The Reduction of Nitro and Azo Compounds by Housefly Microsomes

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Microsomes from housefly abdomens were compared to rat hepatic microsomes in their ability to reduce azo and nitro compounds. Microsomal azo- and nitroreductases from both rat and housefly were stimulated by flavins *in vitro*. However, carbon dioxide inhibited azo- and nitroreductases of rat liver microsomes more than those of housefly microsomes. Cytochrome P-450 and NADPH cytochrome C reductase were found in both microsomal preparations. These studies demonstrated that the enzymatic reduction of azo and nitro compounds may be similar in microsomes from both species.

Comparative studies on the microsomal systems of insects and mammals have shown that both systems catalyze similar reactions, including oxidation of rotenone (Fukami *et al.*, 1969) and epoxidation of aldrin among others (Ray, 1967). Both systems have been shown to require $NADPH_2$ and oxygen as cofactors and to contain cytochrome P-450.

Under anaerobic conditions, mammalian microsomes reduce various azo dyes and nitro compounds (Fouts and Brodie, 1957; Hernandez *et al.*, 1967a). Both azo and nitroreductases are inhibited by carbon monoxide and stimulated by flavins *in vitro* (Hernandez *et al.*, 1967b; Fouts and Brodie, 1957; Fouts *et al.*, 1957).

In an atmosphere of carbon monoxide (CO), mammalian azoreductase is inhibited approximately 40% (Hernandez *et al.*, 1967b). Thus, mammalian microsomes reduce azo dyes *via* a CO-sensitive pathway; *i.e.*, pathway involving cytochrome P-450. Furthermore, a CO-insensitive pathway, for the reduction of azo dye, has been reported which may be identical to NADPH cytochrome C reductase, since purified microsomal NADPH cytochrome C reductase reduces azo compounds and other electron acceptors in the absence of cytochrome P-450 (Hernandez *et al.*, 1967b).

In contrast to the oxidative pathways, there have been no comparative studies on the reduction of azo and nitro compounds in insect microsomes. In the present report, microsomes prepared from rat liver and the housefly abdomens (*Musca domestica* L.) were compared for their ability to reduce a representative aromatic nitro compound (*p*-nitrobenzoic acid) and an azo dye [1,2-dimethyl-4-(*p*-carboxyphenylazo)-5-hydroxybenzene (CPA)] (Figure 1). In addition, the effect of carbon monoxide and flavin on the activity of housefly microsomal reductases was compared to the mammalian system.

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MATERIALS AND METHODS

Reagent grade chemicals were purchased from J. T. Baker Chemical Co. and used without further purification. Nicotinamide, NADP, NAD, FMN, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NED [*N*-(1-naphthylethylenediamine hydrochloride] and *p*-nitrobenzoic acid were obtained from Sigma Chemical Co.; CPA [1,2-dimethyl-4(*p*-carboxyphenylazo-5-hydroxybenzene] was obtained from Nutritional Biochemicals Corp.; crystalline bovine serum albumin came from Armour Pharmaceuticals Co.; Ultra High Purity nitrogen was obtained from Southern Oxygen, Division of Air Products; and carbon monoxide came from Matheson Chemical Co.

ENZYME SOURCES

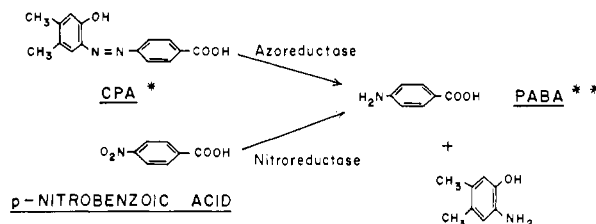
Abdomens from DDT-resistant, 6-day-old female houseflies, which had been fed milk containing 1% sugar, were used as the source of microsomal enzymes. All operations were performed at 0–4° C. The abdomens were homogenized in the ratio of 10 flies/ml of ice cold 1.15% potassium chloride in 0.01 *M* NAH_2PO_4/K_2HPO_4 buffer with 10^{-3} *M* EDTA using a teflon and glass tissue homogenizer, followed by centrifugation at 10,000 × *g* for 15 min in a refrigerated centrifuge. The 10,000 × *g* supernatant was filtered through gauze and then centrifuged at 198,000 × *g* for 45 min in a Spinco ultracentrifuge. The microsomal pellet was resuspended in a volume of 0.05 *M* NAH_2PO_4/K_2HPO_4 buffer pH 7.6 (10^{-3} *M* EDTA) to yield a final concentration corresponding to 30–35 flies/ml.

