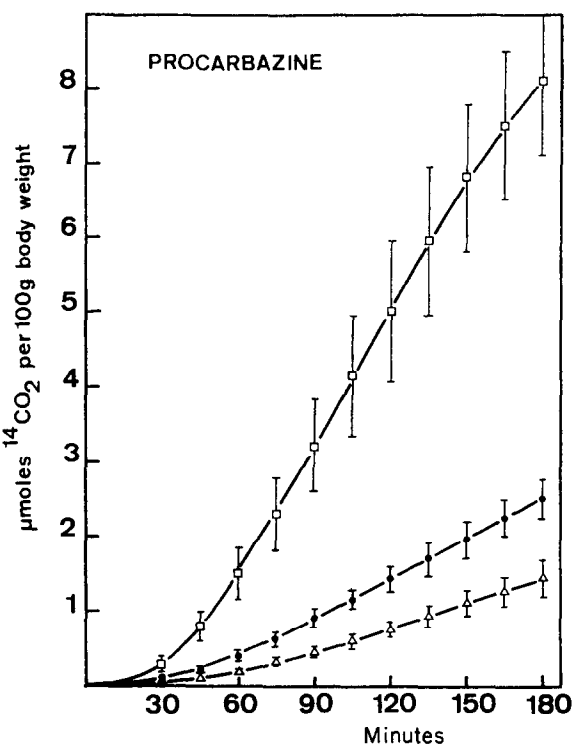

FIG. 3. Demethylation of procarbazine in the isolated perfused rat liver. ●———●, livers without pretreatment; □———□, pretreatment with 3-methylcholanthrene; △———△, pretreatment with SKF 525-A. Each symbol represents the mean value of six to nine experiments. The vertical bars stand for the S.D.

after the administration of the labeled compound. The demethylation rate was calculated for the steady-state period of the CO_2 production curve (see Fig. 3).

Pretreatment with modifiers of the oxidative microsomal activity. A single dose of 20 mg/kg of 3-methylcholanthrene dissolved in peanut oil was injected i.p. 48 hr before the experiment. For some experiments *in vivo* induction was also achieved by pretreatment with phenobarbital, injected for 5 consecutive days at a daily dose of 100 mg/kg. The labeled substrate was given 48 hr after the last phenobarbital injection.

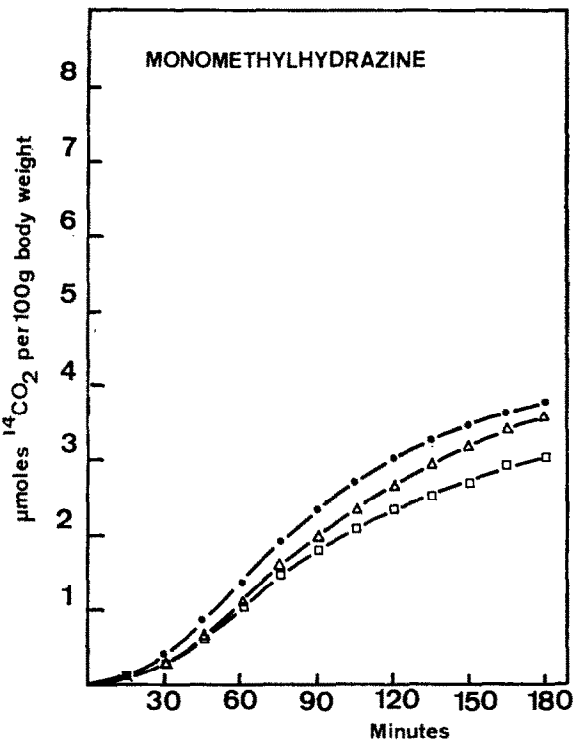

FIG. 4. Demethylation of monomethylhydrazine in the isolated perfused rat liver. ●———●, Livers without pretreatment; □———□, pretreatment with 3-methylcholanthrene, △———△, pretreatment with SKF 525-A. Each symbol represents the mean value of five to seven experiments. S.D. were omitted for graphical reasons but were found to be of the same order as in the case of PCZ (Fig. 3).

