Biochemical Pharmacology, Vol. 20, pp. 2409–2416. Pergamon Press, 1971. Printed in Great Britain

DEFORMYLATION OF 4,4′-DIFORMAMIDODIPHENYL SULFONE (DFD) BY PLASMA OF CERTAIN MAMMALS*

LARRY N. GLEASON† and BETTY P. VOGH

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

(*Received* 7 *August* 1970; *accepted* 12 *February* 1971)

Abstract—Enzymatic deformylation of 4,4′-diformamidodiphenyl sulfone (DFD) to 4,4′-diaminodiphenyl sulfone (DDS) occurs rapidly and completely in the mouse and *in vitro* in mouse plasma. Within 1 hr all DFD is present as DDS. The intermediate product in the stepwise deformylation is 4-amino-4′-formamidodiphenyl sulfone (MFD).

DFD is also deformylated in plasma from other animals with species variation in the rates of deformylation: mouse > rat > guinea pig > rabbit. DFD is not deformylated in plasma from dog or man. Deformylation is inhibited by diethyl *p*-nitrophenyl-phosphate (paraoxon), but not by *p*-chloromercuribenzoate or neostigmine. The reaction also shows marked substrate specificity: of other formamido compounds tested only those linked to the diphenylsulfone structure were substrates.

The rapid deformylation of DFD to DDS indicates that the antimalarial efficacy of DFD in *Plasmodium berghei* in the mouse is due to its conversion to DDS. Reported superiority of DFD to DDS in treatment of *P. berghei* is considered to be related to solubility differences in the two compounds and repository effects of certain routes of administration.

IN SYSTEMS for testing drugs against *Plasmodium berghei* in mouse, 4,4′-diformamidodiphenyl sulfone (DFD) exhibits a greater antimalarial action, on a weight basis, than the parent compound, 4,4′-diaminodiphenyl sulfone (DDS), when the compounds are given subcutaneously or intraperitoneally.[1,2] By the single-dose intraperitoneal route the difference is 12-fold or greater.[2] Low solubility of DFD, ~ 10 μg/ml in water compared to 160 μg/ml for DDS, gives doses of DFD by these routes of administration longer repository action than DDS. Antimalarial efficacy of the two compounds was equal when repository action was eliminated by giving them intravenously in dimethylsulfoxide (DMSO) or by using very low doses so that little remained undissolved.[2] The present studies show that DFD given to mice intravenously in DMSO was rapidly and completely converted to DDS and that the cleavage occurs primarily as a result of enzyme activity in plasma. Data are given on rates of deformylation *in vivo* in mice, *in vitro* in mouse plasma, and *in vitro* in plasma from other species.

* This work was supported by U.S. Army Med. Res. and Dev. Command and N.I.H. Grant GM AI 16934. Results have been summarized in *Pharmacologist*, **12**, abst. No. 397 (1970), and presented at the August, 1970 meeting of The American Society for Pharmacology and Experimental Therapeutics.

† Present address: Department of Biology, Western Kentucky University, Bowling Green, Ky. 42276. Address reprint requests to B. P. Vogh.

MATERIALS AND METHODS

For deformylation studies *in vitro*, DFD was added to plasma separated from heart blood of mouse, rat, guinea pig and rabbit and from venous blood of dog and man. A volume of ~ 0·1 ml of DMSO containing 50 or 100 μg of dissolved drug was added to 1 ml of plasma to give final measured concentrations between 40 and 105 μg/ml. These levels were chosen as being typical of concentrations which were achieved in the studies done for comparison *in vivo* by intravenous administration in DMSO. Plasma containing DFD was incubated in a 37° water bath with continual mixing. Deformylation was stopped at desired times by precipitation of plasma proteins. In one experiment, DFD in DMSO was incubated with mouse whole blood and washed red cells (washed four times with 0·2 M phosphate buffer, pH 7·4, and returned to original blood volume with buffer).

For deformylation studies *in vivo* in the mouse, DFD was injected as a solution of 0·03 ml DMSO into the tail vein. At selected times the mice were anesthetized and heart blood was drawn. To stop enzymatic activity, 2,2-diethyl *p*-nitrophenylphosphate (paraoxon), final concentration 10^{-3} M, was mixed with the blood immediately after removal from the mouse.

