Biochemical Pharmacology, 1965, Vol. 14, pp. 1085–1092. Pergamon Press Ltd., Printed in Great Britain.

THE METABOLISM OF THE HERBICIDE DIPHENAMID* IN RATS

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(*Received* 30 *November* 1964; *accepted* 17 *February* 1965)

Abstract—The *in vivo* metabolism of the herbicide diphenamid (N,N-dimethyldiphenylacetamide) was studied in rats. The compound appeared to be well absorbed and relatively easily metabolized to excretable metabolites. The main route of metabolism was found to be N-dealkylation to nordiphenamid which in turn was excreted as an N-glucuronide. The most interesting metabolite found in urine was the O-glucuronide of N-methyl-N-hydroxymethyl diphenylacetamide. The N-hydroxymethyl compound, often suggested as an unstable intermediate in the N-demethylation reaction, was in this case stabilized by glucuronide formation and excreted in urine. A minor pathway of metabolism of diphenamid was found to be *p*-hydroxylation.

IN A recent study described in this journal[1] it was shown that N-methylated barbiturates and other amides are oxidatively demethylated by mammalian liver microsomes. Among the substrates investigated was the herbicide, diphenamid (N,N-dimethyldiphenylacetamide). This compound, which elicits interesting physiological responses in various plants,[2, 3] was found to be demethylated by both rat and rabbit liver microsomes. Since diphenamid is now widely used in agriculture as a herbicide on food crops, it seemed advisable to study its metabolism in the intact animal. In addition to supplying valuable information concerning the fate of diphenamid, these studies offered an opportunity to compare the *in vitro* and *in vivo* demethylation of this amide. Since the diphenamid molecule is characterized by chemical inertness and by a high degree of water insolubility, it was also of interest to assess the influence of these factors upon the rates of metabolism and transport.

Radiocarbon labeling was utilized to facilitate the experimental work.

MATERIALS AND METHODS

Labeled compounds

Diphenamide-N-methyl-^{14}C was synthesized as follows. Diphenylacetylchloride was synthesized by allowing 132·5 mg (0·625 mM) of diphenylacetic acid to react with 0·113 ml (1·31 mM) oxalyl chloride in 1 ml dry benzene containing two drops of a very dilute solution of pyridine in benzene. Complete solution, indicating complete reaction, was attained after allowing the reaction mixture to stand for 2 hr at room temperature. The solvent was removed *in vacuo*, and a mixture of 40·7 mg (0·5 mc) ^{14}C-dimethylamine hydrochloride was added to the flask. A solution of 2·5 N sodium hydroxide (2 ml) was introduced into the flask and the flask stoppered and shaken vigorously. Colorless crystals separated overnight and were collected by filtration and washed successively with 10-ml portions of water, 1 N hydrochloric acid, and water. The product, after air drying, weighed 105 mg (68% yield) and melted at 120°–127°.

* Dymid 5G (Elanco).

Carbonyl-labeled diphenamide was prepared by the same procedure but starting with diphenylacetic acid-carboxyl-^{14}C and nonradioactive dimethylamine.

Nordiphenamid was synthesized by the general procedure described above, starting with nonradioactive diphenylacetic acid and monomethyl-^{14}C-amine. And finally, carbonyl-labeled nordiphenamid was synthesized from diphenylacetic acid-carboxyl-^{14}C and nonradioactive monomethylamine.

Chromatographic methods

Paper chromatography was carried out on Whatman 1 paper; a BuOH 5 N NH_4OH solution was used for development. Thin-layer chromatography on silica GF (Merck AG) plates employed one of three solvent systems: (1) benzene containing 10% diethylamine, (2) ethyl acetate, or (3) 10% acetic acid in benzene.

Blood-level studies

A polyethylene glycol solution of diphenamid–carbonyl-^{14}C was administered to male rats (200 g, Purdue–Wistar strain) by the i.p., s.c., or oral route (50 mg/kg). At given time intervals, blood samples were taken from the tail vein. Weighed samples (200 mg) were placed in sausage-casing bags, dried for 24 hr, and the radiocarbon content of each determined by the Schöniger combustion method described by Kelly *et al.*[4]

Respired radiocarbon dioxide studies

The rate of *in vivo* demethylation was determined by following the rate of radiocarbon dioxide expiration after administration of methyl-labeled diphenamid or nordiphenamid. For this experiment 150-g male rats were used. The i.p. dose was 50 mg/kg body weight. In order to determine the rate of expiration of radiocarbon dioxide, a radiorespirometer essentially identical with that developed and described by Tolbert and associates[5, 6] was employed. In our instrument both the rat cage and the ionization chamber had a volume of 500 ml. A flow rate of 500 ml/min of air was employed.