SKF 525-A was given i.p. (50 mg/kg) 1 hr before the labeled substrate in the experiments *in vivo*. In the perfusion experiments, SKF 525-A was added to the perfusion medium 1 hr before the addition of the labeled compound in a final concentration of 5×10^{-5} M ($\frac{1}{10}$ of the substrate concentration).

RESULTS

Oxidative demethylation of procarbazine. In the intact rat as well as in the isolated perfused rat liver, the rate of $^{14}CO_2$ formation after the administration of ^{14}C-methyl labeled PCZ is strongly affected by the presence of SKF 525-A and by the pretreatment of the animals with 3-methylcholanthrene (Table 1, Fig. 3). In the perfusion experiments 5×10^{-5} M SKF 525-A ($\frac{1}{10}$ of the PCZ concentration) lowers the $^{14}CO_2$ production rate in the steady-state period to 50 per cent of the control value (Table 1). In order to obtain an inhibition of the same extent in the experiments *in vivo* a relatively higher dose of SKF 525-A is required. In this case, the molar ratio of SKF 525-A/PCZ has to be 1:3. The pretreatment of the rats with 3-methylcholanthrene results in a more than 3-fold increase of the $^{14}CO_2$ production rate as measured in the steady-state period, the induction factor (fold increase over the control value) being 3·5 for the experiments *in vivo* and 3·1 for the isolated perfused liver (Table 1). After pre-

treatment of the rats with phenobarbital, a similar enhancement of the $^{14}CO_2$ formation rate is observed in experiments *in vivo*. In this case an induction factor of 2·5 is found.

Oxidative demethylation of monomethylhydrazine. Under the described experimental conditions, the $^{14}CO_2$ production rate after the administration of ^{14}C-MMH is about three times lower in the intact rat than in the isolated perfused rat liver. Under control conditions, as well as in the presence of SKF 525-A the $^{14}CO_2$ production rate is

TABLE 1. REACTION RATES OF THE DEMETHYLATION OF PROCARBAZINE AND MONOMETHYLHYDRAZINE *in vivo* AND IN THE ISOLATED PERFUSED RAT LIVER*

Pretreatment		Procarbazine	Monomethylhydrazine
None (control)	*In vivo*	1·83 ± 0·08 (100)	0·70 ± 0·10 (100)
	Isolated liver†	1·18 ± 0·19 (100)	2·08 ± 0·14 (100)
SKF 525-A	*In vivo*	0·98 ± 0·07 (54)	
	Isolated liver†	0·60 ± 0·11 (51)	1·80 ± 0·17 (86)
3-Methyl-cholanthrene	*In vivo*	6·39 ± 0·07 (349)	0·70 ± 0·03 (100)
	Isolated liver†	3·66 ± 0·51 (310)	1·54 ± 0·10 (74)
Phenobarbital	*In vivo*	4·57 ± 0·18 (250)	

*The rates are given in μmoles $^{14}CO_2$ per hr per 100 g body weight as calculated from the steady-state period and represent mean values ± S.D. of five to nine experiments. In brackets, relative demethylation rates, control experiments equal 100.

†The demethylation rates are corrected for the very small $^{14}CO_2$ production (1–2 per cent), occuring when the circulating perfusion medium bypasses the liver.

significantly higher with MMH as the substrate than with PCZ. On the contrary, after pretreatment with 3-methylcholanthrene, the rate obtained with MMH is less than one-half of that obtained with PCZ (Table 1).

In the isolated perfused rat liver, both pretreatment with 3-methylcholanthrene and administration of SKF 525-A have little effect on the $^{14}CO_2$ production rate from MMH. In both cases, the steady-state rate is slightly lower than in the control experiments. It must be emphasized that the inducer, 3-methylcholanthrene, causes a 26% decrease of the $^{14}CO_2$ production rate from MMH. In contrast to what has been observed with PCZ as the substrate, the $^{14}CO_2$ production curves are here characterized by an early and relatively short steady-state period. *In vivo*, the pretreatment with 3-methylcholanthrene is without effect.

