Biochemical Pharmacology, Vol. 20, pp. 2401–2408. Pergamon Press, 1971. Printed in Great Britain

DEFORMYLATION OF 4,4′-DIFORMAMIDODIPHENYL SULFONE (DFD) BY MAMMALIAN LIVER HOMOGENATES

C. Y. CHIOU

Department of Pharmacology and Therapeutics, University of Florida, College of Medicine, Gainesville, Fl. 32601, U.S.A.

(*Received* 12 *June* 1970; *accepted* 22 *January* 1971)

Abstract—Deformylation of 4,4′-diformamidodiphenyl sulfone (DFD) was studied in liver homogenates. The rates of deformylation were found to be in the following order: guinea pig ≧ human > mouse = rabbit = rat > dog.

The rates of $^{14}CO_2$ formation from (formyl-^{14}C)-DFD coincided well with that of free arylamino group formation from DFD determined with a modified Bratton–Marshall technique.* These results indicate that DFD is deformylated to produce HCOOH and primary arylamino compounds possibly through hydrolytic reaction.

The rate of HCOOH oxidation by liver homogenates was much faster than that of CO_2 production from DFD, suggesting that the rate limiting step of CO_2 formation from DFD is the deformylation of DFD but not the oxidation of the formate.

It appears that DFD is deformylated in the body to diaminodiphenyl sulfone (DDS) which acts as an active antimalarial agent.

IT HAS been suggested that 4,4′-diformamidodiphenyl sulfone (DFD) is more potent than diaminodiphenyl sulfone (DDS) as an active antimalarial agent.[1] However, the mechanism of action of DFD and the pathways of its metabolism are not clear. During the investigation of the metabolism of DFD, it became apparent that *N*-deformylation might be one of the important pathways of its metabolism.[2–5] It has been shown that homogenates and mitochondria of chick kidney deformylated the aromatic *N*-formyl group of formanilide derivatives at a rapid rate.[6] In this investigation deformylation of DFD by various mammalian liver homogenates was studied. It was found that DFD is deformylated by liver homogenate at widely ranging rates, depending on the species. The products of deformylation of DFD are CO_2 and primary arylamino compounds, DDS and/or monoformyldiaminodiphenyl sulfone (MFD).[2–5] The mechanism by which the formyl group is split off from the formamido group of DFD was investigated.

METHODS

Materials. (Formyl-^{14}C) diformamidodiphenyl sulfone (^{14}C-DFD) with specific activity of 6·84 μc/mg was purchased from New England Nuclear Corp., Boston, Mass. A stock solution ($2{\cdot}5 \times 10^5$ dis./min/400 μg/ml) was made with absolute ethanol because of its poor solubility in aqueous medium. The DFD was dissolved in a small quantity of dimethyl sulfoxide first and then diluted to 400 μg/ml with

* T. H. Maren and L. C. Garg, *J. Lab. clin. Med.* 77, 524 (1971).

absolute ethanol. Sodium ^{14}C-formate ($H^{14}COONa$) with specific activity of 56 mc/mM and sodium ^{14}C-bicarbonate ($NaH^{14}CO_3$) with specific activity of 58 μc/mg were purchased, respectively, from Int. Chem. & Nuclear Corp., Irvine, Calif. and New England Nuclear Corp., Boston, Mass. The radioactivity of these compounds and that of $^{14}CO_2$ released from these compounds were determined in dioxane counting fluid (naphthalene, 100 g; 2,5-diphenyloxazole, 7 g; dioxane, 1000 ml) with Beckman's Liquid Scintillation System.

Preparation of liver homogenates. Guinea pigs (300–500 g), rats (200–300 g), mice (20–25 g) and rabbits (1·0–1·5 kg) of either sex were stunned by a blow on the head and the livers were isolated as soon as possible. Dogs (10–13 kg) were anesthetized with 35 mg/kg of sodium pentobarbital and part of the liver was removed. The human livers were supplied by Dr. M. H. Cho from the Department of Pathology, College of Medicine, University of Florida. The livers were homogenized by hand in normal saline with tissue grinders (Ten Broeck type, A. H. Thomas Co.) to give 17% (w/v) homogenate. All procedures were performed at 0–4°. Two ml of the liver homogenate was incubated with various amounts of ^{14}C-DFD, $NaH^{14}CO_3$ and $H^{14}COONa$ at 37° for period of time indicated in the individual experiments.

