Biochemical Pharmacology, Vol. 19, pp. 443–455. Pergamon Press. 1970. Printed in Great Britain

MODEL SYSTEMS FOR AROMATIC NITRO GROUP REDUCTION—RELATIONSHIPS TO TISSUE CATALYZED REAGENTS*

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(*Received* 1 *March* 1969; *accepted* 6 *June* 1969)

Abstract—The essential biologically functional components of a model system for study of the reduction of *p*-nitrobenzoic acid (PNBA) to *p*-aminobenzoic acid (PABA) appeared to include: reduced nicotinamide adenine dinucleotide phosphate (NADPH) or reduced nicotinamide adenine dinucleotide (NADH) as initial electron donors, a flavin [flavin adenine dinucleotide (FAD), riboflavin-5-phosphate (FMN) or riboflavin], a sulfhydryl donor [e.g. reduced glutathione, (GSH) or cysteine] and a salt. The effects of the flavins and GSH were biphasic, showing activation in lower concentrations but inhibition in higher concentrations. The salt requirement appeared to be essential for the provision of optimal ionic strength. Variations of components of the model system increased the reaction rate to only about one-tenth that observed in the presence of boiled, dialyzed human placental or rat hepatic 104,000 *g* supernatant fractions. The reaction was inhibited (approximately 90 per cent) by carbon monoxide (CO) in incubation flasks containing boiled placental soluble fractions but was not inhibited by CO in model system reactions. Addition of $2{\cdot}5 \times 10^{-3}$ M FMN to reaction vessels containing fresh, nondialyzed rat liver 104,000 *g* supernatant fractions markedly enhanced the reaction rate but addition of the same concentrations (inhibitory in model systems) to vessels containing dialyzed rat liver supernatant markedly inhibited the reaction. The results suggested differing mechanisms of electron transport from NADPH or NADH to PNBA for each of the various tissues studied as well as for homogenate subfractions of a given tissue.

PREVIOUS investigations in our laboratories[1, 2] have shown that the reduction of the aromatic nitro group of PNBA to the corresponding amine (PABA) could be catalyzed by human placental 104,000 *g* supernatant (soluble) fractions only if flavins were added as cofactors. By contrast, the reaction proceeded at an easily measurable rate in the presence of rat or mouse placental soluble fractions or hepatic homogenates from a variety of species without addition of flavins to incubation flasks. Since the flavin content of rat placental or liver homogenates was much higher than that of human placental homogenates, the apparent biochemical differences appeared attributable to dissimilarities in tissue flavin concentrations. Dialysis of rat placental homogenates essentially abolished the reduction reaction and addition of flavins restored the activity, thus supporting this hypothesis.

* This research was supported in part by grants HD-01229-02 and FR-77 Public Health Service.
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Subsequent investigations of the reaction mediated by human placental homogenates revealed that the reduction reaction would proceed as rapidly in the presence of dialyzed, boiled placental soluble fractions as with the freshly prepared soluble fractions if flavins were added as cofactors. In each instance the reaction progressed only about 10 per cent as rapidly under an atmosphere of carbon monoxide as under a nitrogen atmosphere. These observations have led us to investigate various model systems for the study of PNBA reduction. The present paper defines the essential cellular elements of such a system, establishes optimal concentrations of the reactants, and attempts to correlate characteristics of reactions catalyzed by various tissue homogenates with those of reactions in the model systems. The mechanism by which carbon monoxide inhibits the tissue catalyzed reactions was also further investigated. Possible routes of electron transfer for PNBA reduction in hepatic vs. placental tissues are discussed.

MATERIALS AND METHODS

Materials. NADPH, NADP, FMN, Ubiquinone (coenzyme Q-6), PCMB,*l*-ascorbic acid, *l*-glutathione, *l*-cysteine, barbital (Veronal), bovine serum albumin (BSA), and Tris (hydroxymethyl) aminomethane (Trizma) were all obtained from Sigma Chemical Co., St. Louis, Mo.; carbon monoxide (CO) was obtained from the Matheson Co.; PNBA and PABA from Eastman Organic Chemicals and glycylglycine from Calbiochemical Co. Other reagents and solvents were obtained from Fisher Scientific Co. Timed pregnant rats (CD strain) and mice (CD-1) strain as well as adult male animals for the same strains were obtained from Charles River Breeding Laboratories, Wilmington, Mass. Homogenates of liver, kidney, placenta and blood were prepared from these animals. Human placental tissues (8–15 weeks gestation) were obtained from healthy patients at operation (dilatation and curettage or hysterotomy) for therapeutic abortion. Human term placentas were obtained after normal vaginal deliveries and caesarean sections. Human blood was obtained from healthy adult male volunteers.

