Production of a Methemoglobin-forming Metabolite of 3,4-Dichloroaniline by Liver *in vitro*

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Some acylanilides used as herbicides in agriculture have in common 3,4-dichloroaniline (DCA) as the aromatic amine moiety. One of these herbicides is propanil (3,4-dichloropropionanilide) which is widely used in the cultivation of rice, a dietary staple of a major proportion of the world population.

Rice plants possess an acylamidase which hydrolyses propanil to DCA and propionic acid (MATSUNAKA, 1968). STILL and MANSAGER (1969) identified DCA in alkaline hydrolysates of rice grain obtained from experimental rice plots that were treated with propanil, and in hydrolysates of rice grain purchased from the consumer market. In mice, rats, rabbits and dogs, propanil is hydrolysed by a liver acylamidase with the resultant release of DCA (WILLIAMS and JACOBSON, 1966). The fact that DCA may be consumed by animals including man (either per se in rice grains or following enzymatic hydrolysis of the parent acylanilide) is a matter of concern, since it is well known that aniline and its derivatives are capable of producing methemoglobinemia. Evidence for the conversion of aniline derivatives to methemoglobin-formers, phenylhydroxylamines and nitrosobenzene derivatives, in intact animals and by liver microsomes in vitro was reviewed by KIESE (1966) and WEISBURGER and WEISBURGER (1971). SINGLETON and MURPHY (1973) showed that a sublethal dose of DCA administered intraperitoneally to male mice produced substantial methemoglobinemia. Their observation that pretreatment with SKF-525A inhibited and phenobarbital increased DCA-induced methemoglobinemia indicated that DCA, like other aniline derivatives is probably metabolized by microsomal-oxidase enzyme systems to methemoglobin-forming N-hydroxylated derivatives.

The present paper reports the development of a convenient assay method to determine the capacity of liver homogenates to metabolize DCA to an active methemoglobin-forming metabolite. In this method, whole liver homogenates were incubated with DCA, a NADPH-generating system and heparinized rat blood which served as the source of hemoglobin, the reactant with the active metabolite of DCA. The activity was measured in terms of the quantity of methemoglobin formed. Using this method, the relative activities of livers from male and female mice, rats and guinea pigs were compared.

Materials and Methods

Adult Holtzman rats (200-250 gm), Charles River mice (22-27 gm) and guinea pigs (300-350 gm, obtained from a local strain) of both

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sexes were used. Commercial grade 3,4-dichloroaniline was purchased from the Aldrich Chemical Company, Milwaukee, Wisconsin and glucose-6-phosphate and NADP from Sigma Chemical Co., St. Louis, Mo.

Animals were sacrificed by decapitation and exsanguination. Blood samples from rats and guinea pigs were collected in heparinized syringes from the severed vessels exposed following decapitation. Mouse blood was obtained by cardiac puncture. Liver samples were homogenized in a Potter-Elvehjem glass homogenizer with a Teflon pestle, using sufficient cold 1.15% KCl to produce a final homogenate concentration of 20%.

The *in vitro* assay method that was developed and found suitable for assaying the metabolism of DCA to a methemoglobin-forming agent is detailed as follows:

DCA substrate solutions were prepared by adding 83.33 µmole DCA in 0.5 ml N,N,dimethylformamide to 100 ml 0.1 M phosphate buffer, pH 7.4. Solutions of nicotinamide (14.65%, w/v), $MgCl_2.6H_2O$(12.2%), glucose-6-phosphate (7.6%) and NADP (1.38%) were made up with 0.1M phosphate buffer.