Measurement of $^{14}CO_2$. The Warburg apparatus was used to detect the $^{14}CO_2$ released from ^{14}C-DFD, $H^{14}COONa$ and $NaH^{14}CO_3$. The main compartment of 15-ml Warburg flasks contained 2 ml of liver homogenate and 0·05 ml of radioactive compound. The central well contained 0·4 ml of 2-phenethylamine to trap the $^{14}CO_2$ released. The reaction mixture was incubated at 37° for 0·5, 1, 2 and 4 hr. At the end of the incubation, 2-phenethylamine with $^{14}CO_2$ was transferred to the counting vial containing 10 ml of dioxane counting fluid. The central well was washed twice with 0·4 ml and 0·2 ml of methanol and added to the counting fluid.

TABLE 1. ABSORPTION OF $^{14}CO_2$ BY 2-PHENETHYLAMINE*

Time of incubation (min)	CO_2 absorbed Mean ± S.E. ($\times 10^{-7}$ moles)
30	3·32 ± 0·50
60	4·25 ± 0·21

* $NaH^{14}CO_3$, $4{\cdot}76 \times 10^{-7}$ moles; human liver homogenate, 2 ml; 2-phenethylamine, 0·4 ml; temperature, 37°. The maximum amount of $^{14}CO_2$ to be formed from 10 μg/ml of DFD was not more than $1{\cdot}32 \times 10^{-7}$ moles in this incubation system. Number of experiments = 3.

Table 1 shows the capacity of 0·4 ml 2-phenethylamine to absorb $^{14}CO_2$. $NaH^{14}CO_3$ ($4{\cdot}76 \times 10^{-7}$ moles) was incubated with 2 ml of human liver homogenate for 30 and 60 min. The $^{14}CO_2$ released was trapped with 0·4 ml of 2-phenethylamine. The amount of $^{14}CO_2$ absorbed within 30 and 60 min incubation was $3{\cdot}32 \times 10^{-7}$ and $4{\cdot}25 \times 10^{-7}$ moles respectively; the maximum amount of CO_2 to be released from ^{14}C-DFD or $H^{14}COONa$ in these studies did not exceed $1{\cdot}32 \times 10^{-7}$ moles.

The concentrations of ^{14}C-DFD studied were 10 μg/ml ($3{\cdot}3 \times 10^{-8}$ moles/ml), 1 μg/ml and 0·1 μg/ml. The corresponding concentrations of $H^{14}COONa$ studied

were 4·5 μg/ml ($6·6 \times 10^{-8}$ moles/ml) and 0·45 μg/ml since each molecule of DFD produced two molecules of CO_2 or formate.

Measurement of free arylamino group. The free arylamino group formed from DFD was determined with Bratton–Marshall reaction modified by Maren and Garg.[7] Liver homogenate was incubated with DFD at 37° for 15, 30, 60, 120 and 240 min. The concentration of DFD was 10 μg/ml. At the end of the incubation, 30 ml of 3% trichloroacetic acid precooled to 2° was added to precipitate the proteins. One ml of 1 M sodium citrate was then added to 20 ml of the clear filtrate. To each sample 0·3 ml of 0·1% $NaNO_2$ was added and mixed. Five min later 0·3 ml of 0·5% ammonium sulfamate was added, mixed well and allowed to stand for 5 min. Three-tenths-ml of 0·1% coupling agent (*l*-naphthylethylenediamine dihydrochloride) solution was then added and mixed. Ten min later the color developed was extracted with 4 ml of isoamyl alcohol and read at 540 mμ with Coleman Junior Spectrophotometer.