Urinary excretion studies

Carboxyl-labeled drug and 200-g male Purdue–Wistar rats were used for urinary excretion studies. After dosing with 50 mg radiodiphenamid/kg by i.p. injection, the animals were kept in stainless steel metabolism cages. Urine samples were collected at regular time intervals and the radioactive carbon content determined by liquid scintillation counting. This study also included dosing the animals with 50 mg radionordiphenamid/kg.

In order to investigate the nature of the urinary metabolites, urines were incubated for 18 hr with 1·0 ml glusulase solution (mixture of β-glucuronidase and sulfatase; Endo Products, Inc.) per 100 ml urine at pH 5·5 (0·5 M acetate buffer). The liberated metabolites were then extracted with methylene chloride and studied by chromatographic methods.

RESULTS

Figure 1 presents blood-level data obtained after administration of carboxyl-labeled diphenamid by each of three different routes. The results showed that, despite

its water insolubility and relative chemical inertness, diphenamid readily enters the blood stream from the peritoneal cavity. Absorption from the gastrointestinal tract is also relatively rapid, although peak blood levels are only about 60% of those obtained after i.p. administration. Absorption into the blood stream appears to be slowest after s.c. injection. In this case, peak blood levels are reached only after about

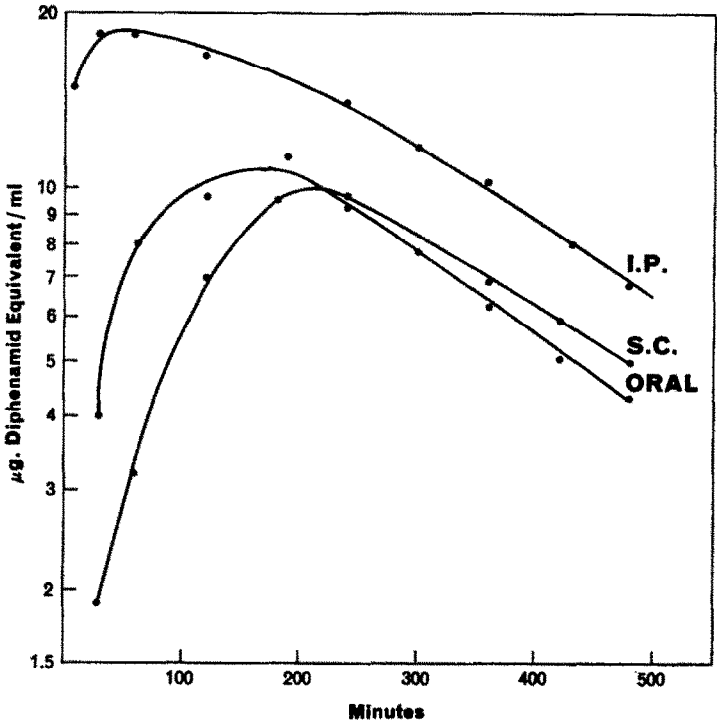

FIG. 1. Plasma radiocarbon levels after administration of diphenamid-carbonyl-^{14}C to rats (50 mg/kg body weight). Each curve represents the mean of three rats.

3 hr. Half-lives were estimated from the curves and found to be 245 min (oral), 235 min (i.p.), and 210 min (s.c.). Thus there is no significant difference in blood half-lives obtained after administration by any of the three routes. Since total radioactivity in blood was measured in these studies, the curve in Fig. 1 represents the rate of decline of diphenamid plus metabolites.

Demethylation *in vivo* was next investigated. The rates of demethylation of diphenamid, nordiphenamid, and a typical lipid-soluble tertiary amine which is readily demethylated *in vivo*[7] are presented in Fig. 2. Diphenamid was found to be extensively demethylated in the rat, thus confirming the earlier *in vitro* finding. Nordiphenamid is also demethylated but to a lesser extent. The interesting aspect of these results is the very slow rates at which the demethylation of the amides occur, in sharp contrast to the rate of demethylation of an ionizable dimethylamine derivative such as 1-propoxyphene, α(-)-4-dimethylamino-1,2-diphenyl-3-methyl-2-propionoxybutane. HCl.