Samples were prepared for determination of unsubstituted arylamine from DDS and 4-amino-4'-formamidodiphenyl sulfone (MFD) as follows: 0·4 ml plasma, whole blood, or red cells in buffer were mixed with 1·6 ml of 100% ethanol to precipitate the proteins; after 10 min the protein was removed by centrifugation; 0·9 ml of the supernatant (half, representing 0·2 ml plasma) was transferred to a tube containing 4·1 ml of a trichloroacetic acid (TCA)-sodium citrate buffer, pH 2·35 (see below); after mixing by inversion 2-ml aliquots were taken for analysis. Buffer was prepared by bringing 245 g TCA and 163 g sodium citrate to 500 ml with distilled water and, after these were in solution 40 ml of 1% Tween 80 in distilled water was added. Choice of pH 2·35 was based on these facts: the color reaction requires pH < 2·5 and pH < 2·0 hydrolyzes formyl groups from DFD and MFD.

Samples were prepared for determination of total arylamine (all DFD, MFD and DDS) as follows: 0·2 ml of plasma, whole blood or red cells in buffer was mixed with 3·8 ml of distilled water; 1 ml of 15% TCA was added; after 10 min, the mixture was centrifuged; 2-ml aliquots were taken for analysis; to these 0·1 ml of 4 N HCl was added to maintain pH ~ 1 despite some loss of TCA during heating; the tubes were covered and heated in a boiling bath for 15 min, then cooled.

Aliquots of 2 ml containing free or total amine were diazotized and coupled[3] by addition of 0·3 ml of 0·1% sodium nitrite, 0·3 ml of 0·5% ammonium sulfamate and 0·3 ml of 0·1% *N*-(1-napthyl)-ethylenediamine dihydrochloride in this order with thorough mixing and a 5 min wait between reagents. After color was allowed to develop for 10 min, 1·0 ml of 100% ethanol was added to assure that all dye formed was in solution. Extinction was determined on a Coleman Junior Spectrophotometer at a wavelength of 540 nm, and the concentrations in the original samples read from a standard curve.

Results are expressed as "per cent deformylation" which is the per cent of all available formyl substituents (2/DFD, 1/MFD) which have been cleaved to expose a free arylamine.

Thin-layer chromatography was used to identify the products of deformylation. Sulfones from 1 ml of plasma incubated with 100 μg DFD were extracted into 1 ml

of ethyl acetate. The extracts were applied to Silica gel plates (Brinkman, F 254, activated by heating at 120° for 1 hr) alongside standards dissolved in ethyl acetate. The plates were developed in ethyl acetate or in methylisobutylketone, and the results were visualized by fluorescence in ultraviolet light (254 nm).

Mice used in these studies were ICR strain of either sex.

RESULTS

Preliminary studies showed that DFD given intravenously in DMSO was converted to DDS in the mouse by the end of 1 hr. Table 1 shows that DFD, incubated *in vitro*, was deformylated rapidly in blood and plasma, but not in red cells. All subsequent experiments *in vitro* were carried out in plasma.

TABLE 1. SITE OF DEFORMYLATION OF DFD IN MOUSE BLOOD

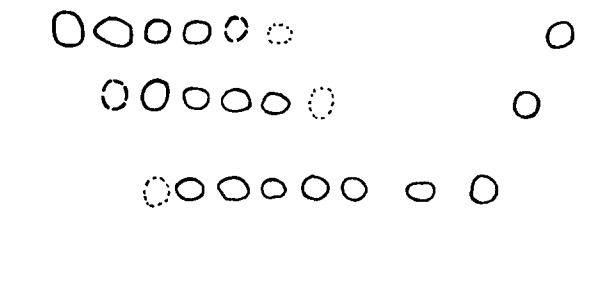
Sample	Incubation time (min)	Total arylamine (μg/ml as DFD)	Free arylamine (μg/ml as DDS)	Deformylation (%)*
Whole blood	30	53·6†	41·5	77
		42·8†	32·2	75
Plasma	30	49·3†	40·0	81
		49·3†	35·0	71
Red cells (in 0·2 M phosphate buffer, pH 7·4)	30	69·4†	< 1	—
		70·0‡	< 1	—

* Per cent of all available groups removed in 30 min (free × 100)/total.
† Measured total after addition of 50 μg in 0·1 ml DMSO to 1-ml sample.
‡ Measured total after addition of 100 μg in 0·1 ml DMSO to 1-ml sample.

