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MECHANISM OF AROMATIC NITRO GROUP REDUCTION IN THE SOLUBLE FRACTION OF HUMAN PLACENTA*†

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Abstract—Investigations were carried out with soluble fractions of human placental tissue to determine components of a catalytic system responsible for the reduction of *p*-nitrobenzoic acid (PNBA) to *p*-aminobenzoic acid (PABA). Partial purification revealed that hemoglobin and/or methemoglobin were the principal catalytic factors present in the placental 104,000 *g* supernatant. Utilizing tissue-free model systems, it was found that several purified heme-containing compounds would catalyze the reduction of PNBA at rates comparable to those observed in placental soluble fractions. Methemoglobin, metmyoglobin, hematin, and dialyzed, lyophilized placental soluble fractions all displayed biphasic effects with variations in concentration. Maximal catalytic activity varied among these heme catalysts but optimal concentrations, when expressed as total monomer heme, were fairly uniform. Carbon monoxide markedly inhibited both the placental tissue catalysis and heme-model system nitroreductase reactions.

NITROREDUCTASE is an incompletely characterized enzyme system which catalyzes the reduction of aromatic nitro groups to the corresponding primary amines. Generally, these amine metabolites possess considerably less biologic activity and are more rapidly excreted by virtue of their increased water solubility. The reduction reaction is presently thought to proceed via the formation of nitroso and hydroxylamino intermediates.[1] Substrates for this enzyme system include several insecticides such as parathion; the fungicide pentachloronitrobenzene; toxic industrial chemicals such as nitrobenzene; the anticonvulsant agent, nitrazepam; and the antibiotics, chloramphenical and azomycin.

Nitroreductase has been detected in a variety of tissues including liver, adrenal, lung and placenta. The components of these various tissues which participate in aromatic nitro group reduction are unresolved. The present investigation seeks to characterize and identify factors present in the soluble fractions of human placental homogenates and elucidate the mechanisms by which nitro group reduction is mediated in such preparations.

MATERIALS AND METHODS

Materials. NADPH, NADH, FMN, bovine methemoglobin, equine heart cytochrome *c*, catalase, and chlorophyllin were obtained from Sigma Chemical Company,

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St. Louis, Mo. Bovine hematin and equine metmyoglobin were purchased from Calbiochem Company, Los Angeles, Cal. *p*-Nitrobenzoic acid (PNBA) and *p*-aminobenzoic acid (PABA) were purchased from Eastman Organic Chemicals. Carbon monoxide (99·5 per cent minimum purity) was purchased from Matheson Company, Inc., Joliet, Ill. All other reagents and solvents used were of analytical grade.

Human term placentas were obtained after normal vaginal deliveries or cesarean sections.

Preparation of placental tissue. Human placentas were freed of adhering connective tissue and rinsed with cold 1·15% KCl. The cotelydons were blotted, weighed and re-homogenized with two parts (w/v) ice-cold isotonic KCl in a Waring blender at high speed for 10 sec. A Potter homogenizer with teflon pestle was used to homogenize the tissue.

The nuclei and cell debris were removed from the 33 per cent homogenates by centrifuging at 800 *g* for 10 min in an IEC refrigerated centrifuge. The 800 *g* supernatant was centrifuged at 9000 *g* for 20 min. The 9000 *g* supernatant was then centrifuged at 104,000 *g* for 1 hr at 4°. The 104,000 *g* supernatant represented the soluble fraction which was either used immediately or stored at −12° to −15°. No effect of storage on nitroreductase activity was detected, even after three months.

Ultrafiltration. Fifty-ml aliquots of placental supernatant fraction were placed in a diaflo cell (Amico Corp.) equipped with a magnetic stirrer. Various ultrafiltration membranes with approximate cut-offs of 30,000, 50,000, 100,000 and 300,000 mol. wt. were used under nitrogen pressure (10–40 psi). Filtrates were assayed as collected. Residues were washed by forcing 200 ml phosphate buffer through the cell under nitrogen pressure, and subsequently reconstituted to the original volume.

Partial purification. The soluble fraction of the placental homogenate was subjected to step-wise ammonium sulfate fractionation. The precipitate retaining the most activity was diluted, dialyzed in 0·1 M phosphate buffer (pH 7·5), and placed on a DEAE-cellulose column (1·0 × 40 cm). Elution was carried out step-wise with 0·1 M, 0·3 M and 0·5 M NaCl solutions at room temperature.