The incubation mixture, in a 25 ml erlenmeyer flask, contained 0.3 ml DCA(0.25 µmole), 0.1 ml $MgCl_2.6H_2O$(60 µmole), 0.1 ml nicotinamide (120 µmole), 0.1 ml glucose-6-phosphate (25 µmole), 0.1 ml NADP (1.8 µmole), 0.2 ml 20% liver homogenate (40 mg fresh liver), 0.2 ml heparinized rat blood (approximately 28 mg hemoglobin) and an appropriate volume of 0.1M phosphate buffer to make a final incubation volume of 4.7 ml. Incubations were carried out aerobically in a shaking (120 oscillations/min) water bath at 37°C. The reaction was terminated by packing the erlenmeyer flasks in crushed ice. Cold saponin solution (0.4 ml of a 0.5% solution) was immediately added to the flask to facilitate rupturing of erythrocytes. The mixture was then centrifuged at 1,000 x g for 10 min and the supernatant solution was assayed for methemoglobin by the procedure of EVELYN and MALLOY, described by HENRY (1964) and modified by SINGLETON and MURPHY (1973). Appropriate tissue blanks (containing all the constituents except DCA) and blood blanks (containing all the constituents except liver homogenate) were incubated in all these assays and their absorbances subtracted from the observed values. Methemoglobin was expressed as percent methemoglobin (percent of total hemoglobin).

Results and Discussion

Figure 1 shows the relative capacities of livers from mice, rats and guinea pigs to produce an active methemoglobin-former from DCA. Livers from mice and guinea pigs were about equally active in producing methemoglobin-forming metabolite from DCA, while rat liver was less effective. Sex differences in liver capacity to activate DCA were not observed in mice and guinea pigs. Male rat livers were significantly more active than female rat livers.

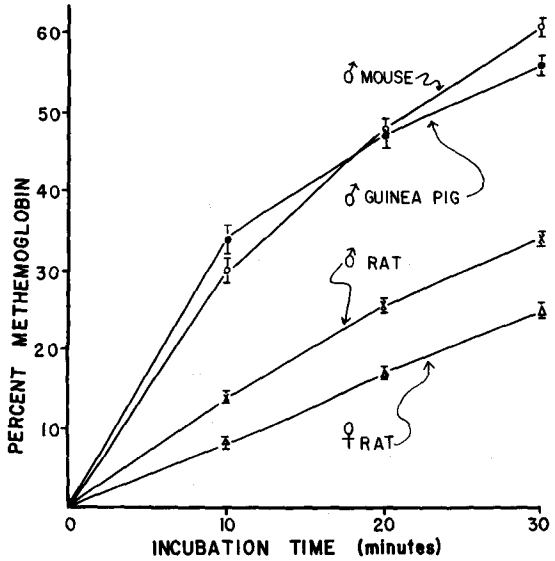

Figure 1. Rates of production of a methemoglobin-forming metabolite of DCA by liver homogenates from mouse, rat and guinea pig with DCA as substrate. Incubation medium is as described in methods. Each point is the mean ± SE for assays on livers from 5 animals. Since there was no significant sex difference in methemoglobin levels with livers from mice and guinea pigs, the results for the female animals are not presented in this graph.

DEBACKERE and UEHLEKE (1964) using liver microsomes prepared from different species of animals studied N-hydroxylation of p-chloroaniline. N-hydroxylated derivatives were determined as p-chloronitrosobenzene according to the method of HERR and KIESE (1959). They found that rates of N-hydroxylation were not linear during the 60 min period of incubation. The calculated Vmax values showed that liver microsomes from guinea pig and mice were about equally active while rat liver microsomes were much less effective. These results are in agreement with those presented in Figure 1 for the metabolism of DCA to an active methemoglobin-forming metabolite.

A major criticism of using microsomes is that the quantity of centrifuged microsomes varies in different species so that the total quantity of microsomal enzymes in the intact liver is not known (DEBACKERE and UEHLEKE, 1964). Since whole liver homogenates were used in our assay procedure, an estimate of the total capacity of livers from different species to produce metabolites capable of oxidizing hemoglobin to methemoglobin could be obtained.

