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OXIDATION OF *p*-(N^1-METHYLHYDRAZINO METHYL) -*N*-ISOPROPYL BENZAMIDE (PROCARBAZINE)* TO THE METHYLAZO DERIVATIVE AND OXIDATIVE CLEAVAGE OF THE N^2–C BOND IN THE ISOLATED PERFUSED RAT LIVER†

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Abstract—The oxidation of *p*-(N^1-methylhydrazino methyl)-*N*-isopropyl benzamide (procarbazine, Natulan) to its azo-derivative (AZO) and the formation of the main inactive metabolite terephthalic acid isopropylamide (TAC) has been studied in the isolated perfused rat liver. In this system, procarbazine was oxidized to AZO at a rate of about 28 μmoles/hr as calculated for 100 g body weight, whereas TAC was produced at a 4-fold lower rate (7 μmoles/hr per 100 g body weight). This results in an accumulation of the lipophilic, cytostatically active AZO. The effect of SKF 525-A, as well as 3-methylcholanthrene, on the rate of TAC production and AZO disappearance indicates that the N^2–C bond of AZO is split by a microsomal hydroxylase. Methyldiimine, which is the theoretically expected product of this reaction beside *p*-formyl-*N*-isopropyl benzamide, is discussed as the possible source of methyl radicals. Such radicals have been suggested by others to be responsible for the cytostatic effect of procarbazine.

AFTER the administration of procarbazine (PCZ), a cytostatically active methylhydrazine derivative, three main metabolic products have been found: (1) The methylazo derivative (AZO); (2) terephthalic acid isopropylamide (TAC); and (3) carbon dioxide. The oxidation of PCZ to AZO proceeds very rapidly *in vivo*, as demonstrated by experiments in dogs and rats as well as by observations in man.[1] Terephthalic acid isopropylamide (TAC) has been found to be the main metabolite eliminated in urine.[1, 2] The conversion of PCZ to AZO has to be considered as a pharmacokinetically relevant step, since AZO is far more lipophilic than PCZ and still has the cytostatic activity of the latter.[3] The large amount of cytostatically inactive TAC excreted in urine demonstrates the importance of the cleavage of the N^2–C bond of PCZ and AZO as an inactivation reaction. This paper describes an investigation of the two metabolic reactions mentioned above since demethylation has been the subject of a previous article.[4] The technique of the isolated perfused rat

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liver was used because it allows a quantitative comparison between the rates of AZO, TAC and CO_2 formation.

MATERIALS AND METHODS

Radioactive materials and other special compounds. *p*-(N^1-methylhydrazino methyl)-*N*-isopropyl-^{14}C benzamide-HCl (^{14}C-carbamoyl-procarbazine) was synthesized by Dr. R. Barner and Dr. J. Würsch from the chemical departments of Hoffman-La Roche, Basle. Unlabelled procarbazine (PCZ), *p*-(N^1-methylazo methyl)-*N*-isopropyl benzamide, the azo-derivative of PCZ (AZO), *p*-(N^1-methylhydrazo methylene)-*N*-isopropyl benzamide (HYDRAZONE), the aldehyde *p*-formyl-*N*-isopropyl benzamide (ALD) and the corresponding acid, terephthalic acid isopropylamide (TAC), were also obtained from Hoffmann-La Roche.

Perfusion experiments. The experiments were performed as described in the previous article.[4] The substrate (^{14}C-carbamoyl-labeled PCZ) was added to the perfusion medium at a concentration of 5×10^{-4} M. At given intervals, aliquots of the perfusion medium were removed and analyzed for substrate and metabolite concentration. The pre-treatment with modifiers of the oxidative microsomal activity (3-methylcholanthrene and SKF 525-A) has been described by Dewald *et al.*[4]

Isolation of procarbazine (PCZ), its azo derivative (AZO) and terephthalic acid isopropylamide (TAC) from the perfusion medium. The concentration of PCZ and its metabolites was determined after isolation from the erythrocyte-free supernatant of the perfusion medium. In all samples protein was precipitated by addition of perchloric acid in a final concentration of 5% (v/v). The acid medium insures stability