Assay procedures. Typical reaction mixtures contained 10^{-3} M NADPH (unless otherwise stated) 5×10^{-4} M FMN, $2{\cdot}6 \times 10^{-3}$ M PNBA, 0·4 ml placental 104,000 *g* supernatant (12–18 mg/ml protein), and 0·1 M potassium phosphate buffer (pH 6·8), in a final volume of 1·9 ml. In tissue-free systems, several heme-containing compounds were added in final concentrations ranging between 10^{-7} and 10^{-3} M. Incubations were carried out in the dark in a Dubnoff metabolic shaker under an atmosphere of nitrogen, or mixtures of nitrogen and carbon monoxide, at 37°. Reaction mixtures were preincubated for 10 min before NADPH was added to wash out residual oxygen. The reaction was stopped after 2 hr by adding 5·0 ml of ice-cold 6·67% TCA. Amounts of PABA formed were assayed colorimetrically by slight modifications of the Bratton–Marshall[2] procedure as previously described.[3]

Protein concentrations were assayed by the method of Lowry *et al.*,[4] with bovine albumin as standard. Hemoglobin concentrations were determined by procedures described by Levere and Granick.[5] Total methemoglobin and hemoglobin were measured by the CO difference spectrum of a dithionite-reduced sample using 570·8 $\mu M^{-1}\ cm^{-1}$ as the extinction coefficient for the difference in absorbance between 418 and 433 nm.

RESULTS AND DISCUSSION

Nitro group reducing activity in the placental soluble fraction was not significantly affected by dialysis, lyophyllization or boiling for 20 min. The catalytic efficiency of the tissue fraction was also stable to repeated freezing and thawing with no apparent loss in activity after prolonged storage (up to 3 months) at −10°. Nitro group reducing activity of the soluble placental tissue fraction could not be extracted into heptane, benzene, chloroform or *n*-butanol. Indeed, nearly 100 per cent of the original activity could be recovered following homogenization of the denatured protein in the aqueous phases.

Ultrafiltration with a diaflo apparatus indicated that the nitroreductase component was associated with a fraction having mol. wts between 50,000 and 100,000. This suggested that the catalytic factor was a protein. Its stability to heat, on the other hand, indicated that tertiary structure was not essential for the transfer of reducing equivalents to PNBA.

Nitroreductase is markedly inhibited by oxygen. This effect has been noted in all tissues studied[6,7] including human placental soluble fractions.[8] Because of its oxygen-binding properties, contaminating hemoglobin was felt to compromise nitroreductase activity in the placental 104,000 *g* supernatant. Furthermore, recent reports have demonstrated that NADPH in the presence of flavin can nonenzymically reduce methemoglobin,[9] indicating that contaminating methemoglobin might compete with PNBA for the NADPH added in these experiments.

Fractional precipitation with ammonium sulfate demonstrated that over 90 per cent of the original activity could be recovered in the 70–80 per cent saturation (Fig. 1). This procedure, however, failed to separate the hemoglobin from the active fraction of the placental supernatant. In addition, the results obtained with DEAE-cellulose column chromatography revealed that the content of ferri- and ferro-hemoglobin in the various fractions collected paralleled nitroreductase activity. This suggestive

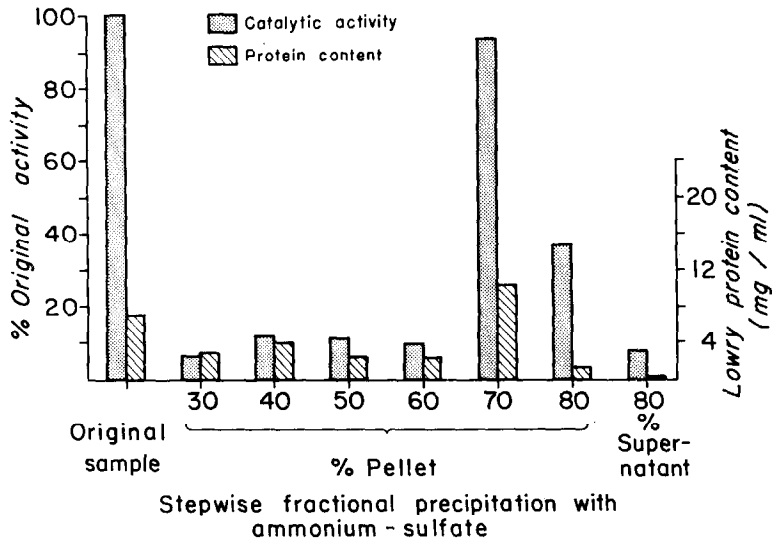

FIG. 1. Reduction of PNBA to PABA in incubation flasks containing ammonium sulfate precipitates of human placental 104,000 *g* supernatant fractions (see Methods).