RESULTS

Deformylation of DFD by various mammalian liver homogenates. Figure 1 shows the rate of $^{14}CO_2$ formation from ^{14}C-DFD by liver homogenates of guinea pig, rabbit, human, mouse, rat and dog. The liver homogenates were incubated with 1 μg/ml of ^{14}C-DFD at 37° for 0·5, 1, 2 and 4 hr. Deformylation proceeded more rapidly during the first hour of incubation and leveled off after 2 hr of incubation. It was obvious that different mammalian livers deformylated DFD at a different rate. The rates of deformylation in the species studied were found to be in the following order: guinea

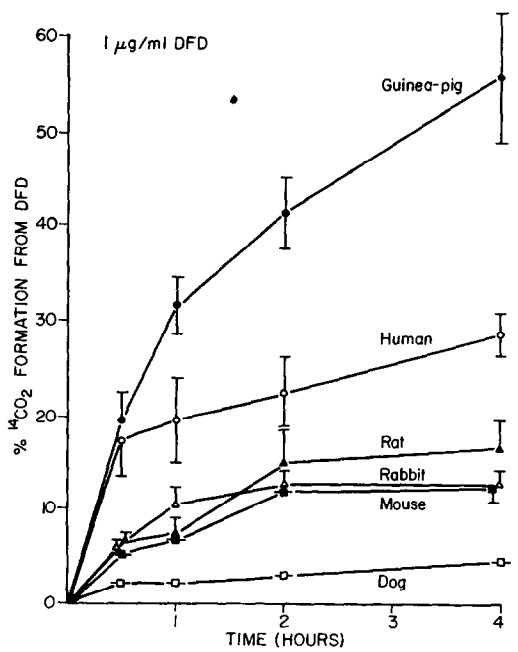

FIG. 1. Patterns of $^{14}CO_2$ formation from DFD by liver homogenates of guinea pigs, humans, rats, rabbits, mice and dogs as a function of time. Liver homogenate, 2 ml; DFD, 1 μg/ml. Each point is a mean of 3 values and the bar represents the standard error.

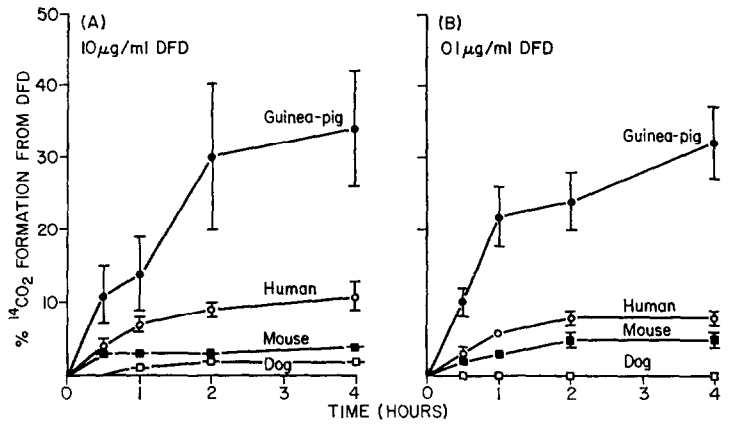

FIG. 2. Evolution of $^{14}CO_2$ as a function of time from ^{14}C-DFD during its hydrolysis by liver homogenates of guinea pigs, humans, mice and dogs. Liver homogenate, 2 ml; DFD, 10 μg/ml (A); DFD, 0·1 μg/ml (B). Each point is a mean of three values and the bar represents the standard error.

pig ≧ human > rabbit = mouse = rat > dog. DFD deformylation rates at concentrations of 10 μg/ml and 0·1 μg/ml of DFD were also studied. The results were essentially the same as those obtained at 1 μg/ml of DFD (Fig. 2).

Since pentobarbital anesthesia was used only for the dog, a question might arise as to whether or not this anesthetic agent would inhibit the enzymes that deformylate DFD. Guinea pig liver was used to investigate this problem because it induces rapid deformylation (Figs. 1 and 2). Guinea pigs were either anesthetized with 35 mg/kg of pentobarbital given intraperitoneally or stunned by a blow on the head. Liver homogenates were made and incubated with 1 μg/ml of ^{14}C-DFD for 30 and 60 min. It is clearly shown in Table 2 that there is no significant inhibition of the enzymes involved in the metabolism of DFD by pentobarbital anesthesia. Accordingly, the exceptionally low rate of deformylation of DFD by dog liver is not due to the inhibition of the deformylation enzymes by pentobarbital.