TABLE 1. SPECTROPHOTOMETRICAL EVIDENCE FOR THE QUANTITATIVE CONVERSION OF AZO AND HYDRAZONE INTO ALD

Amounts of AZO and HYDRAZONE in test mixtures* (nmoles)			O.D. 253 in ethylene dichloride extract	Amount of ALD in extract (nmoles)	Percentage recovery
AZO	HYDRAZONE	Total			
200	0	200	0·676	204	102
0	200	200	0·660	200	100
100	100	200	0·646	196	98
40	200	240	0·788	238	99
200	40	240	0·792	240	100
200	200	400	1·290	390	97·5

* Test mixtures were prepared by dissolving the listed amounts of AZO and HYDRAZONE in 2 ml of 30 mM Na phosphate buffer, pH 7·4. These solutions were acidified by the addition of 0·5 ml of 25% (v/v) perchloric acid, and, after 5 min, extracted in 5 ml of ethylene dichloride. The u.v. spectrum of the ethylene dichloride phase was recorded and the O.D. 253 was measured.

of PCZ against autoxidation during the extraction procedures. Furthermore, the acidification results in an immediate and quantitative isomerization of AZO to HYDRAZONE followed by hydrolysis of the latter to ALD and methylhydrazine.[2] This rapid conversion of AZO and HYDRAZONE to ALD is demonstrated in Fig. 1 and Table 1, as well as by the fact that infra-red spectra of the compound which is extracted with ethylene dichloride from solutions of AZO and HYDRA-

ZONE in 5% perchloric acid coincide with that of ALD.* The isomerization and hydrolysis of AZO at room temperature were also followed on silica gel plates by the method of Baggiolini and Dewald.[5] Consequently, the determination of AZO is based on the measurement of the amount of ALD formed after addition of perchloric acid. The AZO estimation is not affected by the presence of ALD resulting from the metabolism of PCZ since ALD is very rapidly oxidized to TAC by an aldehyde dehydrogenase present in the system (see Results).

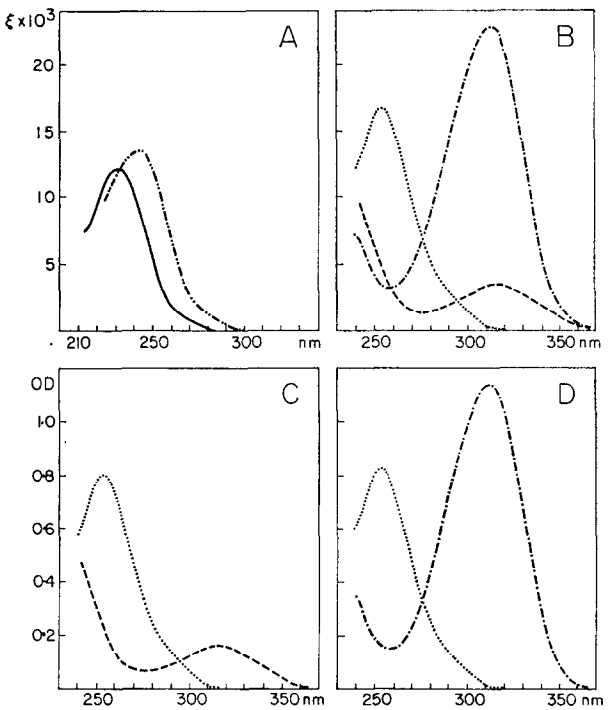

FIG. 1. Absorption spectra of procarbazine and metabolites. A. ————, procarbazine·HCl in water; —··—··—, TAC in 1 N NaOH. B. — — — —, AZO; —·—·—·—, HYDRAZONE; and ....., ALD in ethylene dichloride. C. AZO extracted in ethylene dichloride at pH 7·0 (— — — —) and 5 min after addition of perchloric acid (.....). D. HYDRAZONE extracted in ethylene dichloride at pH 7·0 (—·—·—·) and 5 min after addition of perchloric acid (.....). The concentration of AZO and HYDRAZONE in C and D is 5×10^{-5} M.