evidence, however, implicating hemoglobin as the major factor, appeared to be in conflict with previous investigations in several laboratories,[6,10,11] including our own,[8] which were unable to demonstrate significant activity with whole blood or erythrocytes.

In one attempt to resolve these seemingly contradictory observations, purified bovine methemoglobin (twice recrystallized) was substituted in varying concentrations for the placental tissue fraction in reaction flasks. Somewhat unexpectedly, methemoglobin was found to catalyze an easily observable reduction reaction. The catalytic activity of methemoglobin showed biphasic effects with increasing concentrations (Fig. 2). Optimal activity was observed at about 2×10^{-5} M, and was comparable

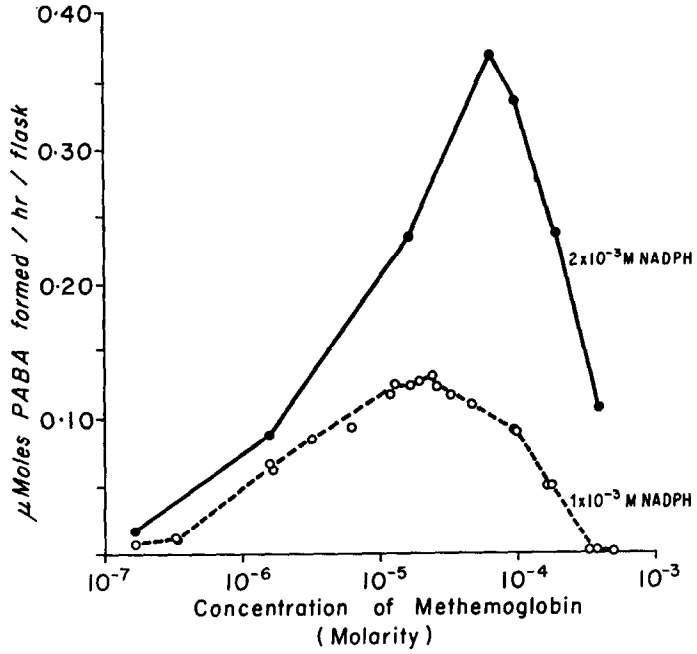

FIG. 2. Biphasic effects exhibited by methemoglobin utilized as a catalyst for aromatic nitroreductase. Increased concentration of electron donor resulted in a shift of the optimal methemoglobin concentration from 2×10^{-4} M to approximately 10^{-4} M.

with the rate of PABA formation observed with placental tissue soluble fractions. Additions of solutions of purified methemoglobin with heme concentrations equivalent to that of whole blood (yielding a final concentration of $4{\cdot}9 \times 10^{-4}$ M methemoglobin in reaction mixtures) resulted in the detection of only very slight activity. Addition of washed erythrocyte membranes did not significantly influence the activity of purified methemoglobin. Dilution of hemolyzed human red cells to a hemoglobin concentration of 2×10^{-5} M in reaction mixtures resulted in amounts of PABA formed which were comparable to those obtained with purified methemoglobin. It appeared likely that the biphasic nature of methemoglobin catalysis could be responsible for the low levels of activity previously reported with whole blood or erythrocytes.

Buhler and Rasmusson[11] reported that blood of rainbow trout exhibited only slight PNBA-reductase activity. They noted, however, that prolonged centrifugation

of the fish liver soluble fraction at 105,000 *g* for 8 hr resulted in increased activity associated with a flocculent reddish precipitate. It is considered possible that the increase in specific activity observed by these workers may have been due to the development of a more favorable hemoglobin concentration.