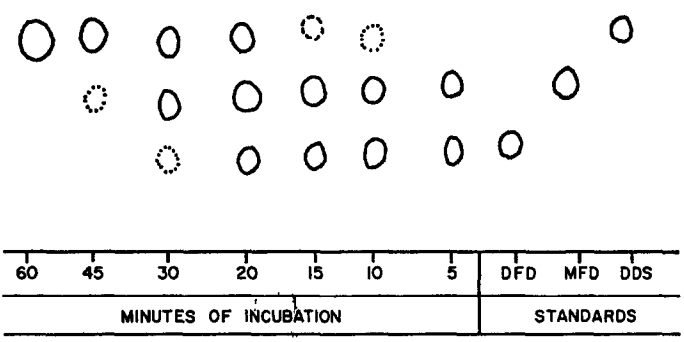
To eliminate the possibility that DMSO was mediating the deformylation, DFD dissolved in acetone or crystalline DFD obtained from acetone-DFD solution was added to plasma. (DFD can exist in several crystal forms depending on the solvent from which it is crystallized; that prepared from acetone is small thin leaflets which dissolve readily.) After 10 min of incubation there was 41 per cent deformylation of the DFD added in acetone and 34 per cent deformylation of the DFD added as crystals as compared to 34 per cent deformylation of the DFD added in DMSO.

As shown by thin-layer chromatography (Fig. 1, a and b), appreciable amounts of MFD were formed after 5 min of incubation. After 10 min, traces of DDS were present, in addition to DFD and MFD. Most of the DFD had been deformylated to MFD and DDS after 30 min. By 45 min no DFD and only traces of MFD remained. All MFD was converted to DDS by 60 min.

Comparison of rates of deformylation of DFD *in vivo* and in plasma *in vitro* is shown in Fig. 2. Of the formyl groups available, 50 per cent was removed in 7 min *in vivo* and in 18 min *in vitro*. This difference, if real, may involve liver activity *in vivo* (see Discussion). Plasma concentration of total sulfone, i.e. original DFD, ranged between 50 and 100 μg/ml (0·15–0·3 mM) both *in vivo* and *in vitro*. Concentrations in this range were achieved *in vivo* by giving a dose of 200 mg/kg, i.v. in DMSO by tail vein. These levels were well below saturation of the enzyme system. Using concentrations between 7 and 1130 μg/ml plasma (24–3700 μM) we established from a Lineweaver–Burk plot an apparent K_m for the system of ~ 10 mM.



(a)



(b)

FIG. 1. Tracing of thin-layer chromatograph showing products of incubation with mouse plasma at 37°. Separate 1-ml samples, to which 100 μg DFD in DMSO were added, were removed at time intervals shown, and products extracted into 1 ml of ethyl acetate. This extract was applied to Silica gel plates. (a) Plate developed in methyisobutylketone; R_fs of DFD, MFD and DDS are 0·21, 0·40 and 0·53 respectively. (b) Plate developed in ethyl acetate; R_fs of DFD, MFD and DDS are 0·18, 0·35 and 0·48 respectively. Closed circle implies amount > broken > dotted circle.

A single study of deformylation *in vitro* of original MFD in pooled plasma of 13 mice at a concentration of 98 μg/ml was made (Fig. 3). This showed the rate for removal of formyl groups from MFD and DFD to be the same. At 5 min, a time shown by Fig. 1 to be prior to deformylation of MFD to DDS when starting with DFD, 40 per cent of the single formyl group was removed from MFD (Fig. 3) as compared to 20 per cent of the two formyl groups from DFD (Fig. 2).

Experiments were also conducted to determine if DFD could be deformylated by plasma of other mammalian species. These showed that the plasma of rat, guinea pig and rabbit contained an enzyme system capable of cleaving the formyl groups from DFD, but the plasma of man and dog did not. Where enzymatic activity was present, there were species differences in the rates of deformylation (Fig. 4). After 1 hr of incubation of DFD in plasma, mouse deformylated 100 per cent, rat 58 per cent, guinea pig 25 per cent and rabbit 3 per cent. There was no deformylation of DFD in the plasma of dog or man at the end of 6 hr of incubation.