The methods used by other workers[1, 2, 6] for the separation of PCZ, AZO and TAC have been modified. All extraction steps have been tested spectrophotometrically and in some cases by radioactivity measurement with PCZ, AZO, HYDRAZONE, ALD and TAC dissolved in the erythrocyte-free supernatant of the perfusion medium at concentrations varying between 5×10^{-5} and 5×10^{-4} M. The per cent recovery of these compounds in the phase used for their determination was in every instance 97 to 101 per cent.

* The infra-red spectra were recorded by Dr. W. Michaelis, Research Institute, Wander Corp., Berne, Switzerland.

In the perfusion experiments, two extraction procedures were used; one for the separation of PCZ and TAC, the other for AZO (determined as ALD). In the first case, 1 ml of erythrocyte-free supernatant was mixed with 1 ml of 10% perchloric acid and extracted twice with 5-ml portions of a water-saturated mixture of chloroform : ethanol (6:1); 1 ml of the aqueous phase was used for the determination of PCZ In order to isolate TAC from the organic phase, 3-ml aliquots of each chloroform: ethanol extract were pooled and re-extracted with 2 ml of 1 N NaOH; 1 ml of the NaOH phase was then used for the measurement of TAC. For the determination of AZO, 0·5 ml of the supernatant was acidified by addition of 1·5 ml of 7% perchloric acid and then extracted with 5 ml of ethylene dichloride. The organic phase was subsequently re-extracted with 2 ml of 1 N NaOH to remove minor amounts of TAC. Three ml of the ethylene dichloride phase was used for the determination of AZO. All extractions were carried out for 10 min at room temperature in 1·5 × 15 cm glass centrifuge tubes with polyethylene caps.

In preliminary liver perfusion experiments, the AZO and TAC fractions, isolated by the described extraction procedure, were characterized spectrophotometrically. In these experiments, no Aureomycin was added to the perfusion medium to avoid interference with the spectrophotometrical determination of TAC. Spectrophotometrical analysis of the ethylene dichloride extract used for the determination of radioactive AZO showed a unique peak with a maximum at 253 nm, coinciding with that of ALD (Fig. 1). In the NaOH phase used for the determination of radioactive TAC, a slightly asymmetrical peak, with maximum at 245 nm and almost identical to that of TAC (Fig. 1) was recorded. For both ALD and TAC a good correlation of spectrophotometry and radioactivity measurement was observed. PCZ could not be identified directly in the perfusion medium since it is partially oxidized to AZO during extraction in organic solvents at neutral pH.

Measurement of radioactivity. The radioactivity was measured in a Packard scintillation spectrometer, model 314 EX. The following two scintillation solutions were used: SI, 5 parts of toluene containing BBOT (4 g/l.) and 1 part of methanol; SII, *m*-xylene (1 part), dioxane (3 parts) and cellosolve (3 parts with addition of naphthalene (50 g/l.) and BBOT (5 g/l.). The activity of 3-ml aliquots of the ethylene dichloride phase (determination of AZO) was measured after addition of 12 ml of solution SI. The activity of 1-ml aliquots of the two aqueous phases containing either PCZ or TAC was measured in 15 ml of the more polar solution SII. For each experiment, the specific activity of the substrate solution was determined in these quenched mixtures. A further correction for quenching was made by means of the channel ratio method.

Experiments in vitro. Experiments *in vitro* were made with rat liver homogenate, a 250,000 *g* min supernatant containing microsomes and soluble fraction but practically free of mitochondria and a 6,000,000 *g* × min supernatant. The protein content of these preparations was determined by the Kjeldahl method. ALD was isolated from the acidified reaction mixture by extraction with 5·0 ml of ethylene dichloride. The organic phase was re-extracted with 2·0 ml of 1 N NaOH to remove minor amounts of TAC and finally re-extracted with 2·0 ml of 0·1 N HCl to remove traces of alkali. ALD was then determined spectrophotometrically at 253 nm in the ethylene dichloride phase. AZO and HYDRAZONE were estimated spectrophotometrically at 312 nm in the ethylene dichloride extract obtained at neutral pH.