The methemoglobin levels obtained when erythrocytes are exposed to an oxidant depends on both the relative rates of oxidation of hemoglobin and its subsequent reduction by methemoglobin reductase, back to hemoglobin (JAFFE, 1964; STOLK and SMITH, 1966). Figure 2 shows the rates of methemoglobin formation when 0.2 ml of heparinized male rat, mouse and guinea pig blood were incubated in an incubation mixture containing 0.2 ml 20% male mouse liver homogenate, 0.25 µmole, DCA, cofactors and phosphate buffer as described in methods. At the various incubation periods shown, the percent methemoglobin was highest with rat blood followed by guinea pig and then mouse blood. The possibility that these differences were due to different rates of methemoglobin reduction is supported by results shown in Figure 3. In this study, sodium nitrite pretreated erythrocytes from male mice, rats and guinea pigs containing about the same percent methemoglobin were incubated with mouse liver, cofactors and phosphate buffer. In these incubations DCA was omitted. At various incubations periods residual percent methemoglobin was determined. The rate of decline of methemoglobin was most rapid in mouse erythrocytes followed by guinea pig and then rat erythrocytes. The results obtained for the mouse and rat erythrocytes are in agreement with previous reports by STOLK and SMITH (1966) showing that mouse erythrocytes have higher rates of methemoglobin reductase activity than rat erythrocytes. Guinea pig erythrocytes apparently have intermediate reductase activity. This experiment indicates that heparinized rat blood is preferred in the assay method to determine the capacity of liver to metabolize DCA to a methemoglobin-former primarily because of the lower methemoglobin reductase activity.

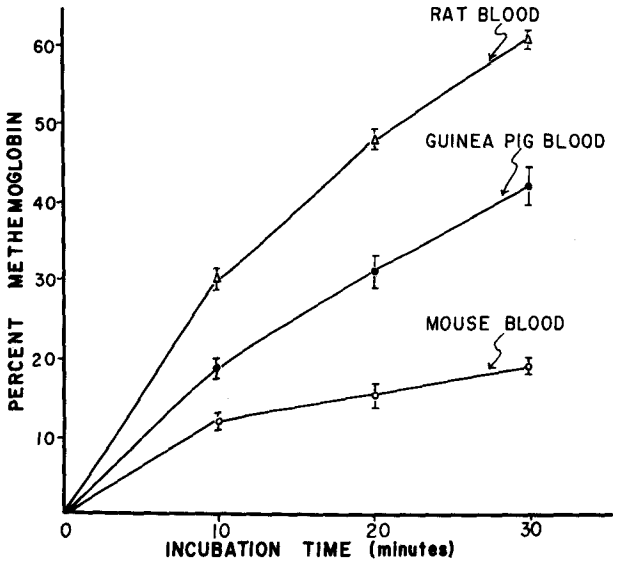

Figure 2. Rates of methemoglobin production by male mouse liver homogenate with DCA and incubated in the presence of heparinized blood from male mouse, rat and guinea pig. Other components of the incubation medium are as described in methods. Each point is the mean $\pm$ SE from assays on 5 mouse livers.

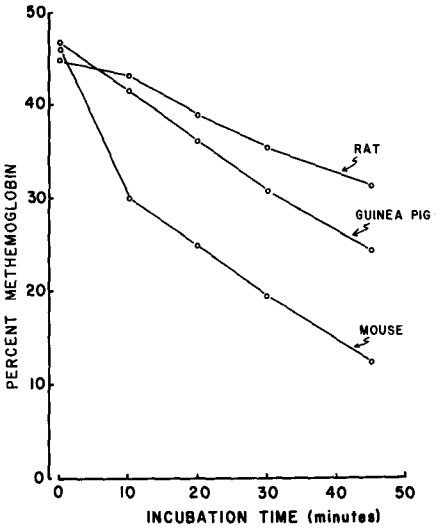

Figure 3. Rate of methemoglobin reduction following in vitro incubation of sodium nitrite pretreated erythrocytes from male mice, rats and guinea pigs. The results are the mean of 2 separate experiments.

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