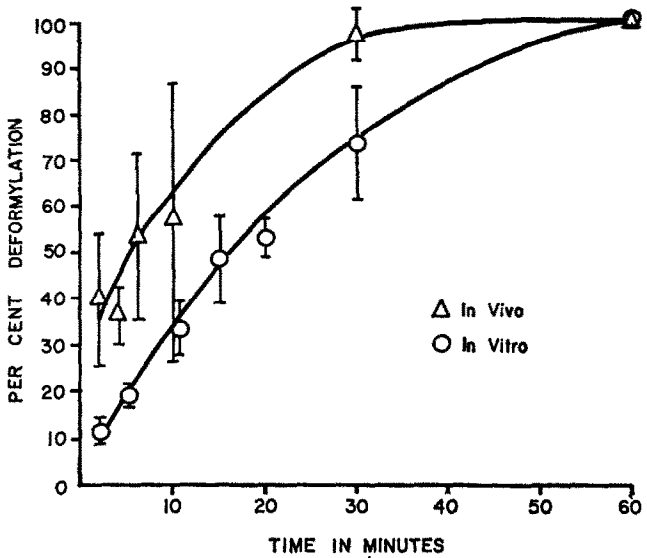


FIG. 2. Comparison of rates of deformylation of DFD *in vivo* in mice and *in vitro* in mouse plasma at 37°. Per cent deformylation is calculated from free arylamine formed, determined as DDS. Each point represents the mean, with standard deviation, of four to six experiments in which plasma from one mouse or from two mice pooled was taken for each time interval. For experiments *in vivo* 200 mg/kg DFD were given i.v. in DMSO; for experiments *in vitro* 50 μg DFD were incubated with 1 ml plasma. Final plasma concentrations were similar for both studies.

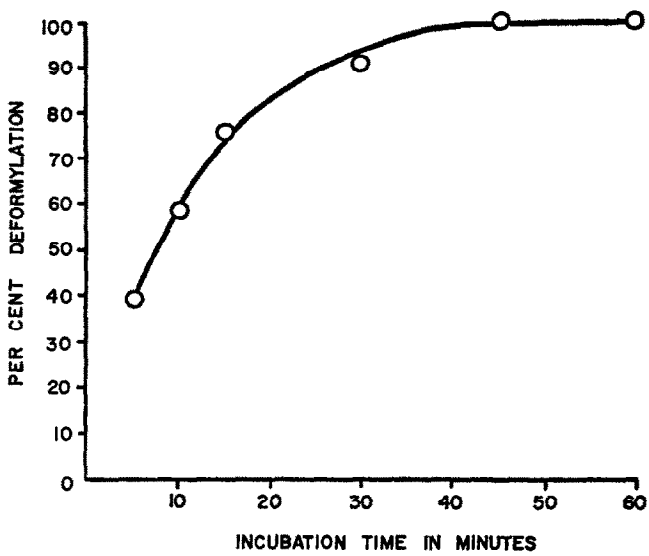


FIG. 3. Rate of deformylation of MFD *in vitro* to DDS by mouse plasma incubated at 37°. Each point represents duplicate determinations on pooled mouse plasma in a single experiment in which 100 μg DFD were added to tubes containing 1 ml plasma.

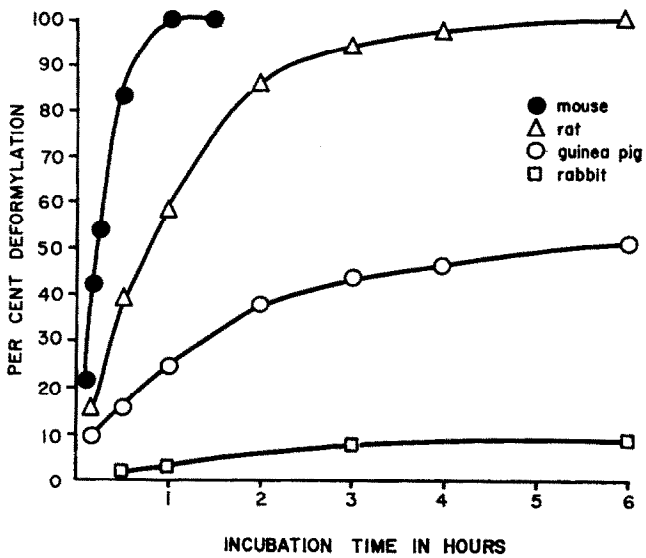


FIG. 4. Rates of deformylation of DFD *in vitro* in mouse, rat, guinea pig and rabbit plasma incubated at 37°. Separate 1-ml samples of plasma to which 50 μg DFD in DMSO were added were analyzed after indicated times. Plasma from dog (two experiments) and man (three experiments) did not deformylate DFD and are not shown. Number of animals for data shown: 12 mice pooled; three rats pooled; two guinea pigs; one rabbit.