Previous studies in our laboratory[8] have indicated that reaction velocity increased linearly as NADPH concentration is increased. As shown in Fig. 2, a 2-fold increase in NADPH concentration in reaction mixtures containing purified methemoglobin was paralleled by a marked enhancement of reaction velocity. In addition, it appeared that increased NADPH concentrations would result in a shift in methemoglobin concentration optima inasmuch as maximal reaction rates were observed at higher methemoglobin concentrations. This phenomenon implied a stoichiometric dependence between the concentration of electron donor and the methemoglobin catalyst in the transport of electrons to the substrate. Further studies, however, will be necessary to confirm and define these relationships.

Subsequent studies with other heme-containing compounds revealed that metmyoglobin, oxidized cytochrome *c*, and even hematin were capable of catalyzing the reduction of PNBA at significant rates in the presence of FMN and NADPH or NADH (see Methods). In fact, hematin on an equimolar basis (i.e. equivalent concentrations of total monomer heme) was more effective than the placental soluble fraction or methemoglobin (Fig. 3). Metmyoglobin was considerably less active than methemoglobin. The activity of each of these compounds, as with methemoglobin and placental soluble tissue, was stable to boiling. Preliminary observations indicated that concentrations of catalase equivalent to optimal methemoglobin concentrations

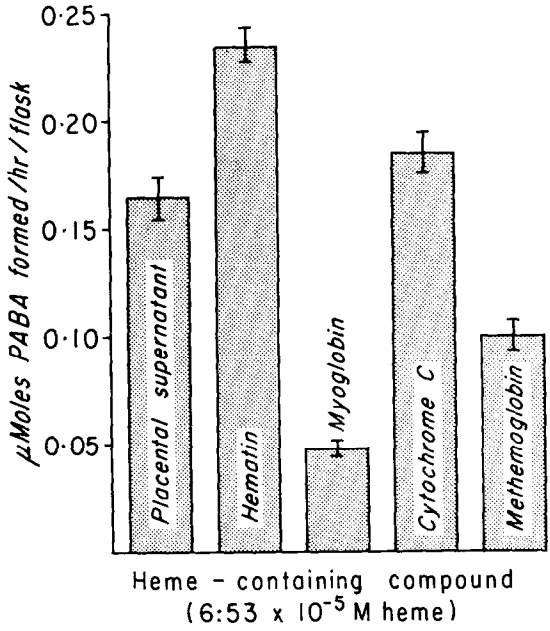

FIG. 3. A comparison of the catalytic efficiency of human placental 104,000 *g* supernatant fractions with several heme-containing compounds with respect to the reduction of PNBA. Conditions are described in Methods.

were only slightly active. Subsequent experiments, however, revealed that such preparations were not pure, and it now appears that catalase is a highly efficient catalyst for the reaction. Chlorophyllin, a magnesium analog of heme, was totally inactive at several concentrations ranging between 10^{-6} and 10^{-3} M. Ferrous or ferric ions, either in the presence of absence or EDTA, did not exhibit detectable activity. It would appear from these observations that the porphyrin structure is essential to catalytic function.

In further experiments we were able to establish that hematin, metmyglobin, and placental soluble fractions all displayed biphasic effects with variations in heme concentration. Placental soluble fractions were dialyzed and concentrated by lyophyllizing and resuspending in appropriate volumes of buffer. The optimal activities of each compound were observed at heme concentrations closely approximating those of methemoglobin.

Carbon monoxide is a potent inhibitor of aromatic nitroreductase. Gillette *et al.*[1] have suggested that reduction of PNBA in rat liver microsomes is mediated by cytochrome P-450 and that CO blocks hepatic microsomal nitroreductase by binding to the cytochrome. Previous studies in our own laboratory have demonstrated that the rates of PABA formation with placental subfractions or boiled homogenates were severely retarded under a carbon monoxide gas phase.[3,8] In the present study it was demonstrated that an atmosphere consisting of one part CO and seven parts N_2 markedly inhibited PNBA reduction in the presence of each heme-containing compound tested (Table 1). In view of the high affinity of cytochrome P-450 and of hemoglobin for CO, it was considered possible that carbon monoxide competes with the nitro substrate (or an intermediate) for the heme binding site. As indicated in Table 1, however, the 12·5% CO atmosphere inhibited more than 90 per cent of the PNBA-reductase activity of cytochrome *c*, which does not bind CO in either its Fe^{2+} or Fe^{3+} forms at the pH of the incubation medium. In earlier studies,[8] we were unable to demonstrate a significant accumulation of nascent formaldehyde in reaction mixtures incubated under CO. It is unlikely, therefore, that a competition for reducing equivalents is responsible for the profound inhibitory effect of this gas on nitroreductase activity. The detailed mechanism of CO inhibition, therefore, remains obscure, but presents an interesting and important problem for further study.