RESULTS

TAC production as a measure of the N^2*–C bond cleavage.* Experiments *in vitro* show that ALD, the primary product formed by the cleavage of the N^2–C bond of PCZ or AZO, is rapidly converted into the corresponding acid (TAC). This reaction is catalyzed by an NAD-linked dehydrogenase occurring in the soluble fraction of rat liver homogenate (Fig. 2). The rate of dehydrogenation of ALD is not affected by the presence of PCZ and/or AZO (10^{-3} to 5×10^{-3} M). Its activity is 50–80 times higher than the rate of TAC production from PCZ. Therefore, TAC formation is a measure of the rate at which the N^2–C bond of PCZ or AZO is split, as the rate of CO_2 production is a measure of demethylation.[7]

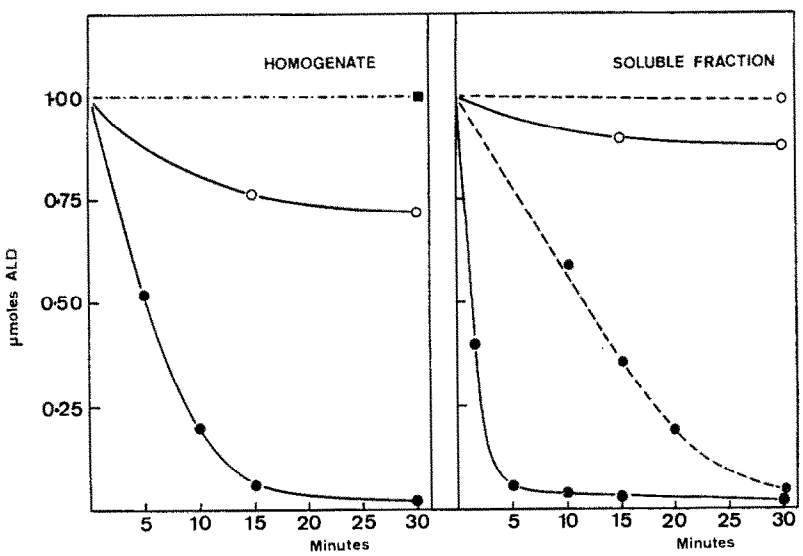

FIG. 2. Dehydrogenation of *p*-formyl-*N*-isopropyl benzamide (ALD) to terephthalic acid isopropylamide (TAC) by rat liver homogenate and soluble fraction (6,000,000 *g* × min supernatant) *in vitro*. Homogenate corresponding to 40 mg of fresh liver (————); blank, without biological material (— · — · — · —). Soluble fraction corresponding to 3 mg (————) and 0·6 mg (— — — —) of protein respectively. Filled symbols represent incubation with, open symbols without, addition of NAD 1 mM. Initial concentration of ALD was 0·5 mM.

Oxidation of PCZ to AZO and cleavage of the N^2*–C bond.* As in the experiments on demethylation,[4] the rates of PCZ oxidation and TAC formation have been measured in the isolated perfused rat liver under three different experimental conditions: (1) In livers without treatment (control experiments); (2) in livers treated with SKF 525-A; and (3) in livers of animals pretreated with 3-methylcholanthrene.