To examine the specificity of the reaction, N^4-formyl derivatives of *p*-aminobenzoic acid, sulfadiazine and sulfanilamide, and 4-formamido-4′-nitrodiphenylsulfone were incubated at 100 μg/ml in mouse plasma for 30 min. Of these compounds, only the diphenylsulfone structure was deformylated (55 per cent in 30 min). There was no deacetylation of 4,4′-diacetamidodiphenyl sulfone (DAD) *in vitro*. *In vivo*, drug present following 200 mg/kg, i.v. DAD in DMSO was 40 and 49 per cent deacetylated (two experiments) at 1 hr, with no increase in the proportion of free amine to total in the second hour.

The deformylating activity present in mouse plasma could be completely abolished (no free amine produced in 60 min) by preincubation of the plasma at 56° for 30 min. This proof that DFD was cleaved enzymatically led to studies to determine if the reaction could be inhibited by some common inhibitors of plasma enzymes. It was found that the activity could be completely inhibited by 10^{-4} M diethyl *p*-nitrophenylphosphate. However, there was no inhibition in the presence of 10^{-3} M of *p*-chloromercuribenzoate or neostigmine.

A comparison of enzyme activity at room temperature and at 37° by TLC showed that after 60 min at 25° only traces of DDS had been formed while MFD and DFD were present in approximately equal concentrations.

DISCUSSION

Upon first observing the rapidity of the conversion of DFD to DDS after intravenous injection in DMSO into mice, we considered that either blood or some very active system in liver must be the agent of deformylation. Simple aqueous hydrolysis

at neutral pH is very slow.[4] Our experiments *in vitro* showed that enzymatic cleavage takes place in plasma at a rate which could account for the entire phenomenon *in vivo*. However, liver may and probably does account for some deformylation *in vivo*.[5,6] This may be the reason for the modest difference observed in Fig. 2 between rates *in vivo* and *in vitro*.

Hoffmann *et al.*[5] and Chiou[6] found that liver homogenates from various animals will deformylate DFD. Hoffmann *et al.*[5] found the process to be stepwise through MFD, hydrolytic rather than oxidative, and the rates for guinea pig > mouse > rat. Chiou extended this study to other species and used longer incubation times. His results show liver deformylation of DFD in guinea pig > man > rabbit = mouse = rat > dog. A point which remains unresolved is that deformylation is not appreciable in dog plasma, liver or kidney,[6] yet we have observed that the level of DDS in dogs given 30 mg/kg DFD i.v. in DMSO rises to a peak at 6 hr[7] equal to 65 per cent of all drug present and thereafter accounts essentially for all drug in plasma as drug level decays (data not shown). It may be that dog liver *in vivo* deformylates DFD more rapidly than *in vitro*, or that still another tissue is involved. In the case of man, in whom DFD has been shown to have antimalarial properties,[8] about 10 per cent of an oral dose is recovered in urine as total arylamine or ^{35}S. Free DDS, its acetyl derivatives or other conjugates arising from the free amine, account for 9 per cent indicating that essentially all of the drug absorbed is deformylated.[9] In plasma free DDS is $\sim$ 60 per cent of the total drug present between 1 and 48 hr.* This DDS can be accounted for by liver deformylation,[6] but may also be formed in the gastrointestinal tract by acidic hydrolysis or as the result of enzyme action by intestinal flora.

