Biochemical Pharmacology, Vol. 17, pp. 1969–1979. Pergamon Press. 1968. Printed in Great Britain

STUDIES ON REDUCTION OF AZO-LINKAGES IN HUMAN PLACENTAL HOMOGENATES*†

MONT R. JUCHAU, JOSEPH KRASNER and SUMNER J. YAFFE

Department of Biochemical Pharmacology, School of Pharmacy
and Department of Pediatrics, School of Medicine,
State University of New York, Buffalo, N.Y., U.S.A.

(*Received* 29 *November* 1967; *accepted* 23 *February* 1968)

Abstract—Investigations of the capacity of human placental tissue to metabolize exogenous substrates revealed a system present in the soluble fraction of placental homogenates which increased the NADPH-dependent reduction of the azo-linkage of neoprontosil. Characterization of the enzyme system involved indicated striking differences in comparison to the azo-reductase system(s) present in hepatic tissues. Activity of the placental system was not affected by additions *in vitro* of magnesium ion, nicotinamide, NADP or NAD, and was only very slightly inhibited when incubated under atmospheres of pure oxygen of carbon monoxide. Placental azo-linkage reduction was enhanced by addition *in vitro* of EDTA, reduced glutathione and glucose 6-phosphate. The addition *in vitro* of flavins resulted in a decreased rate of conversion of neoprontosil to sulfanilamide. Flavin mononucleotide appeared to possess the greatest inhibitory effect. The system was also inhibited by sulfhydryl reagents. Comparisons of specific activities from homogenates of tissue obtained early in the gestational period vs. that obtained at term revealed no statistically significant differences. Studies on the mechanism of increased azo-linkage reduction revealed that an enzymic–nonenzymic system involving D-glucose 6-phosphate:NADP oxidoreductase (EC 1.1.1.49) and 6-phospho-D-gluconate:NADP oxidoreductase (EC 1.1.1.44) could account for this increase in azo-linkage reduction.

DESPITE the fact that the placenta is capable of catalyzing the biotransformation of a wide variety of endogenous substrates,[1,2] the role of this organ in the metabolism of xenobiotic substances has not been extensively investigated. The possible relationship of placental drug metabolism to the subsequent effects of drugs on pregnant women, fetuses and newborn infants has prompted us to undertake a systematic study of drug metabolic processes at this site with techniques *in vitro* and *in vivo*.

Previous preliminary investigations in this laboratory[3] revealed that homogenates of human placental tissue (at 9–12 weeks of gestation) could increase the reduction of several xenobiotic substrates. Such substrates included 2,6-dichlorophenol-indophenol (DCPIP), potassium ferricyanide and neoprontosil, but not *p*-nitrobenzoic acid. Cytochrome C was likewise enzymically reduced by placental homogenates under similar incubation conditions. Cytochrome C reduction and diaphorase activity utilized reduced nicotinamide adenine dinucleotide (NADH) in preference to reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a hydrogen donor in

* This research was supported in part by Grants FR-05493, FR77 and HD-01219, Public Health Service and G-67-UB-27, United Health Fund.

† Presented in part before the American Society for Pharmacology and Experimental Therapeutics, Washington, D.C., July 1967 (*Pharmacologist* **9**, 190, 1967).

reaction mixtures. Reduction of the azo-linkage of neoprontosil, however, appeared to be much more efficient if NADPH were employed as the electron donor in such reactions. Many of the systems which normally catalyze drug oxidation and conjugation reactions in adult animal liver homogenates appeared to be absent from homogenates prepared from placentas obtained from weeks 9–12 of gestation.

The present report represents a more thorough investigation of enzymic azo-linkage reduction by human placental homogenates. Optimal incubation conditions for NADH- and NADPH-dependent reactions have been evaluated. The effects of several inhibitors and activators have been studied in order to partially characterize the placental enzyme(s) involved. Comparisons have been drawn between azo-linkage reduction by homogenates of early placentas (8–12 weeks of gestation) and such reduction in homogenates of full-term placentas. Finally, we have shown that the difference between the amounts of neoprontosil converted to sulfanilamide in preparations containing placental soluble fraction vs. those containing no enzyme can be attributed solely to the presence of components of an NADPH-generating system in the homogenates.