Preparation of tissue homogenates. Preliminary experiments indicated that repeated freezing and thawing of placental tissues or homogenates did not affect their catalytic activity with respect to PNBA reduction. Therefore placental tissues were homogenized and the homogenates were frozen until assayed. Homogenates could be stored at -12 to $-15°$ for several weeks without a significant loss of activity. Liver, kidney, and blood, however, were analyzed in the fresh state. All tissues were prepared as 33% homogenates in ice-cold, isotonic KCl solutions (placentas were first freed of all adhering connective tissue). They were blotted, weighed and homogenized, using a Potter homogenizer with a Teflon pestle. Human placental tissues (cotyledons) were prehomogenized in a Waring blender at high speed for 10 sec.

To sediment nuclei, cell debris and connective tissue, homogenates were centrifuged at 800 *g* for 10 min in a Sowall refrigerated centrifuge. Mitochondrial enriched fractions were obtained by centrifuging the 800 *g* supernatant at 9000 *g* for 20 min. Soluble and microsomal fractions were obtained by centrifuging the 9000 *g* supernatant at 104,000 *g* for 1 hr. Supernatants derived from centrifugation of placental soluble fractions at 400,000 *g* for 1 hr retained approximately 90 per cent of the original specific activity indicating that the catalytic activity of the placental 104,000 *g* supernatant fraction was not due to the presence of "light microsomes".

For dialysis experiments, 8–10 ml of the various homogenates or homogenate subfractions were dialyzed against 700 ml of phosphate buffer (0·1 M, pH 7·4) for 24 hr with three changes of the dialysate. In boiling experiments, homogenates were placed in boiling water for 5 min, rehomogenized and reconstituted to the original volume.

Assay procedures. In the preparation of incubation mixtures, NADPH or NADH was employed as the initial electron donor in the final concentrations ranging between 10^{-4} to 3×10^{-3} M. Substrate (PNBA) concentrations varied from $2{\cdot}6 \times 10^{-4}$ M to $7{\cdot}6 \times 10^{-3}$ M (final concentrations). For tissue catalyzed reactions, 0·4 ml of 33% homogenates were added to the incubation flasks. For model system reactions, reduced glutathione, cysteine, or mercaptoethanol (10^{-4} to 10^{-2} M final concentrations) or 0·4 ml bovine serum albumin (5 mg/ml) were substituted for tissue homogenates. Flavins (FAD, FMN, or riboflavin) were required as cofactors in model system reaction and in incubation mixtures containing human placental homogenates or blood and plasma homogenates. These were added in final concentrations between 10^{-5} M and 5×10^{-2} M. For model system reactions addition of a metal salt (in addition to the buffer salt present) appeared to enhance the reaction in some cases but was not required as a component in the mixture. $MnCl_2$ was usually included in model system reaction flasks. Concentrations of $MnCl_2$ varied from 2×10^{-4} M to $6{\cdot}4 \times 10^{-3}$ M (final concentration). Certain incubation flasks contained an NADPH generating system rather than pre-reduced NADP. Glucose 6-phosphate (7·7 μmoles), NADP (0·18 μmoles), nicotinamide 33·3 μmoles) and magnesium sulfate (8·3 μmoles) were added to these flasks. Zero-time flasks contained 0·17 μmoles PABA but no electron donor was employed as standard. Appropriate tissue and reagent blanks were also prepared. Total volume of each incubation flask was 1·7 ml.

Incubations were carried out under atmospheres of nitrogen, oxygen or carbon monoxide at 37°. Incubation times varied between 15 min and 6 hr and pH of the incubation mixtures varied from 4·0 to 9·4. Potassium phosphate, sodium phosphate, Tris , barbital, glycylglycine, sodium citrate, and sodium borate were employed as buffering agents. Mixtures were incubated with shaking in a Dubnoff metabolic incubator (50–60 rpm). At the conclusion of the incubation period, 5·0 ml of ice-cold 6·67% trichloroacetic acid (TCA) was added to each incubation flask. The PABA present in each flask was then determined quantitatively by slight modifications of the Bratton–Marshall[3] procedure as previously described.[1] In some experiments the amount of formaldehyde formed in incubation flasks was assayed by the method of Cochin and Axelrod.[4] The total flavin content of various tissue homogenates was assayed by a method described by Levy and Jusko.[5] FMN was employed as the reference standard. Protein concentrations were determined by the method of Lowry *et al.*,[6] using bovine serum albumin as standard.