Since DFD was not deformylated by dog plasma[5,8] nor by its liver homogenate, it was thought that the kidney might be responsible for the reaction because formani-

TABLE 2. EFFECT OF PENTOBARBITAL SODIUM ON THE ENZYME ACTIVITY TO DEFORMYLATE DFD

Time of incubation (min)	Per cent $^{14}CO_2$ formation from DFD* Normal liver	Pentobarbital treated liver†	P value‡
30	13·8 ± 0·8	14·3 ± 1·2	>0·5
60	37·9 ± 2·2	40·4 ± 2·8	>0·5

* One μg per ml of DFD, Mean ± S.E., $n = 3$.
† Thirty-five mg per kg i.p.
‡ Calculated with Student's t-test.

lides were deformylated by chick kidney homogenate quite rapidly.[6] The homogenate of dog kidney was made with the same method used to make liver homogenate and was incubated with 10 μg/ml of ^{14}C-DFD for 30, 60 and 180 min. The result showed that there was no significant amount of $^{14}CO_2$ produced from ^{14}C-DFD by dog kidney.

It has been shown that DFD is also deformylated by certain mammalian plasmas notably from rodents and rabbits.[5,8] Thus, it is important to find out whether the rate of deformylation by these liver homogenates is due solely to the enzyme in the liver or to the blood content in it. Since no deformylation was found when DFD was incubated with human and dog plasma,[5,8] the rate of deformylation by human and dog livers must be due entirely to the enzymes present in the liver. The rate of deformylation by the plasma of other species was found to be in the following order: mouse > rat > guinea pig > rabbit.[5,8] The livers of rat and guinea pig were perfused with physiological saline to wash away the blood. The livers without perfusion served as control. Liver homogenates were made and incubated with 1 μg/ml of ^{14}C-DFD for 0·5, 1, 2 and 4 hr. The results indicate that the blood content in the liver does not significantly affect the rate of deformylation by liver homogenate (Table 3).

TABLE 3. EFFECT OF BLOOD CONTENT IN THE LIVER ON THE DEFORMYLATION OF DFD BY LIVER HOMOGENATES

Time of incubation (hr)	Per cent $^{14}CO_2$ formation from DFD*		P value†
	Normal liver	Perfused liver	
0·5	6·0 ± 1·0	5·3 ± 0·3	>0·5
1	12·0 ± 1·0	8·8 ± 1·0	>0·3
2	10·0 ± 0·8	8·0 ± 0·3	>0·1
4	12·7 ± 0·6	11·3 ± 1·2	>0·5

* One μg per ml of DFD, Mean ± S.E., $n = 3$.
† Calculated with Student's t-test.

Oxidation of H^{14}COONa by various liver homogenates. It has been reported that DFD is deformylated stepwise to MFD and DDS by guinea pig and mouse liver homogenates studied using thin-layer chromatography.[2] There are several possibilities by which CO_2 could be produced from the formamino group of DFD. The most probable way would be the hydrolysis of formamino group to liberate HCOOH as its intermediate:

$$\text{DFD} \xrightarrow{\text{(a)}} \text{MFD} + \text{HCOOH} \xrightarrow{\text{(c)}} CO_2$$

$$\text{MFD} \xrightarrow{\text{(b)}} \text{DDS} + \text{HCOOH} \xrightarrow{\text{(d)}} CO_2.$$

If this is the case, the liver homogenates should oxidize HCOOH to CO_2 at a rapid rate. To test this, 4·5 μg/ml ($6{\cdot}6 \times 10^{-8}$ moles/ml) and 0·45 μg/ml of H^{14}COONa were incubated with liver homogenates from guinea pigs, rats, mice and dogs at 37° for 0·5, 1, 2 and 4 hr. There was rapid release of CO_2 for 30–60 min, slowing considerably thereafter (Fig. 3). The rate of $^{14}CO_2$ formation from H^{14}COONa was much faster than from DFD, indicating that the rate-limiting step of $^{14}CO_2$ formation is