Figure 3 shows the time-dependent changes of the concentration of PCZ and its metabolites, AZO and TAC, in the perfusion medium. Since these compounds are neither accumulated in liver nor excreted to any significant extent in the bile, their concentration in the perfusion medium can be regarded as representative for their metabolism. In all three types of experiments, the initial phase is characterized by a rapid decrease of the PCZ concentration and the concomitant increase of the AZO

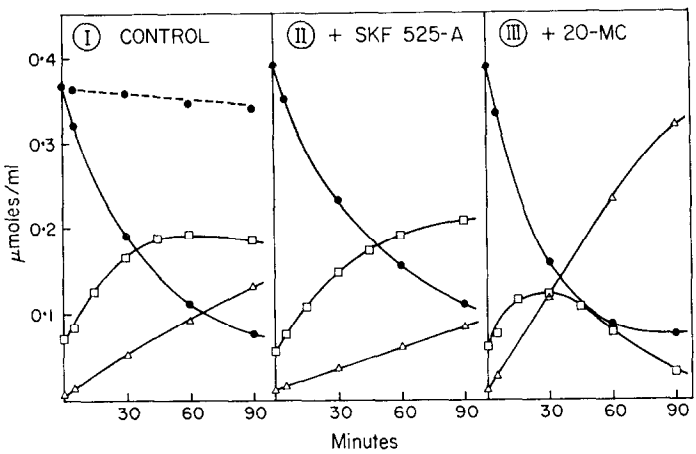

FIG. 3. Oxidation of procarbazine and formation of terephthalic acid isopropylamide in the isolated perfused rat liver. ●——●, PCZ; □——□, AZO; △——△, TAC; ●- - - -●, blank curve for PCZ. The symbols represent mean values of three experiments: I. Control experiments, liver without treatment; II. SKF 525A (5×10^{-5} M) added to the perfusion medium 60 min before the substrate; III. Livers of rats treated with 3-methylcholanthrene (20 mg/kg i.p.) 48 hr before the experiment.

level. This reaction only occurs when the liver is perfused. If the experiment is conducted in a perfusion circuit bypassing the liver, the rate of PCZ disappearance does not exceed 2 to 3 per cent of the value obtained in experiments under control conditions (Fig. 3, I). Modifiers of the activity of microsomal hydroxylases have only small effects on the rate of disappearance of PCZ. The rate is slightly decreased in the presence of SKF 525-A and slightly increased after treatment with 3-methylcholanthrene. On the contrary, both agents have strong effect on the rate of TAC formation. In the presence of SKF 525-A, the rate of TAC production is reduced to one-half of the control and after 3-methylcholanthrene treatment an almost 3-fold increase of this rate is observed (Table 2).

Under the three selected experimental conditions, the AZO concentration curves exert a different shape (Fig. 3). In the control experiments, the maximal AZO level is reached after about 45–60 min and is followed by a slow but constant decrease. In the presence of SKF 525-A, the AZO concentration is continuously increasing during the 2 hr of perfusion. After the treatment with 3-methylcholanthrene, a brief increase in the AZO concentration is followed by a rapid decrease to an extremely low level.

DISCUSSION

The metabolism of PCZ in the isolated perfused rat liver is characterized by the high rate at which PCZ is oxidized to AZO. Under control conditions, the calculated rate of about 28 μmoles/hr per 100 g body weight (Table 3) is several times higher than that of the other reactions studied. The mechanism of the PCZ oxidation cannot be completely explained by the present results. SKF 525-A slightly decreases and 3-methylcholanthrene slightly increases the rate at which PCZ disappears from the perfusion medium (Table 3). The inhibitory effect of SKF 525-A is also reflected by a diminished rate of AZO formation (Fig. 3). (An effect of 3-methylcholanthrene on

TABLE 2. INCUBATION OF AZO AND HYDRAZONE WITH RAT LIVER PREPARATIONS*

Additions to:	Substrate	
33 mM Na, K phosphate buffer, pH 7·4 40 mM KCl 10 mM $MgCl_2$ 0·5 mM substrate	AZO	HYDRAZONE
	(O.D. 312 of ethylene dichloride extract)	
None	0·655	3·975
Homogenate + NAD	0·650	3·875
250,000 g × min supernatant + NAD	0·645	3·775
250,000 g × min supernatant + NAD + NADPH generating system	0·495	3·400
6,000,000 g × min supernatant + NAD	0·660	3·880

* One μmole of substrate, dissolved in 0·05 ml of methanol, was added to 2 ml of final mixture and incubated at 37° for 30 min. The reaction was stopped by extracting with 5 ml of ethylene dichloride. Homogenate corresponded to 40 mg of liver, 250,000 g × min supernatant to 11 mg of protein, 6,000,000 g × min supernatant to 8 mg of protein. NAD was 1 mM, the NADPH generating system was composed of 10 mM G6-P, 0·25 mM NADP and 0·1 i.u. of G6-P dehydrogenase.