RESULTS

Characteristics of the model system reaction. The observation that PNBA reduction proceeds essentially as rapidly in dialyzed, boiled human placental soluble fractions as in the corresponding fresh homogenate was confirmed (Fig. 1) and led us to investigate the possibility that the reduction reaction could be catalyzed in the absence of tissue homogenates or slices. Since we found that the placental reaction could be inhibited by additions of *p*-chloromercuribenzoic acid (PCMB) we attempted to

substitute a sulfhydryl donor (reduced glutatione, 10^{-3} M, final concentration) for boiled, dialyzed human placental soluble fraction. An easily measurable reaction occurred but at a considerably (approximately 90 per cent) less rapid rate than in the presence of the placental tissue. It was subsequently discovered that mercaptoethanol, cysteine and cystine in equimolar concentrations were equally as effective as reduced glutathione in carrying the reaction. Bovine serum albumin (BSA, 5 mg) could also substitute for homogenate but was not as effective as cysteine, cystine, glutathione and mercaptoethanol. Since boiling of BSA uncovers additional sulfhydryl groups we ran the reaction in the presence of 5 mg of boiled (5 min) BSA. No increase in the reaction

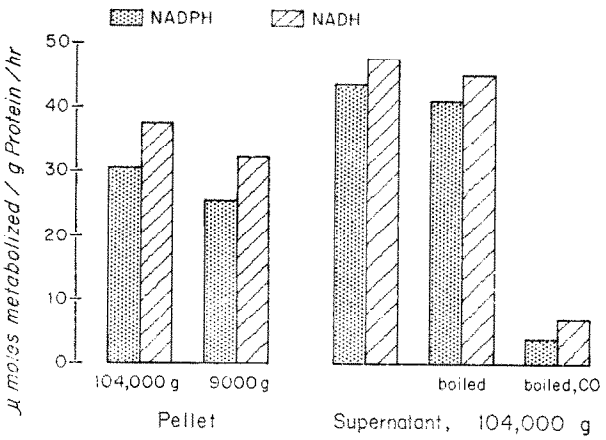

FIG. 1. Reduction of PNBA in incubation flasks containing human placental (term) homogenates. Flasks were incubated anaerobically at 37° for 2 hr. PNBA, $2{\cdot}6 \times 10^{-3}$ M, 0·4 ml homogenate 10^{-3} M FMN, $1{\cdot}8 \times 10^{-3}$ M NADPH or NADH was present in the flasks (final concentrations). Sufficient potassium phosphate buffer (0·1 M, pH 7·35) to give a total volume of 1·7 ml was added.

rate was observed, however. Human serum albumin (fraction V-Pentex) exhibited the same effect as BSA in the reaction mixture. None of the above reactions proceeded measurably in the absence of an added flavin (FMN, FAD or riboflavin) or in the absence of either NADPH, or NADH. NADH appeared to be slightly more efficient than NADPH as an electron donor in the model system reaction but the difference was very slight and possibly attributable to differences in the purity of the commercial preparations. Ascorbate did not substitute for NADPH or NADH thus indicating that reduced glutathione, cysteine, mercaptoethanol, bovine serum albumin and ascorbate could not serve as the initial electron donors in PNBA reduction. The reaction was not observable if distilled water was substituted for buffer. Lack of a reaction in distilled water was not due to a pH change since initial pH of the reaction mixtures was approximately 6·8 and the final pH was approximately 6·6. Thus it was established that the only essential cellular components for catalysis of PNBA reduction were NADPH and NADH as initial electron donors, a flavin (or related compounds), a sulfhydryl donor and a salt. Since a variety of salts could substitute for potassium phosphate (e.g. sodium phosphate, Tris, veronal, sodium borate, etc.) the effect of the salt was assumed to be for the provision of optimal ionic strength.

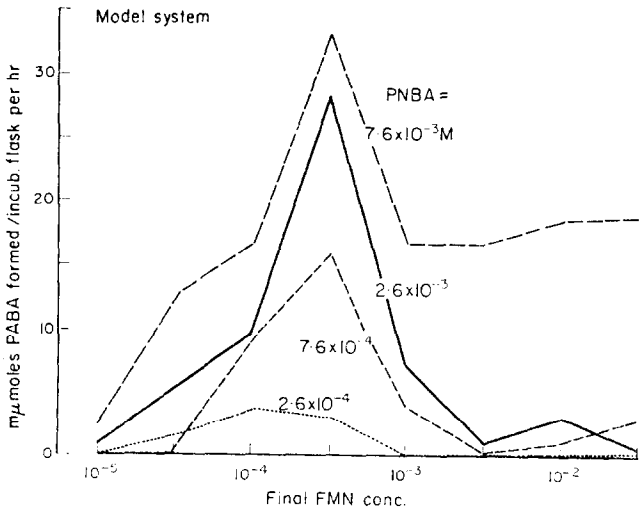

FIG. 2. Effect of variation of substrate and flavin (FMN) concentrations. Flasks contained no homogenate, 10^{-3} M NADPH, 10^{-3} M GSH, and phosphate buffer (0·1 M, pH 7·35), and were incubated under nitrogen gas. Determinations were made at 10^{-5}, 5×10^{-5}, 10^{-4}, 5×10^{-4}, 10^{-3}, 5×10^{-3}, 10^{-2} and 5×10^{-2} M FMN (final concentrations).