In experiments with bovine methemoglobin, we noted that a considerable amount of the ferri-heme compound was reduced during anaerobic incubation, although quantitative determinations were not carried out. Diversion of electrons to the chelated iron atoms of methemoglobin or other ferri-heme catalysts possibly could account for the biphasic nature of the reactions observed in these experiments. A relatively high content of oxidized heme in reaction mixtures could act as an electron sink. If such were the case, it might be expected that reduced forms of heme catalysts would not exhibit the characteristic bell-shaped activity curve.

To test this hypothesis we reduced a concentrated stock solution of bovine methemoglobin with sodium dithionite and employed a Sephadex G-200 column to separate completely the reducing agent and contaminants from the hemoglobin. Subsequent analysis revealed that at final concentrations of 2×10^{-5} M (optimal for methemoglobin), the reduced form of hemoglobin surprisingly was slightly less efficient than its ferric counterpart (Table 2). Only very little PABA was formed in reaction flasks containing 4×10^{-4} M hemoglobin, suggesting that ferrous forms of heme also are

TABLE 1. EFFECT OF CARBON MONOXIDE ON THE CATALYTIC REDUCTION OF PNBA*

Catalyst†	PABA (nmmoles formed/hr/flask)‡ 100% N_2	PABA (nmmoles formed/hr/flask)‡ 12·5% CO + 87·5% N_2	Inhibition with CO atmosphere (%)
Dialyzed placental soluble fraction	169	8	95
Bovine methemoglobin	129	11	91
Hematin	175	18	90
Mouse liver 9000 *g* supernatant	217	19	91
Cytochrome *c*	168	10	94

* The reaction mixtures contained heme-containing catalyst, NADPH (10^{-3} M), FMN (5×10^{-4} M), PNBA ($2{\cdot}6 \times 10^{-3}$ M) and were incubated under an atmosphere of pure N_2 or an atmosphere of 7 parts N_2 and 1 part CO.

† All catalysts, except the mouse liver supernatant, were added to reaction mixtures to produce 10^{-4} M monomer heme (corresponding to an optimum concentration of 2×10^{-5} M for methemoglobin). Fresh mouse liver homogenate (0·4 ml of the 9000 *g* supernatant), containing 56 mg protein/ml, was added to reaction mixtures with a total volume of 1·9 ml.

‡ Amounts of PABA formed were determined as described in Methods. The values represent the mean of triplicate determinations. The experiment was repeated twice with essentially identical results.

inhibitory to the reaction at high concentrations. It should be pointed out, however, that the present techniques do not preclude some oxidation of hemoglobin in reaction mixtures prior to anaerobic incubation. More definitive comparisons of ferrous and ferric forms of heme compounds are currently under way. Dithionite will itself reduce PNBA to PABA in incubation mixtures. However, addition of a hemoprotein and/or flavin did not appear to accelerate the dithionite mediated reduction.

TABLE 2. COMPARISON OF THE CATALYTIC EFFICIENCY OF METHEMOGLOBIN AND HEMOGLOBIN AT CONCENTRATIONS OPTIMAL AND INHIBITORY FOR METHEMOGLOBIN*

	Ferrous iron (%)	Final concn. (M)	PABA (nmoles formed/hr/flask)†
Methemoglobin	2	2×10^{-5} M	132
		4×10^{-4} M	2
Hemoglobin	93	2×10^{-5} M	122
		4×10^{-4} M	2

* Reaction mixtures containing oxidized or reduced forms of purified bovine hemoglobin, NADPH (10^{-3} M), FMN (5×10^{-4} M) and PNBA ($2{\cdot}6 \times 10^{-3}$ M) were incubated under an atmosphere of N_2 for 2 hr.

† Amounts of PABA formed were determined as described in Methods. The values represent the means of triplicate determinations.