DISCUSSION

The rate of CO_2 production from PCZ and MMH is affected in different ways by modifiers of the activity of the microsomal hydroxylases such as SKF 525-A and 3-methylcholanthrene. The CO_2 production rate from PCZ is also enhanced by the pretreatment with phenobarbital. These rates of CO_2 production can be taken as a measure of the metabolism of the methyl group of PCZ and MMH since in these experiments the oxidation of formaldehyde to CO_2 is not a rate-limiting step. Under control conditions, the rate of formaldehyde and formate oxidation to CO_2 in rat liver is several-fold higher than the measured CO_2 production rate from PCZ and MMH.[10] As demonstrated by Werner *et al.*,[11] hydrazine derivatives do not inhibit the oxidation of either formaldehyde or formate. Finally, SKF 525-A and 3-methyl-

cholanthrene appear to have little, if any effect on the oxidation of formaldehyde to CO_2, as shown by experiments on the demethylation of methylamine.[4]

The fact that after treatment with 3-methylcholanthrene the rate of CO_2 production from PCZ is considerably higher than that obtained from MMH suggests that MMH is not an intermediate of the main pathway of CO_2 formation from PCZ. Since under identical conditions, the *N*—*N* bond of PCZ is not split to a measurable extent,[4] the formation of CO_2 from PCZ appears to involve the direct cleavage of the N^1—C bond. The increase of the CO_2 production rate from PCZ induced by the treatment with 3-methylcholanthrene or phenobarbital, as well as the decrease after the administration of SKF 525-A, indicates, that the cleavage of the N^1—C bond is catalyzed by a microsomal hydroxylase. A similar mechanism could be demonstrated for the splitting of the N^2—C bond, as shown in the following paper.[12]

PCZ is rapidly oxidized to the corresponding AZO derivative *in vivo*[13] as well as in the isolated perfused rat liver.[12] Because of its rapid formation and accumulation,[12] the AZO compound, which exhibits the same cytostatic activity as PCZ itself, has therefore to be considered as the main substrate of the N^1—C splitting microsomal hydroxylase. Direct demethylation of the parent compound, PCZ, is possible but considering the rapidity by which the AZO derivative is formed, its contribution to the overall rate is probably small.

SKF 525-A and 3-methylcholanthrene have only minor effects on the CO_2 production rate from MMH. These data may suggest that CO_2 is formed from MMH and PCZ by different mechanisms and that MMH may not be demethylated by a microsomal hydroxylase. The effect of 3-methylcholanthrene on the CO_2 production rate from MMH could in fact also be due to the induction of the competitive pathway by which MMH is converted to methane.[14] Dost and Reed, however, found no increase in methane formation from PCZ after pretreatment with phenobarbital.[7]

Dost *et al.*,[7, 14] as well as Schwartz *et al.*,[15] found significant amounts of labeled methane in the expiratory air of rats after administration of both $^{14}CH_3$-MMH and $^{14}CH_3$-PCZ. The amount of methane formed in 24 hr from MMH is three to four times higher than that of CO_2. In the case of PCZ, however, CO_2 is the major volatile metabolite. A CO_2/CH_4 ratio of about 2 has been found *in vivo* 90 min[15] and 8 hr[7] after administration of PCZ. Methane formation from PCZ has been interpreted by Dost and Reed[7] as evidence for the intermediary occurrence of MMH, in accordance with the metabolic pathway of PCZ proposed by Raaflaub and Schwartz[13] and by Oliverio *et al.*[16] Results presented in the following paper[12] do not support this metabolic scheme but do not rule out the formation of small amounts of MMH by the oxidative cleavage of the *N*–^{2}C bond of PCZ, which is discussed as a minor pathway in PCZ metabolism.[12]

In the isolated perfused rat liver, MMH is demethylated (CO_2 production) at a 3-fold higher rate than *in vivo*. This discrepancy can hardly be explained by the different tissue distribution of the substrate in both systems even if one takes into account the rapid renal excretion of MMH.[6] It could, however, be that the rapid conversion of MMH to methane occurs extrahepatically, and that therefore only small amounts of substrate *in vivo* would be available for demethylation.

Acknowledgements—The authors are indebted to Mr. Jakob Trachsel for the construction of the perfusion apparatus and to Mr. Daniel Burkhard for valuable technical assistance.

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