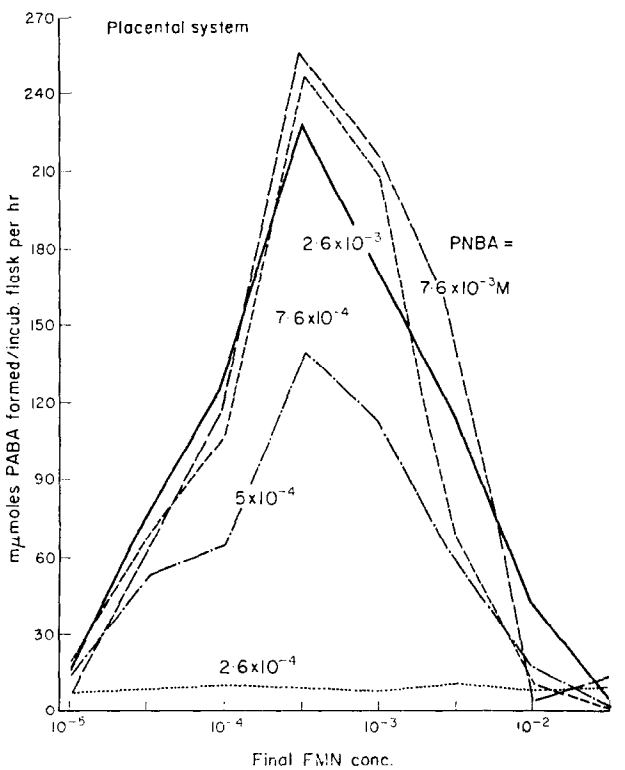

FIG. 3. Effect of variation of substrate and FMN concentration in the presence of human placental soluble fraction. Flasks contained 0·4 ml homogenates; other conditions were same as in Fig. 2 except that no GSH was added.

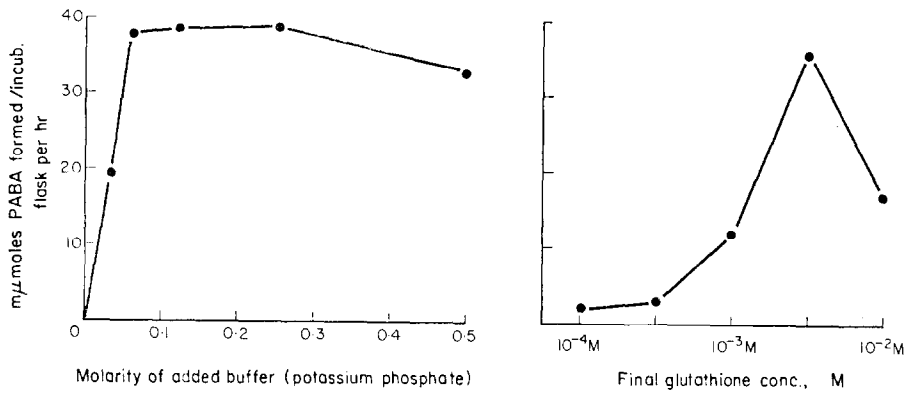

FIG. 4. Effect of ionic strength and GSH concentration in the model system reaction. Conditions were same as in Fig. 2 except that GSH concentration was 5×10^{-3} M in the graph at the left FMN concentration was 5×10^{-4} M, PNBA concentration was $2{\cdot}6 \times 10^{-3}$ M.

Alteration of the flavin concentration (as well as the substrate concentration) in the model system reaction indicated that the optimal concentration was approximately 5×10^{-4} M (Fig. 2). As is shown, the effect of the flavin was biphasic, showing activation in lower and inhibition in higher concentrations. A similar effect was observed when the reaction was run in the presence of human placental soluble fraction (Fig. 3).

Manipulation of the concentration of the sulfhydryl donor as well as the ionic strength of the system (Fig. 4) and the pH (Fig. 5) established that the optimal glutathione concentration was approximately 5×10^{-3} M, optimal ionic strength approximately 0·25, and pH optimum approximately 6·8. However, when the reaction was run under these conditions together with optimal flavin concentrations, activity

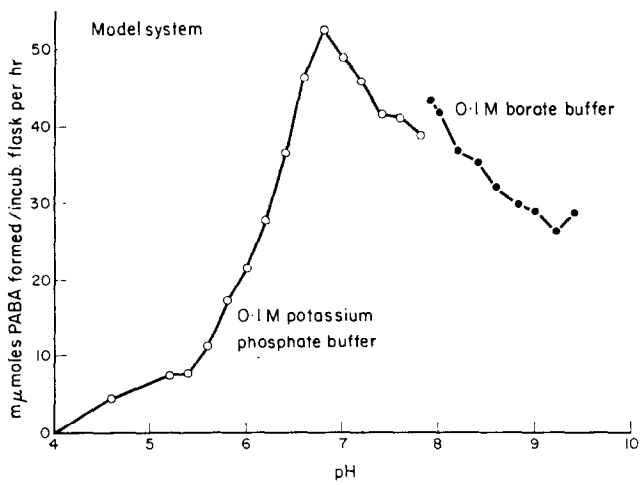

FIG. 5. Determination of optimum pH for the model system. NADPH, 10^{-3} M, 5×10^{-4} M FMN, 5×10^{-3} M GSH, and 10^{-3} M $MnCl_2$ were present in a total volume of 1·7 ml. Other conditions were same as Fig. 2.