Since part of the label in diphenamid-N-methyl-^{14}C is lost through respired radiocarbon dioxide, urine studies were carried out with carboxyl-labeled drug. The rate of excretion of radioactive metabolites in urine is summarized in Table 1.

For the identification of metabolites, the combined 0 to 34-hr urines were used. Preliminary paper chromatography showed that nearly all the radioactivity was present as slow-moving polar materials (presumably conjugated metabolites). Only a trace of nonpolar materials (amides) was present. No diphenylacetic acid was detected. We found in our preliminary experiments that diphenylacetic acid is excreted

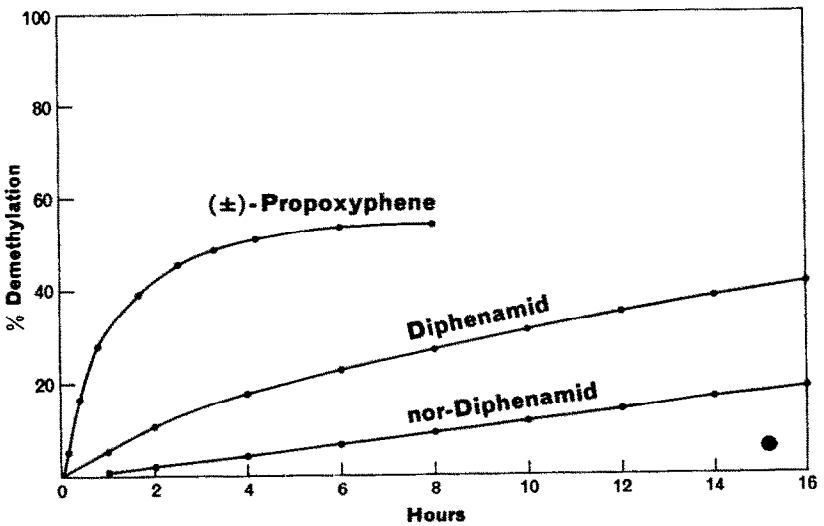

FIG. 2. Rates of demethylation of diphenamid-N-methyl-^{14}C, nordiphenamid-N-^{14}C, and *dl*-propoxyphene-N-methyl-^{14}C in rats. The i.p. dose in each case was 50 mg/kg. Each curve represents the mean of three individuals.

unchanged in the urine of this species. Therefore the absence of this acid as a urinary metabolite demonstrates that diphenamid is not metabolized by hydrolysis of the amide linkage.

TABLE 1. EXCRETION OF DIPHENAMID-CARBOXYL-^{14}C METABOLITES IN RAT URINE

Time (hr)	Accumulated recovery (%)	Time (hr)	Accumulated recovery (%)
2	2·6	26	72·2
6	8·8	34	82·1
10	16·1	38	83·9
14	27·9	42	87·4
22	50·5	46	89·0

Conditions: 200-g male rats were used. The dose was 50 mg/kg body weight by the i.p. route; average values from three individuals.

Since most of the radioactivity in urine appeared to be present in conjugated form, the urine was hydrolyzed with a mixture of β-glucuronidase and sulfatase at pH 5·5 before extraction. After this enzyme treatment the liberated metabolites were readily extracted into dichloromethane at pH 6. When the dichloromethane extract was

extracted in turn with 5% sodium hydroxide solution, about 30% of the radioactivity present passed into the aqueous layer. This base-soluble material was back extracted into chloroform after neutralizing the alkaline solution with carbon dioxide. The observed behavior of this material suggested strongly that it was phenolic in nature. The u.v. spectra of this metabolite showed maxima at 224 and 279 mμ, which shifted to 243 and 297 mμ in alkali. This corresponded well with the spectrum of *p*-cresol. which has maxima at 222 and 278 mμ that shift to 244 and 296 mμ in alkali. These data thus served to identify the acidic metabolite as the product of *p*-hydroxylation of diphenamid. *p*-Hydroxynordiphenamid and *p*-hydroxydiphenylacetamide may have been present in this fraction also, but no attempt was made to confirm this. Further proof of the identity of the phenolic metabolite came from hydrolysis studies. Hydrolysis of the crude phenolic fraction led to the formation of a phenolic acid which was chromatographically identical with *p*-hydroxydiphenylacetic acid in both the paper chromatographic system and in the thin-layer system (3).

Most of the metabolites obtained after enzyme treatment were neutral in nature and remained in the dichloromethane solution after the alkaline extraction. By the use of thin-layer chromatography in system (1), it was found that this material was predominantly nordiphenamid together with a small amount of diphenylacetamide (corresponding to about 5% of the dose) and a trace of unchanged diphenamid (corresponding to 1–2% of the dose).