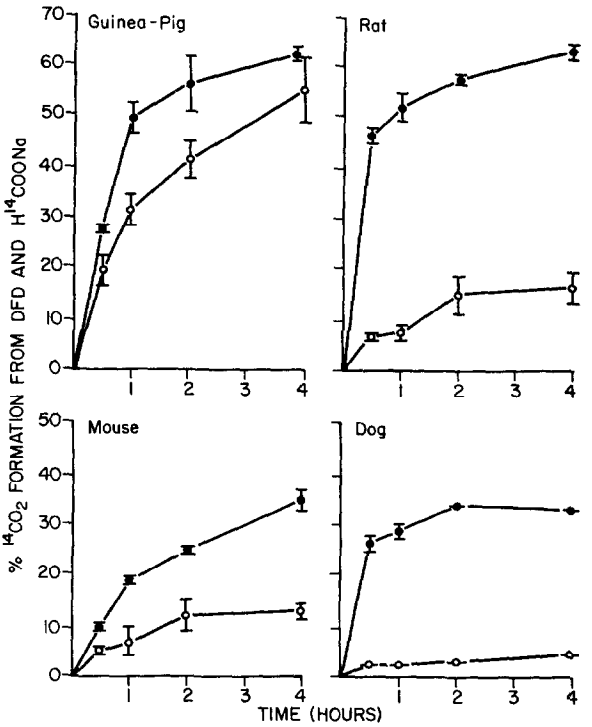

FIG. 3. Comparison of $^{14}CO_2$ evolution as a function of time from deformylation of ^{14}C-DFD (○) and oxidation of $H^{14}COONa$ (●) by liver homogenates of guinea pigs, rats, mice and dogs. Liver homogenate, 2 ml; DFD, 1 μg/ml ($3{\cdot}3 \times 10^{-9}$ moles/ml); $H^{14}COONa$, 0·45 μg/ml ($6{\cdot}6 \times 10^{-9}$ moles/ml). Each point is a mean of three values and the bar represents the standard error.

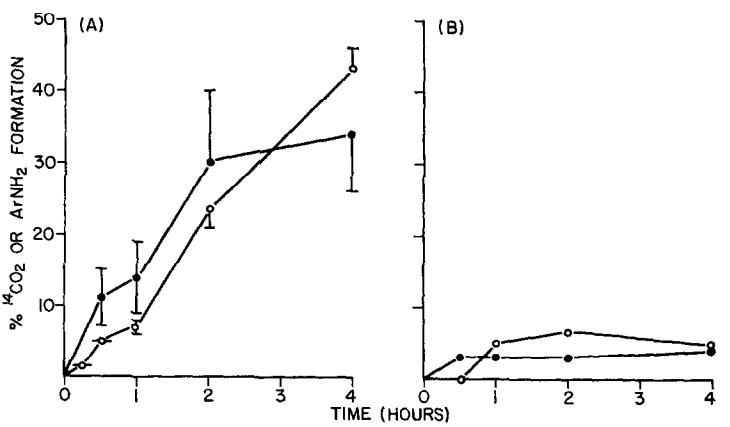

FIG. 4. Comparison of $^{14}CO_2$ evolution from ^{14}C-DFD (●) and free arylamino group formation from DFD (○) as a function of time by guinea pig (A) and mouse (B) liver homogenates. The $^{14}CO_2$ was trapped by 2-phenethylamine and determined with Liquid Scintillation System (Beckman). The free arylamino group was determined with a modified analytical method of Bratton–Marshall.[7] Liver homogenate, 2 ml; DFD, 10 μg/ml. Each point is a mean of three values and the bar represents the standard error.

the deformylation of DFD (steps a and b) but not the oxidation of HCOOH (steps c and d).

Determination of free arylamino group. Determination of free arylamino group with the modified analytical method of Bratton–Marshall[7] provided further information concerning the deformylation of DFD. If steps a and b are correct, we should be able to detect the same quantity of arylamino group and CO_2 formed from DFD.

Ten micrograms per ml of DFD was incubated with liver homogenates of guinea pigs and mice at 37° for 15, 30, 60, 120 and 240 min. The amount of arylamino group formed after incubation was determined with a modified method of Bratton–Marshall.[7] The rates of free arylamino group formation were about the same as those of CO_2 formation from DFD by liver homogenates of guinea pigs [Fig. 4(a)] and mice [Fig. 4(b)].