could be increased to only about one-tenth that observed in the presence of placental soluble fraction. Carbon monoxide did not inhibit the model system reaction under the above described conditions (Fig. 6) but the reaction was completely inhibited under an atmosphere of pure oxygen. Increases in concentration of NADPH at optimal concentrations of FMN, GSH and buffer increased the reaction rate linearly up to the highest concentration tested (3×10^{-3} M, final concentration).

A variety of inorganic anions and cations were tested to observe whether they could increase the reaction rate in the model system. Anions added in 10^{-3} M final concentration (in addition to 0·1 M potassium phosphate) included sulfate (as the copper and

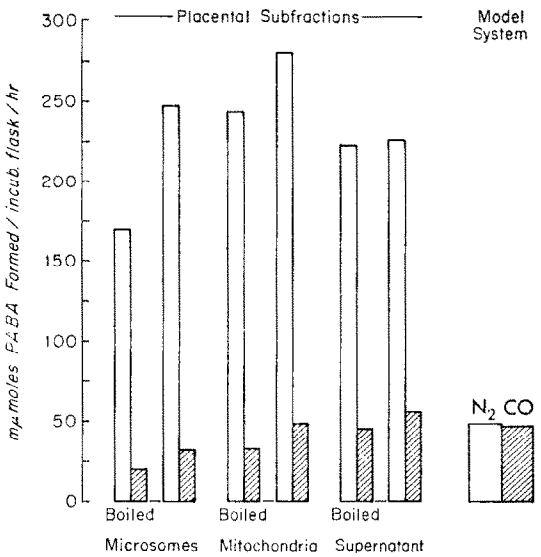

FIG. 6. Comparison of the effects of CO on human placental (term) homogenates subfractions, the corresponding boiled fractions and on the model system. Conditions for the model system were same as Fig. 5. Conditions for placental subfractions were same as Fig. 3. FMN concentration was 5×10^{-4} M. Incubation flasks were adjusted to contain 6 mg of protein.

ferrous salts), molybdate (as the sodium salt), chloride (as manganese, magnesium, ferric, ferrous, sodium, calcium, zinc and nickel salts), carbonate (as the sodium salt), and oxalate (as the potassium salt). Preliminary observations indicated that additions of $MnCl_2$ or Na_2MoO_4 would enhance the reaction. Subsequent experiments, however, indicated that, when direct comparisons were made, other salts (including sodium chloride) were equal in their effects in the presence of 0·1 M phosphate buffer and that the increase in reaction rate was only slight. These observations indicated that the only effects of added salts were probably due to differences in ionic strength of the reaction mixture. Sodium phosphate, Tris, barbital, glycylglycine, citrate and borate buffers were also directly compared to potassium phosphate buffer (all 0·1 M, pH 7·35) and no significant differences in reaction rates were observed. The reaction in model systems as well as in the presence of placental homogenates proceeded linearly for a period of 4 hr.

Characteristics of the reaction in the presence of tissue homogenates. The character-

istics of PNBA reduction in the presence of liver homogenates (and to a lesser degree in certain other tissues) have been studied in some detail. We were particularly interested in determining whether only parenchymal tissues would catalyze the reaction at a more rapid rate than that observed in model systems or whether the increased reaction rate could be observed in all tissues. We first established that the reaction proceeded at comparatively rapid rates in incubation systems containing rodent liver, kidney and (at a slower but easily observable rate) placental homogenates. Addition of flavins markedly enhanced these reactions but they were readily catalyzed without flavin additions. We had already established that PNBA reduction in the presence of human placental homogenates was measurable only upon the addition of flavins to incubation flasks. We next investigated blood to determine if the reaction was catalyzed by this tissue (Fig. 7). In the absence of flavins no reaction could be detected

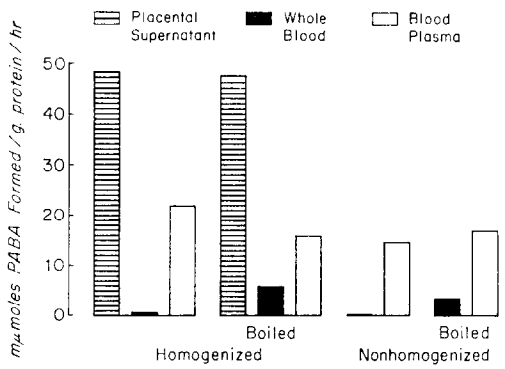

FIG. 7. Comparison of PNBA reduction in human placental soluble fraction, whole blood and blood plasma. Conditions for the reactions were same as Fig. 6.

but when FMN was added to incubation mixtures the reaction was easily observable. Separation of red cells from the plasma by centrifugation markedly enhanced the reaction rate in the presence of plasma. This was apparently due to the fact that red cells retain oxygen which profoundly inhibits this reaction. Both rodent and human blood homogenates catalyzed an observable reaction only when flavins were added as cofactors. The activity in plasma was much higher than that of a corresponding model system, making it appear unlikely that plasma albumin could be entirely responsible for the enhanced reaction rate.