TABLE 3. RATES OF DISAPPEARANCE OF PROCARBAZINE AND FORMATION OF TEREPHTHALIC ACID ISOPROPYLAMIDE IN THE ISOLATED PERFUSED RAT LIVER*

Treatment	Experiment No.	PCZ disappearance	TAC formation
		(μmoles/hr per 100 g body weight)	
None (control)	1	26·29	6·86
	2	30·72	7·52
	3	28·16	6·72
	Avg.	28·39 (100)	7·03 (100)
Pretreatment with 3-methylcholanthrene	4	31·66	18·72
	5	39·52	17·74
	6	40·00	20·00
	Avg.	37·06 (130)	18·82 (268)
Pretreatment with SKF 525-A	7	25·44	3·68
	8	21·54	4·46
	9	25·81	3·47
	Avg.	24·26 (85)	3·87 (55)

* The data were calculated from the experiments illustrated in Fig. 2. In brackets, relative activity (control experiments = 100).

the same rate cannot be shown because under these conditions AZO rapidly undergoes further metabolization.) This observation would be in accordance with results of experiments *in vitro*, which have shown that liver microsomes oxidize PCZ to AZO in the presence of NADPH and that this reaction is strongly inhibited by SKF 525-A.* However, the fact that neither SKF 525-A nor 3-methylcholanthrene significantly affect the rate of PCZ oxidation suggests that a major part of the oxidative activity cannot be accounted for by an enzyme behaving like a microsomal hydroxylase.

Addition of SKF 525-A to the perfusion medium reduced the rate of TAC formation to 55 per cent of the control, whereas pretreatment of the animals with 3-methylcholanthrene increased the same rate 2·7-fold. These results suggest that the N^2–C bond of PCZ or AZO is split by a microsomal hydroxylase. The mechanism of this reaction appears to be very similar to that of the cleavage of the N^1–C bond previously described.[4] In fact, under the same experimental conditions, the relative decrease after inhibition of the microsomal hydroxylases, as well as the relative increase after their induction, is of the same extent for both TAC and CO_2 formation. The absolute rate, however, is five to six times higher for TAC production than for CO_2 formation.†

Theoretically, both PCZ and AZO can be regarded as substrates of the N^2–C splitting hydroxylase. The interrelationship between the AZO and TAC concentration curve shows that AZO is, in fact, a substrate. When the TAC production rate is low (Fig. 3, II) the AZO level increases steadily during the 2 hr of perfusion, whereas at a five times higher TAC production rate, an early and rapid decrease of the AZO concentration is observed (Fig. 3, III). A further indication that AZO has to be considered as the main substrate of the N^2–C splitting hydroxylase is given by its early formation at a rate similar to that of PCZ disappearance. The concomitant oxidative cleavage of PCZ cannot be excluded by the present results. It appears, however, to be quantitatively of minor importance because of the rapid oxidation of PCZ to AZO.

It finally has to be shown that AZO itself and not its isomer HYDRAZONE is the precursor of TAC. The pathway AZO → HYDRAZONE → ALD → TAC[1,2] would require an AZO isomerizing enzyme, as well as a hydrolase splitting the HYDRAZONE formed. A nonenzymatic mechanism can be excluded because both AZO and HYDRAZONE remain unchanged during several hours of incubation in saline at pH 7·4 and 37°. Experiments *in vitro* with rat liver preparations (Table 2) fail to give any evidence for the isomerization of AZO. The conversion of even small amounts of AZO into HYDRAZONE would result in a measurable and significant increase in O.D. at 312 nm since the molar extinction coefficient of HYDRAZONE is several times higher than that of AZO (Fig. 1). Further evidence against the AZO → HYDRAZONE → ALD → TAC pathway is given by the observation that the formation of TAC is strongly inhibited by SKF 525-A and highly enhanced by 3-methylcholanthrene, which

* M. Baggiolini and B. Dewald, in preparation.