DISCUSSION

It has been shown that aromatic formamide compounds are deformylated by kynurenine formamidase of mammalian liver[9,10] and of *Neurospora crassa*[11] and by homogenate and mitochondria of the chick kidney.[6] The cleavage of the molecules of formanilide and its derivatives is quite well established in these systems.[6] There is no reason to believe that the DFD molecule should behave in any other way during deformylation.

The formation of MFD and DDS from DFD by liver homogenates of guinea pig and mouse was reported briefly by Hoffman *et al.*[2] using thin-layer chromatography.[2] However, the mechanism by which the formyl group was split off from DFD was not determined. Since free arylamino group was detected with the modified method of Bratton–Marshall[7] and the equivalent amount of $^{14}CO_2$ was collected from the incubation mixture of DFD and liver homogenates, it appears that DFD was deformylated via hydrolytic reaction to liberate HCOOH and primary arylamino group. This is further supported by the fact that formate was oxidized to CO_2 by liver homogenates rapidly. The rate of free arylamino group formation and that of the $^{14}CO_2$ formation from the DFD were very close to each other suggesting that most, if not all, of the formyl group deformylated from DFD was converted into CO_2. Since the CO_2 formation from formate was much faster than that from DFD, it indicates that the rate limiting step of CO_2 formation from DFD is the deformylation step (steps a and b) but not the oxidation step (steps c and d).

The DFD was deformylated by various liver homogenates at different rates. The order of the rate of deformylation by the liver homogenates of the species studied was found to be: guinea pig $\geqq$ human > rabbit = mouse = rat > dog. This order agrees with that reported by Hoffman *et al.*:[2] guinea pig > mouse > rat. The exact activity of the human liver is not known because these livers were available only several hours post-mortem. The rate of deformylation with human liver was about the same as that with guinea pig liver at the first 30 min. It leveled off quickly thereafter. It is possible that the actual activity of the enzymes in the fresh human liver would be greater than that in the guinea pig liver. The deformylation of DFD *in vivo* by human has been studied by Maren *et al.*[3] A significant amount of $^{14}CO_2$ and free arylamino group have been recovered, respectively, from the expired air and the urine.

It is interesting to note that mouse and rat deformylated DFD slowly by their

liver homogenates but rapidly by their plasma enzymes.[5,8] The reverse is true for the deformylation of DFD by guinea pig and human. Consequently, the over-all results are the deformylation of DFD either by liver (guinea pig and human) or by plasma (mouse and rat) or by both (rabbit) to produce DDS which acts as an active antimalarial agent. It has been reported that chick kidney homogenate deformylates formanilide derivatives rapidly.[6] However, DFD was not deformylated by dog kidney homogenate, although $^{14}CO_2$ and primary arylamino group were detected, respectively, in the expired air and the urine after intravenous administration. The DFD was not appreciably deformylated by dog liver homogenate nor by its plasma enzymes. The reason for this exception is not known and is to be investigated.

Acknowledgement—The author is grateful to Dr. Thomas H. Maren for his suggestion to carry out this experiment and his critical review of this manuscript. This work was supported in part by Research Grant from NIH GM 16934.

REFERENCES

1. D. M. AVIADO, *Expl Parasit.* **20,** 88 (1967).
2. P. HOFFMANN, J. KLEINMAN, C. MILROM and K. P. DUBOIS, *Fedn Proc.* **26,** 568 (1967).
3. T. H. MAREN, A. C. SONNTAG and V. C. STENGER, *Pharmacologist* **12,** 272 (1970).
4. A. C. SONNTAG and T. H. MAREN, *Pharmacologist* **12,** 272 (1970).
5. L. N. GLEASON and B. P. VOGH, *Pharmacologist* **12,** 272 (1970).
6. R. H. NIMMO-SMITH, *Biochem. J.* **75,** 284 (1960).
7. T. H. MAREN and L. C. GARG, *J. Lab. clin. Med.* **77,** 524 (1971).
8. L. N. GLEASON and B. P. VOGH, *Biochem. Pharmac.* **20,** 2409 (1971).
9. A. H. MEHLER and W. E. KNOX, *J. biol. Chem.* **187,** 431 (1950).
10. R. S. S. SANTTI and V. K. HOPSU-HAVU, *Biochem. Pharmac.* **17,** 1110 (1968).
11. W. B. JAKOBY, *J. biol. Chem.* **207,** 657 (1954).