In further experiments we were able to establish that differences between reduction of PNBA by rodent vs. human placental soluble fractions could not be ascribed entirely to differences in the flavin content of the tissue homogenates. Pooled human placental supernatant contained a $1{\cdot}5 \times 10^{-7}$ M total flavin content whereas pooled mouse placental supernatant contained $1{\cdot}2 \times 10^{-6}$ M. Addition of 2×10^{-6} M FMN (final concentration) to human placental soluble fractions, however, did not result in an observable reaction even after 6-hr incubation. This contrasts to our previous conclusions with respect to the apparent biochemical differences between rat and human placental homogenates and suggests that additional factors may be present in rat placenta.

In studies with rat hepatic soluble fractions we discovered that flavins markedly activated the reaction in nondialyzed fractions but very strongly inhibited the reaction if these fractions were dialyzed (Fig. 8). This phenomenon was observed in experiments in which only a small portion of the 104,000 *g* supernatant fraction was carefully aspirated from the center of the tube (supernatant A) or in which the entire supernatant was simply poured from the microsomal layer (supernatant B). Boiling of fortified rat liver soluble fractions decreased specific activity by approximately 50 per cent.

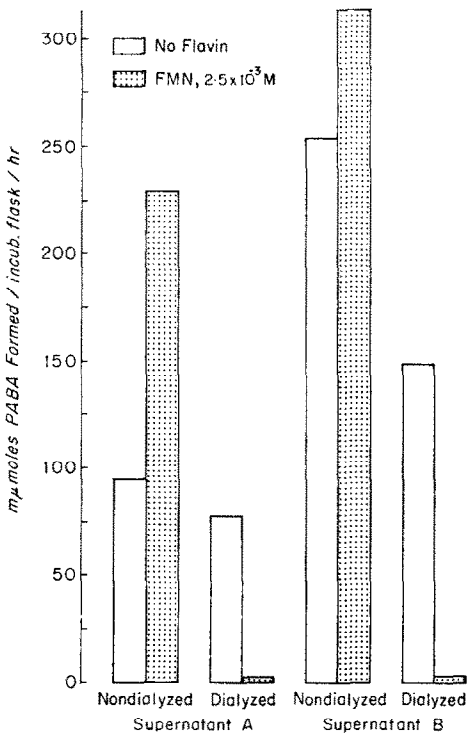

FIG. 8. Effect of FMN on PNBA reducing capacity of rat liver 104,000 *g* supernatant fraction (see Methods). NADPH, 10^{-4} M, $2{\cdot}6 \times 10^{-3}$ M PNBA, 0·4 ml homogenates and a NADPH generating system (see Methods) were added to the flasks. Incubation flasks (a) contained 4·1 mg of protein, flasks (b) contained 7·3 mg of protein.

Attempts were made to alter the reaction in the presence of human placental soluble fraction with a variety of reagents. It can be seen from Table 1 that most of these agents had little if any effect on the placental reaction. EDTA consistently inhibited the reaction but, even at 10^{-3} M (final concentration), only 26 per cent inhibition could be achieved. Citrate appeared to enhance the reaction slightly in homogenates with low activity, but no effect or slight inhibition was observed in homogenates with higher activity. Sulfhydryl reagents (PCMB) inhibited only slightly and other reagents had either no effect or inconsistent effects. Addition of coenzyme Q-6 (ubinquinone) to placental homogenates or model systems produced no observable effect on the reaction rates.

It was considered possible that the inhibitory effect of CO on the reaction rate in placental soluble fractions could be due to competition of CO with PNBA for electrons. If such were the case, it could be expected that significant amounts of formaldehyde would be formed in mixtures incubated in an atmosphere of CO. Experiments performed (by analysis with the Nash reagent) to test this hypothesis, however, indicated that no more formaldehyde was formed in mixtures incubated under an atmosphere of CO than in those incubated under nitrogen.