The results of these studies left one unsolved puzzle; i.e. the amounts of nordiphenamid and diphenylacetamide, the products of demethylation, appeared to be greater than would be expected from the amount of radiocarbon dioxide collected in earlier experiments. The explanation for this apparent anomaly resulted from the following experiments. Urine was collected from a rat which had been dosed with diphenamid-N-methyl-^{14}C. To this urine was added 10 μmoles semicarbazide/ml. The urine was then incubated at pH 7 with β-glucuronidase (bacterial). The presence of the semicarbazide served to trap any formaldehyde that might form during the incubation. After incubation, a sample of the treated urine was treated first with 10% zinc chloride then with saturated barium carbonate. The precipitate was then removed by centrifugation and the supernatant treated with Nash reagent in order to convert any radioformaldehyde to 2,6-dimethyl-3,5-diacetyl-4-^{14}C-3,4-dihydropyridine (cf. Cochin and Axelrod[8] and Nash[9]). The pyridine derivative so formed was extracted with chloroform and its u.v. spectra determined. It had a maximum at 411 mμ which corresponded exactly with that of known material. When subjected to thin-layer chromatography (system 2) a bright yellow radioactive spot with the same R_F value as authentic 2,6-dimethyl-3,5-diacetyl-3,4-dihydropyridine was observed. When a mixture of known and unknown was chromatographed, no separation occurred.

When the described experiment was repeated with urine from rats receiving diphenamid-carboxyl-^{14}C, the same pyridine derivative was obtained, but in this case it was not radioactive. Control urine from rats which had not received diphenamid did not yield formaldehyde by this treatment.

Thus urine from rats receiving diphenamid apparently contains an unusual metabolite which, when treated with β-glucuronidase, breaks down to form formaldehyde and nordiphenamid. The formaldehyde is radioactive when methyl-labeled diphenamid is used but nonradioactive when carbonyl labeling is employed, and it thus clearly originates from one of the N-methyl groups of diphenamid. These results are best

explained if it is assumed that the metabolite is the glucuronide of N-methyl-N-hydroxymethyldiphenylacetamide (compound 4, Fig. 3). This compound would upon treatment with β-glucuronidase be hydrolyzed to the parent N-hydroxymethyl compound which is a chemically unstable structure and would be expected to dissociate readily into nordiphenamid and formaldehyde.

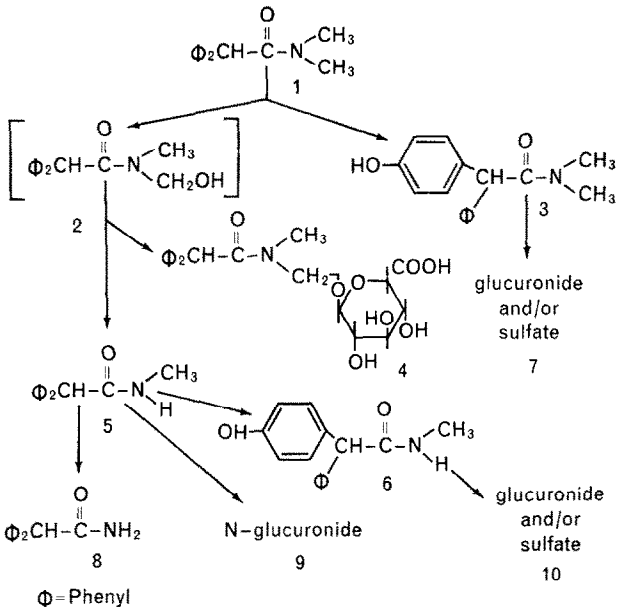

FIG. 3. Summary of pathways by which diphenamid and nordiphenamid are metabolized.

The *in vivo* metabolism of nordiphenamid-carboxyl-^{14}C in the rat was also investigated. When nordiphenamid-carboxyl-^{14}C was administered to rats by the i.p. route (50 mg/kg), about 60% of the radioactivity appeared in urine in 24 hr. Through the use of the methods described above, it was found that the major portion (≈80%) of this radioactivity was present as *p*-hydroxynordiphenamid (compound 6, Fig. 3). About one half the phenol appeared in urine in the unbound form while the remainder occurred as a conjugate which was readily cleaved with a mixture of β-glucuronidase and sulfatase.