† Since demethylation reactions take place simultaneously with PCZ oxidation and N^2-C bond cleavage, the production of demethylated analogues of either PCZ or AZO has to be considered. These two compounds, still carrying the ^{14}C label in the carbamoyl group, are extracted together with the corresponding methylated substances. Therefore, their presence does not appreciably change the overall rate of either PCZ oxidation or TAC formation. From the CO_2 formation rate, it can be calculated that the highest amount of demethylated metabolites to be expected does not exceed 15 per cent of the total radioactivity of the fractions. For this reason, no distinction between PCZ and demethylated PCZ, as well as AZO and demethylated AZO, was made in the present study.

implies the existence of a rate limiting hydroxylation step between AZO and TAC.

In the light of the discussed results (see also reference 4), a scheme of the PCZ metabolism can be proposed in which the oxidation of PCZ to the AZO derivative and the N^2–C cleavage of the latter compound appear to be the most important steps (Fig. 4). The demethylation reactions and the breakdown of the methylhydrazine are discussed by Dewald *et al.*[4]

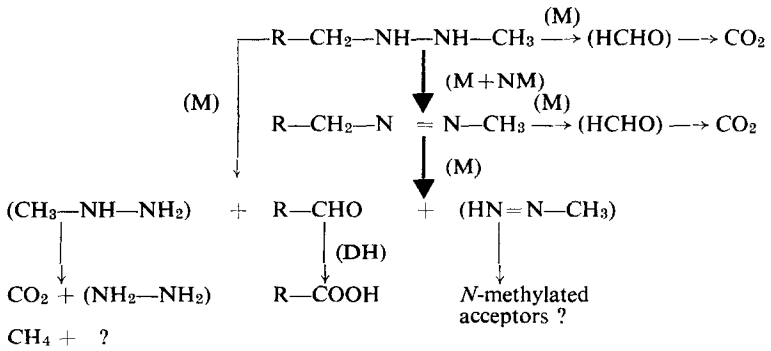

FIG. 4. Pathways of procarbazine metabolism. Products in brackets have not been identified. The other compounds have been identified in the perfusion experiments or in related assays *in vitro*. Methane has been detected by Dost and Reed[8] as well as by Schwartz *et al.*[9] Symbols: R = $(CH_3)_2$-CH-NH-CO-C_6H_4-; (M) = microsomal hydroxylase; (NM) = oxidation other than by microsomal hydroxylase; (DH) = NAD-linked dehydrogenase.

The pharmacokinetical parameters of PCZ determined in this study demonstrate that AZO is the active metabolite[3] with the longest half-life. Under control conditions (Fig. 3, I) AZO is rapidly formed and rather slowly broken down; moreover, AZO, owing to its high liposolubility, is not excreted through the kidney.[2, 8] The mixed-function oxidative cleavage of the N^2–C bond of AZO, which has been shown here to be the main pathway of metabolism implies the formation of ALD (*p*-formyl-*N*-isopropyl benzamide) and methyldiimine (HN-N-CH_3). The metabolism and the biological effects of methyldiimine are not known. This compound, however, spontaneously or after oxidation to diazomethane, could provide methyl radicals for the methylation reactions reported by Kreis *et al.*[10, 11] The same kind of radicals could also arise from the homolytic fission of AZO itself, as discussed by Schwartz *et al.*[9] This view, however, finds little support in the experimental results presented here, which clearly show that AZO is quite stable when incubated at 37° and pH 7·4 in phosphate buffer as well as with certain rat liver preparations, but is degraded by a microsomal hydroxylase both *in vitro* (Table 2) and in the isolated liver (Fig. 3).

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