TABLE 1. EFFECT (PER CENT CHANGE) OF ADDED REAGENTS ON REDUCTION OF PNBA IN PLACENTAL SOLUBLE FRACTIONS*

	Final concn		
	10^{-5}M	10^{-4}M	10^{-3}M
Oxalic acid	+2	+12	+15
Potassium oxalate	−14	−8	−8
Citric acid	+2	+1	−8
Sodium citrate	+2	−7	−10
Sodium azide	−4	−3	0
Disodium EDTA	−5	−8	−22
Sodium citrate†	+28	+34	+32
Potassium oxalate†	+14	+32	+12
Disodium EDTA†	−1	−3	−17
p-Chloromercuribenzoate†	−3	−16	−26

* Incubations were run at 37° for 2 hr. Flasks contained pooled term human placental 104,000 *g* supernatant fractions (0·4 ml), FMN (10^{-3}M), NADPH (10^{-3}M), PNBA ($2{\cdot}6 \times 10^{-3}$M) and potassium phosphate buffer (0·1 M, pH 7·35) to make a final volume of 1·7 ml.

† Activity of control flasks in these four experiments was 170 mμmoles PABA formed per flask per hour. In the remainder of the experiments, activity of controls was 280 mμmoles/flask/hr. Assays were in triplicate.

DISCUSSION

Aromatic nitro compounds of pharmacologic interest include nitrofurantoin, chloramphenicol, nitrobenzene, parathion, paraoxon, *O*-ethyl *O*-(4-nitrophenyl) benzene thiophosphonate (EPN) and PNBA. Such compounds ordinarily are converted in the body to the corresponding amines which are more rapidly excreted by virtue of their higher water solubility. The pharmacological and toxicological potencies of the amine forms are also markedly reduced. It has been postulated that aromatic nitro compounds are converted first to a nitroso intermediate, to a hydroxylamine, and finally to the amine.[7]

Although the reaction has been studied extensively in hepatic tissues,[7–12] characteristics of aromatic nitro group reduction in other mammalian tissues have been investigated only superficially.[1, 8, 13, 14] Our results appeared to be in conflict with those of Hitchcock and Murphy[13] who reported a low level of activity in erythrocytes but no activity in blood plasma. A possible explanation for low activity in whole blood vs. higher activity in blood plasma might be that hemoglobin-bound oxygen in whole blood is inhibiting the reaction. This supposition is further supported by the observation that boiled, homogenized whole blood catalyzed the reaction at a much more rapid rate than fresh whole blood. Early experiments indicated that human placental homogenates from early (8–16 weeks) gestation catalyzed PNBA reduction more efficiently than term placental homogenates. The presence of a higher blood

content in term placental homogenates probably account for this observation. The experiments with blood, however, indicate that the placenta (as well as other tissues) possess catalytic factors for PNBA reduction which are independent of those found in blood.

It should be emphasized that although oxygen markedly inhibits PNBA reduction anaerobic conditions are not essential for the reaction. The fact that the reaction is almost totally inhibited in an atmosphere of pure oxygen but is readily measurable in air[1, 8] indicates that the inhibition is concentration dependent and that the reaction would proceed *in vivo*, particularly in tissues which have relatively low oxygen tension. In certain bacterial systems the reaction is not markedly inhibited by oxygen[15, 16] and the possibility remains that certain mammalian tissues may possess systems with similar properties. The fact that gastrointestinal fluid reduces aromatic nitro groups[17] creates additional interest in these aspects of the problem. The fact that the model system described in this study is totally inactive in an atmosphere of oxygen would tend to imply that the bacterial systems which catalyze the reduction possess an additional electron carrier which is not easily autoxidiazable. Autoxidizability of carriers in various tissues might well play a prominent role in the metabolism of these substrates *in vivo*.

Studies of the model system reaction have provided considerable insight into tissue catalyzed mechanisms. The finding that CO does not block the model system reaction and that no formaldehyde is formed in reaction mixtures could indicate that CO may block only by virtue of its chemical interaction with various electron carriers (CO does not block the reduction of flavins[18]). Thus, in addition to its ability to block metalloenzymes (such as xanthine oxidase, tyrosinase and β-phenethylamine hydroxylase) and cytochromes (microsomal P-450 and cytochrome oxidase) CO appears able to inhibit nonenzymic electron transfer, possibly by interaction with electron carriers. The nature of these carriers remains an important item for future investigation.

Gillette *et al.*[7] demonstrated very recently that addition of boiled hepatic microsomes to nonboiled microsomes enhanced the reaction but, since relatively low cofactor concentrations were employed, these workers did not observe that the entire reaction would proceed in the presence of boiled hepatic microsomes alone. They did observe that the activation of the reaction with boiled microsomes was inhibited by CO. This tends to corroborate the results observed in this study. The view presented that oxygen blocks nitro-reductase activity by blocking P-450, however, appears somewhat less likely in light of the fact that oxygen also blocks the reaction in a very similar fashion in the model system. Hepatic microsomal cytochrome P-450 and NADPH cytochrome *c* reductase may play an additional role in the catalysis of PNBA reduction in the liver. The present studies tend to indicate, however, that inhibition of the reaction by CO does not necessarily imply a role for cytochrome P-450.