As can be seen from Fig. 3 and Table 1, bovine methemoglobin was only about 65–75 per cent as efficient as the placental soluble fraction at equimolar, optimal heme concentrations. Pre-oxidation of the ferro-hemoglobin present in the placental soluble

fraction did not compensate for these observed differences in catalytic activity. Possibly, the higher specific activity of the placental soluble fraction was due in part to a greater catalytic efficiency of human fetal hemoglobin as compared to the bovine methemoglobin employed in these experiments. Although the heme nucleus is itself an efficient catalyst, the globin portions of the various protoheme molecules apparently can influence reactivity. Optimal concentrations of bovine methemoglobin, for example, were found to be twice as active as the optimum observed with equine myoglobin (Fig. 3). In addition, it is possible that other cofactors may be present in the placental 104,000 *g* supernatant which may participate to enhance heme-catalysis. For example, the flavin-containing diaphorase, ferri-hemoglobin reductase, has been shown by other investigators[12] to promote the reduction of nitrosobenzene to phenylhydroxylamine in red blood cells. Kato *et al.*[13] have suggested that reduction of PNBA by rat liver 105,000 *g* supernatant is mediated partially by dicoumarol-sensitive DT-diaphorase. We were unable, however, to demonstrate more than a slight inhibition of placental soluble nitroreductase activity with 10^{-4} M dicoumarol.

The results of the present study suggest that the only essential biological components of a heme-catalyzed reduction of PNBA to PABA include (in addition to any of several heme compounds) an initial electron donor and a flavin. None of the individual cofactors of coenzymes catalyzed a measurable reaction alone. Moreover, exclusion of any one of these functional elements precluded a demonstrable formation of the primary arylamine product.

It is not known at the present whether the nitroreductase systems of various mammalian tissues and subfractions of a given tissue homogenate also involve iron porphyrin and flavin cofactors. Fouts and Brodie[6] have reported that hepatic nitroreductase includes a flavoprotein, and addition of FMN, FAD or riboflavin markedly accelerates the normal reduction velocity. More recently, other groups of investigators have postulated the participation of certain FAD-containing microsomal reductases, cytochrome b_5 reductase[14] and cytochrome *c* reductase.[15] With regard to the proposed involvement of DT-diaphorase in hepatic soluble fractions, it is considered possible that the flavin prosthetic group of this enzyme serves as a non-specific carrier of electrons between reduced pyridine nucleotide and substrate. The flavin-containing enzyme, xanthine oxidase, has been reported to catalyze the reduction of a number of nitro compounds.[7,16] Recent reports,[6,17] however, have indicated that purified xanthine oxidase does not catalyze the reduction of chloramphenicol or PNBA.

Gillette *et al.*[1] have recently provided indirect evidence that the remarkably efficient reduction of PNBA by hepatic microsomes is mediated by cytochrome P-450. Yoshida,[14] on the other hand, has postulated the participation of cytochrome b_5. It is highly significant that both these microsomal enzymes contain prosthetic heme. Interestingly, addition of bovine methemoglobin (1×10^{-5} M) to the 9000 *g* supernatant fraction of human liver homogenates (20-hr post-mortem) caused a significant decrease in nitroreductase activity (Table 3). In contrast, addition of methemoglobin enhanced PNBA reduction by the 104,000 *g* liver supernatant. However, optimal concentrations of heme compounds of microsomes for aromatic nitro group reduction have not yet been determined.

In consideration of the present findings and the ubiquitous nature of protoheme and flavoprotein in mammalian tissues, it is tempting to speculate that several of the various tissue nitroreductase systems require these components. Investigations are cur-

TABLE 3. EFFECT OF ADDITION OF BOVINE METHEMOGLOBIN TO SUBFRACTIONS OF HUMAN LIVER HOMOGENATES*

Subfraction	μmoles PABA formed/hr/flask†	
	No addition of methemoglobin	Addition of methemoglobin (1×10^{-5} M)
9000 *g* supernatant	2·02	1·64
104,000 *g* supernatant	0·71	0·91

* Subfractions were prepared from human liver (20 hr post-mortem). 0·4 ml of the 9000 *g* supernatant or the 104,000 *g* supernatant (32·5 and 29·3 mg protein/ml, respectively) were added to reaction mixtures containing G-G-P ($4·6 \times 10^{-3}$M), nicotinamide (1×10^{-5} M), $NADP^+$ (10^{-3} M), FMN (5×10^{-4} M) and PNBA ($2·6 \times 10^{-3}$ M).

† Values represent the means of triplicate determinations.

rently under way to isolate the hepatic microsomal cytochromes and flavin-containing reductases, and compare the nitro-reducing activity of reconstituted systems with the microsomal fraction and heme-model systems.

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