Rat liver microsomes from adult male Sprague–Dawley rats (150–300 g) were prepared as previously described (Hernandez *et al.*, 1967a).

ENZYME ASSAYS

Microsomal NADPH cytochrome C reductase activity and cytochrome P-450 were determined by the method of Hernandez *et al.* (1967a). Protein was determined by the method of Lowry *et al.* (1951) as modified by Miller (1959) using crystalline bovine serum albumin as standard.

Housefly azo- and nitroreductase was determined as follows. The reaction mixture contained 0.5 μmol of NADP, 50 μmol of glucose-6-phosphate, 100 μmol of nicotinamide, housefly microsomes (equivalent to 30–35 fly abdomens), rat liver



* 1,2-DIMETHYL-4-(p-CARBOXYPHENYLAZO)-5-HYDROXYBENZENE

** p-AMINOBENZOIC ACID

Figure 1. Azoreductase and nitroreductase pathways in housefly microsomes. Both pathways require NADPH

Table I. Requirements for Microsomal Housefly Azo- and Nitroreductase

Conditions	*p*-Aminobenzoic acid formed (nmol/30 min) Nitro-reductase	Azo-reductase
(1) Housefly microsomes + housefly abdomen soluble fraction	46	34
(2) Housefly microsomes + rat liver soluble fraction	15	17
NADP omitted	6	12
NAD substituted for NADP	5	0
(3) Housefly microsomes + glucose-6-phosphate dehydrogenase (5 units)	8	13

Enzyme source, microsomes from female housefly abdomens (equivalent to 25 abdomens or 10 mg protein). Rat liver soluble fraction equivalent to 125 mg wet weight of liver. The results are the means of duplicate incubations from two separate experiments. Incubation conditions are described in Materials and Methods. All values have been corrected for nonenzymatic activity due to the soluble fraction.

Table II. Comparison of Microsomal NADPH–Cytochrome C Reductase and Cytochrome P-450

Microsomes	NADPH–cytochrome C reductase (mμmol/min/mg protein)	Cytochrome P-450 (nmol/mg protein)
Housefly	86.5	0.096
Rat	40.6	0.480

NADPH cytochrome C reductase and cytochrome P-450 were determined by the procedure described in Materials and Methods.

Table III. Comparison of Housefly and Rat Azo- and Nitroreductase

Microsomes	Nitrogen atmosphere	Diff %	Carbon monoxide atmosphere	Diff %
Azoreductase[a]				
Housefly + FMN[b]	7.12	+405	6.84	+242
	36		23.20	
Rat + FMN	185	+434	51	+1270
	987		698	
Nitroreductase[a]				
Housefly + FMN	7.12	+84	5.10	+45
	12.80		7.40	
Rat + FMN	139	+91	35	+209
	266		108	

[a] mμmol *p*-aminobenzoic acid formed/mg protein/30 min. [b] FMN = 2×10^{-5} *M*. The results are the means of duplicate incubations from two separate experiments. All values have been corrected for nonenzymatic activity due to the soluble fraction. Incubation conditions are described in Materials and Methods.

soluble fraction (equivalent to 125 mg wet weight liver), and substrate. For azoreductase, 10 μmol of CPA, and for nitroreductase, 6.0 μmol of *p*-nitrobenzoic acid in a final volume of 2.0 ml 0.05 *M* NAH_2PO_4/K_2HPO_4 pH 7.6 (10^{-3} *M* EDTA) buffer. The reactants were placed in serum bottles with screw tops and rubber diaphragms. While on ice, the bottles were flushed for 5 min with nitrogen or carbon monoxide which had been passed through a deoxygenizer mixture (0.5% sodium dithionite, 0.05%, 2-anthroquinone sodium sulfonate in 0.1 *N* NAOH). The bottles were incubated with shaking for 30 min at 32° C while continuously flushed with the respective gas. The reaction was stopped by the addition of 2.0 ml of 10% TCA. A 2.0-ml aliquot of the TCA supernatant was removed and the amount of *p*-aminobenzoic acid (PABA) formed was determined by the method of Bratton and Marshall (1937).