Purified liver formamidase (or formylase) of several species[10–12] and extracts of *Neurospora*[13] have been shown to deformylate formylkinurenine and other formyl or formamido compounds with marked specificity for formylkinurenine. *Escherichia coli* extracts deformylate *N*-formylmethionine.[14] (DFD) has not been tested in these systems as far as we know, nor to our knowledge has a formamidase been described for plasma. The response to inhibitors tested in our mouse plasma system, the species specificity and the concentration of diethyl *p*-nitrophenylphosphate giving 50 per cent inhibition (5×10^{-5} M) all show marked similarity to the aliesterase or B esterase of plasma as described by Augustinsson[15] in his study of plasma esterases in several species. In view of the substrate specificity that we observed, it may be that the diphenylsulfone skeleton provides the basis for action of an esterase on the formamido linkage, an action which is lacking for the other ring structures tested in our experiments. Or it may be that a true plasma formamidase exists in some species. The lack of deformylation of N^4-formyl *para*-aminobenzoic acid by mouse plasma is similar to the low deformylating activity of the liver enzyme for this compound.[10] Deacetylation by the liver enzyme is also minimal,[10,11] in agreement with the inability of mouse plasma to deacetylate DAD.

The number of animals used in the study of deformylation by plasma of several species (Fig. 4) may leave some doubt about comparing exact rates of deformylation. It is firm, however, that mouse has the fastest rate of all animals tested and that neither human nor dog plasma can deformylate DFD.

The stepwise deformylation of DFD, with removal of the first of two groups from DFD proceeding at the same rate as removal of the remaining group from MFD,

* Maren *et al.*, unpublished observations.

implies the absence of large shifts in charge properties or electron densities by removal of one group from the skeleton. This neutrality is reflected in the fact that DFD does not respond to titration by either acid or base and DDS responds only minimally as a base with $pK_a \sim 2$[2].

It has been shown that removal of the repository action of DFD reduces the antimalarial action of DFD against *P. berghei* to that of DDS. It is now clear that the reason for this is rapid removal of formyl substituents upon contact with plasma. Earlier observed superiority of DFD in *P. berghei* over DDS on a weight basis, when given subcutaneously or intraperitoneally,[1,2] can be presumed to result from the repository effect of the routes of administration and the difference in solubilities of the two compounds in body fluids. While higher plasma levels of DDS would be expected from injecting the more soluble DDS by these routes, the poor solubility of DFD results in a lower but sustained and effective level of DDS in the plasma.[2]

Acknowledgements—We are indebted to Dr. Arch C. Sonntag of the Department of Pharmacology and Therapeutics, College of Medicine, University of Florida, for the preparation of the compounds bearing an N^4-formyl substituent which we used in the study of substrate specificity.

REFERENCES

1. D. M. AVIADO, *Expl Parasit.* **20,** 88 (1967).
2. B. P. VOGH and L. N. GLEASON, *J. Pharmac. exp. Ther.* **177,** 301 (1971).
3. A. C. BRATTON and E. K. MARSHALL, JR., *J. biol. Chem.* **128,** 537 (1939).
4. L. C. GARG, *J. pharm. Sci.* **60,** 606 (1971).
5. P. HOFFMAN, J. KLEINMAN, C. MILGROM and K. P. DUBOIS, *Fedn Proc.* **26,** 568 (1967).
6. C. Y. CHIOU, *Biochem. Pharmac.* **20,** 2401 (1971).
7. A. C. SONNTAG and T. H. MAREN, *Pharmacologist* **12,** abstr. 396 (1970).
8. D. F. CLYDE, C. C. REBERT, V. V. MCCARTHY, A. T. DAWKINS and S. A. CUCINELL, *Milit. Med. suppl.* **135,** 527 (1970).
9. T. H. MAREN, A. C. SONNTAG and V. G. STENGER, *Pharmacologist* **12,** abstr. 395 (1970).
10. A. H. MEHLER and W. E. KNOX, *J. biol. Chem.* **187,** 431 (1950).
11. R. S. S. SANTTI and V. K. HOPSU-HAVU, *Biochem. Pharmac.* **17,** 1110 (1968).
12. R. S. S. SANTTI and V. K. HOPSU-HAVU, *Acta chem. scand.* **24,** 791 (1970).
13. W. B. JAKOBY, *J. biol. Chem.* **207,** 657 (1954).
14. H. WEISSBACH and B. REDFIELD, *Biochem. biophys. Res. Commun.* **27,** 7 (1967).
15. K. B. AUGUSTINSSON, *Acta chem. scand.* **13,** 571 (1959).