In human placental soluble fraction, the carrier (or carrier system) appears to be heat stable, nondialyzable and carbon monoxide sensitive. The system in rat hepatic soluble fraction, however, appears considerably more complex. The latter homogenates readily catalyze the reaction in the absence of added flavins. Addition of flavins markedly stimulates the reaction in nondialyzed preparations but profoundly inhibits in dialyzed fractions (in concentrations which inhibited in model systems and in placental soluble fraction). This may indicate the presence of a dialyzable factor in rat liver supernatant which is activated by flavins. The reaction in rat hepatic soluble

fractions was also inhibited (approximately 50 per cent) by boiling, indicating the presence of enzymic components. The electron transport mechanisms in rat placental soluble fraction now also appears to be more complex since the difference between activity in human vs. rat placental homogenates cannot be explained solely on the basis of tissue flavin content. Adamson *et al.*[19] have also concluded that species differences in hepatic nitro-reductase activity were not due to differences in flavin content.

Both cystine and mercaptoethanol would substitute for reduced glutathione or cysteine in the reaction mixture for model systems. These observations indicate that amino acids and peptides are not essential components and that the sulfhydryl donor need not be in the reduced form. The biphasic effect of glutathione in the model system further indicates that the increased reaction rate in the presence of tissue homogenates is not attributable to an abundance of sulfhydryl donors. Results obtained with boiling of BSA also tend to support this view. It thus appears that the unknown catalytic factor(s) present in human placental soluble fractions is not a sulfhydryl donor, but is heat stable, nondialyzable and carbon monoxide sensitive. Its non-enzymic nature is further supported by the fact that the placental reaction did not follow the usual enzyme kinetics.

Previous investigators have suggested that NADPH is much more effective than NADH as an electron donor for PNBA reduction in liver homogenate subfractions. Initial observations in placental homogenates, likewise, suggested a similar donor specificity. However, when generation or regeneration of the electron donor in the reaction mixture was eliminated as a factor, it became apparent that NADH was at least equally as effective as NADPH. The presence of hexose monophosphate shunt enzymes and substrates in incubation mixtures provide not only generation of the required electron donor but regeneration after oxidation as well. This provides for the maintenance of high concentrations of the donor in the reaction and frequently results in misinterpretation of results. Very recently Peters and Fouts[20] have provided a graphic example of this in which they demonstrated that certain investigators were unable to show induction of PNBA reduction with DDT because of suboptimal concentrations of electron donors in the reaction mixtures. Other investigators have failed to demonstrate significant activity in microsomal fractions,[14] quite possibly for similar reasons. The fact that nitro-reductase activity is enhanced in starvation may be a reflection on this point, since starvation is known to enhance glucose 6-phosphate dehydrogenase activity. Developmental studies on nitro-reductase activity may also be colored by these observations as well as the fact that PNBA can be reduced non-enzymically. The present studies tend to re-emphasize and enlarge the point that in the situation *in vivo*, tissue concentrations of the electron donors may well represent the rate limiting factor for PNBA reduction in many tissues.

It is important to note that none of the individual essential cofactors in the model system would reduce PNBA alone. GSH has been shown to reduce nitrate to nitrites nonenzymically but its function in the model system is more difficult to explain, particularly in view of its biphasic effect. It may be that GSH functions to maintain the flavin in its reduced form which in turn reduces PNBA nonenzymically. If so, then one must postulate that NADPH or NADH is capable of reducing cystine to cysteine nonenzymically since cystine is equally as effective as cysteine in the model system reaction.

Nonenzymic reduction of the aromatic nitro group of 4-nitroquinoline *l*-oxide to the aminoquinoline derivative by NADH has been demonstrated by Sugimura *et al.*[21] Relatively high concentrations ($6{\cdot}7 \times 10^{-3}$ M, final concentration) by NADH and an alkaline pH were required to demonstrate the reaction. At the concentrations employed in most of our experiments (10^{-3} M) we were unable to demonstrate nonenzymic reduction of PNBA at pH 7·4. Our pH curve, however, appeared to be skewed to the alkaline side which may reflect a contribution of nonenzymic conversion at higher pH's.

An interesting observation is that PNBA reduction in placental soluble fractions is markedly inhibited by sodium hydrosulfite and carbon monoxide, both of which are reducing agents. It would be of interest to determine whether other reducing agents also inhibit the placental reaction. Boiling did appear to significantly inhibit the reaction in the placental microsomal fraction. This is another interesting observation which merits further investigation. The presence of cytochrome P-450 in placental mitochondria and microsomes was recently reported by Meigs and Ryan[22] and may have some bearing on this point.

Acknowledgements—The authors wish to acknowledge the valuable technical assistance to Mrs. Carolyn Tocha and the cooperation of Dr. Kenneth Niswander in obtaining placental material.

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