RESULTS

The data in Table I indicate that insect microsomes, in common with mammalian hepatic enzymes, required NADPH for the reduction of *p*-nitrobenzoic acid and the azo dye. When the 10,000 × *g* supernatant of housefly homogenates was used as the source of enzyme, the reductase activities were greater than the combination of housefly microsomes and an NADPH-generating system (rat liver soluble fraction plus NADP plus glucose-6-phosphate). Measurement of the glucose-6-phosphate dehydrogenase activity of the fly soluble fraction used in the incubation flask containing the fly 10,000 × *g* supernatant was shown to possess 0.83 Kornberg units/ml, whereas the rat liver soluble fraction (Table I) contained 0.25 Kornberg units/ml. However, the differences in the glucose-6-phosphate dehydrogenase cannot account for the greater activity seen with the fly 10,000 × *g* supernatant, since the activity contributed by the soluble fraction alone (nonenzymatic activity) of both rat and fly systems was the same. Thus, the greater activity observed with fly 10,000 × *g* implies the presence of some other factor in the soluble fraction which enhances reductase activity other than the NADPH-generating system.

Similar to rat liver microsomes, the microsomes from housefly abdomens displayed NADPH cytochrome C reductase activity and contained the carbon monoxide binding pigment, cytochrome P-450 (Table II). When based on specific activity (μmol/min/mg protein) NADPH cytochrome C reductase was higher in fly abdomen microsomes than in microsomes obtained from rat liver.

Housefly microsomal azo and nitroreductases were found to be qualitatively similar to the mammalian reductase system (Table III). Specific enzyme activities (μmol/min/mg protein) were less in microsomes from fly abdomens than from rat liver. This could be due to dilution of those specific microsomal enzymes by nonactive microsomal protein, since the enzyme source included the microsomes obtained from the entire fly abdomen. Furthermore, Ray (1967) observed that fly microsomes were $^1/_{10}$ as active as the mammalian system in the epoxidation of aldrin.

Under a nitrogen atmosphere, FMN stimulated housefly and rat azoreductase equally (Table III). All enzyme activities were corrected for any nonenzymatic reduction due to the soluble fraction (less than 10%). FMN at 2×10^{-5} *M* does not affect the nonenzymatic component (Shargel, 1967). Carbon monoxide (CO) was more effective in inhibiting rat hepatic azoreductase than housefly azoreductase. However, total FMN-stimulated activity was less under CO than under nitrogen in both species.

Nitroreductase was stimulated to the same extent by FMN in both species (Table III). Furthermore, CO was less effective in inhibiting housefly nitroreductase than rat liver nitroreductase. Similar to azoreductase, total FMN-stimulated nitroreductase activity was less under carbon monoxide than under nitrogen. Furthermore, although CO inhibited the activities of both reductases, the degrees of inhibition in the presence of FMN were similar for both species. The decrease in reductase activities under CO and the decreased FMN-stimulated activity under CO, as well as the presence of cytochrome P-450, would indicate the presence of a CO-sensitive or cytochrome P-450 mediated pathway.

DISCUSSION

The studies on the reductive enzyme systems of housefly microsomes have indicated that the metabolism of xenobiotic compounds is not unique in vertebrate systems. Adamson *et al.* (1965) investigated azo- and nitroreductase activities in a variety of vertebrates and concluded that these reductive enzymes might be more primitive than the drug metabolizing oxidative enzymes. Our studies demonstrated that microsomal preparations from houseflies and rat livers contained several reductive enzymes. These include NADPH cytochrome C reductase, azo- and nitroreductase. Furthermore, inhibition of azoreductase and nitroreductase by CO and the presence of cytochrome P-450 suggested the existence of an electron transport system in insect microsomes resembling that in liver microsomes.

The ability of flavins, *in vitro*, to stimulate azo- and nitroreductase activities in hepatic microsomes was reported by several investigators (Fouts and Brodie, 1957; Fouts *et al.*, 1957; Shargel, 1967). That azo- and nitroreductases of housefly microsomes were stimulated by FMN and inhibited by CO would imply that these reductive enzyme systems are widespread in various species.

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