The remainder of the radioactivity in urine after removal of the phenols consisted of a mixture of nordiphenamid and diphenylacetamide, the demethylated metabolite of nordiphenamid.

DISCUSSION

Diphenamid is a neutral, non-ionizable compound characterized by high lipid solubility and extremely low chemical reactivity. Despite these rather unpromising properties, diphenamid was found to be readily absorbed into the blood stream both from the gastrointestinal tract and from injection sites. Diphenamid and its metabolites also appeared to be rather easily eliminated from the body, as was shown by the rate of disappearance of radioactivity from the blood (half-life = 4 hr) after administration of labeled diphenamid. The rate of appearance of metabolites in urine (Table 1)

also demonstrated that the intact animal can handle this compound adequately and that accumulation of this herbicide or its metabolites in the body would not be anticipated.

The various pathways by which diphenamid undergoes biotransformation are summarized in Fig. 3. Pathways for the further metabolism of nordiphenamid are also included. The most interesting aspect of this study was the demonstration of the *in vivo* demethylation of diphenamid. Although N-demethylation of amines *in vivo* is well known, the first thorough studies of the *in vivo* demethylation of the methylated amide nitrogen were the researches of Butler and associates[10, 11] of the demethylation of N-methyl barbiturates. The first *in vitro* report appears to be the study of Hodgson and Casida[12] on the demethylation of N-methyl carbamates by rat liver microsomes. Recently the N-de-ethylation of an amide, benzquinamide, has also been reported,[13] and both the *in vivo*[14] and *in vitro*[15] demethylation of N-methyl sulfonamides have been reported in the current literature.

It is generally thought that the N-demethylation reaction proceeds though the intermediate formation of an N-hydroxymethyl intermediate.[16] In the case of amines, the resulting N-hydroxymethylamine is extremely unstable and breaks down to form the dealkylated amine and formaldehyde. N-hydroxymethylamides are known to be somewhat more stable. In the present study of the demethylation of diphenamid the intermediate N-hydroxymethyl compound (compound 2, Fig. 3) apparently was sufficiently stable so that a part of it could be "trapped" by the glucuronyl transferase system and converted to the stable O-glucuronide (compound 4, Fig. 3) which was excreted in urine. Evidence for the occurrence of a similar O-glucuronide in the metabolism of an N-methyl imide has been reported by Keberle *et al.*[17] Very recently an unconjugated N-hydroxymethyl compound has been detected as a urinary metabolite of the insecticide, 1-naphthyl N-methylcarbamate.[18]

These studies thus lend support to the suggestion that the N-hydroxymethyl compound is an intermediate in the microsomal demethylation reaction. The major question yet to be answered is whether this hydroxymethyl intermediate arises directly by hydroxylation of the methyl group or through intermediate formation of an N-oxide which then rearranges to the N-hydroxymethyl intermediate.[16] Recently [19]we have presented data which argue against the N-oxide mechanism for the demethylation of l-propoxyphene. The N-oxide mechanism seems at present to be unlikely in the case of amides as well, particularly since N-oxide derivatives of tertiary amides have never been reported in organic chemical studies. The most likely possibility is that the demethylation of diphenamid proceeds by direct hydroxylation of the N-methyl group by a mechanism similar to that of the many other hydroxylations of which the microsomal enzymes are capable.

Although part of the N-hydroxymethyl intermediate was converted to O-glucuronide, the major part dissociated to form nordiphenamid. However, nordiphenamid was not extractable from urine until after β-glucuronidase treatment, thus suggesting that it was present as an N-glucuronide, a class of glucuronides only recently discovered. Ring hydroxylation of diphenamid also occurred but was a minor route of metabolism.

It was interesting to note that the rate of demethylation of nordiphenamid was considerably slower than that of diphenamid. This difference in rate is similar to that observed between secondary N-methylamines and the corresponding N,N-dimethyl tertiary amines.[20] The alternative pathway for the metabolism of these two amides is

ring hydroxylation and, since ring substitution is the same in each, the rate of hydroxylation of each should occur at about the same rate. The net effect of these rate considerations is that demethylation is the major route of metabolism of diphenamid, whereas hydroxylation is more important in the case of nordiphenamid.

Acknowledgement—We wish to express our appreciation to Dr. F. J. Marshall for his assistance with the preparation of the labeled compounds; to Dr. Q. F. Soper for supplying *p*-hydroxydiphenylacetic acid; and to Dr. T. Golab for advice on the thin-layer chromatography methods used.

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