MATERIALS AND METHODS

Preparation of placental homogenates. For studies of enzymic activity in placentas of 8–12 weeks' gestation, tissue was obtained from healthy patients at operation (dilation and curretage or hysterotomy). Term placentas were obtained after normal vaginal deliveries or caesarean sections. Stability studies indicated that freezing and thawing of placental tissue or of placental homogenates did not materially affect the azo-reductase activity of the high speed (104,000 *g* for 1 hr) supernatant. (Boiled homogenates were inactive.) It was also shown that such frozen preparations could be stored (−10 to −20°) for several weeks without significant loss of activity. Therefore, placental tissues were obtained fresh and stored at approximately −10° until required for performance of assays for enzymic activity (usually not more than 2–3 days). The tissues were then thawed, placed in a large volume of isotonic (1·15%) KCl solution, minced with scissors and rinsed several times with separate volumes of KCl solution. The desired parts of the placenta (cotyledenous tissue) were carefully dissected away and fibrous tissue was discarded. Tissue of 8–12 weeks' gestation obtained in this fashion was essentially free of blood, but term placental tissue appeared to retain some blood subsequent to the above procedure (as judged by the color of the tissue). The tissue was then blotted, weighed and homogenized in 2 vol. of ice-cold isotonic KCl in a Waring–Blendor at high speed for 30 sec. The resulting homogenate was centrifuged at 9000 *g* for 20 min in a Servall refrigerated centrifuge. The pellets were discarded and the supernatant was centrifuged at 104,000 *g* for 1 hr in a Spinco, model L, refrigerated ultracentrifuge. The pellets were again discarded and the supernatant fraction (hereafter referred to as the soluble fraction) was employed as the enzyme source. Pooled placental supernatant fractions were employed in all experiments unless otherwise indicated. Assays were run in triplicate.

Assay procedures. Incubation mixtures consisted of the following components: 10^{-4} M NADPH (final concentration), $1{\cdot}5 \times 10^{-3}$ M neoprontosil (final concentration), 1·0 ml placental supernatant fractions as the enzyme source and sufficient 0·1 M phosphate or Tris buffer to give a total volume of 5·0 ml. Zero time flasks contained 10^{-4} M sulfanilamide but no NADPH and were employed as standards.

Appropriate tissue and reagent blanks were also prepared. With each incubation, flasks in which only the enzyme source was omitted were incubated simultaneously with test flasks. This allowed calculation and correction for the contribution of non-enzymic reaction.

Mixtures were incubated with shaking in a Dubnoff metabolic incubator (50–60 rpm). Incubation times varied between 0 and 6 hr, pH of phosphate buffer was varied from 4·5 to 8·0 and incubation temperatures ranged from 28° to 47°. Incubations were carried out in atmospheres of air, 100% oxygen, 100% nitrogen and 100% carbon monoxide. At the conclusion of the incubation period, 15·0 ml of ice-cold 6·7% trichloroacetic acid (TCA) was added to each incubation flask. The amount of sulfanilamide present in each flask was then determined quantitatively by slight modifications of the Bratton–Marshall procedure[4] as follows: 0·5 ml of a fresh solution of 0·2% sodium nitrite was added to a 4·0-ml aliquot of the cold TCA mixture and mixed well. After allowing the solution to stand in the cold for 10 min, 0·5 ml of a 1% ammonium sulfamate solution was added and the mixture shaken vigorously. At least 3 min was allowed for the reaction to equilibrate. One ml of 30% sodium acetate was then added with mixing. Diazotization of sulfanilamide was accomplished by addition of 0·5 ml of a 0·2% solution of Bratton–Marshall reagent (*N*-(1-naphthyl)-ethylenediamine dihydrochloride). After mixing, the reaction was allowed to go to completion at room temperature (10 min). The diazotized sulfanilamide was then extracted by shaking the mixture with 3·0 ml of washed isoamyl alcohol. After centrifugation, 2·0 ml of the alcohol layer was added to 0·1 ml of a 25% solution of TCA in washed ethylene dichloride. The resultant solution was mixed with a vortex mixer, sometimes resulting in cloudiness which disappeared upon standing. Color was stable and solutions could be read the following day. Optical densities were determined at 540 mμ on a Beckman DU spectrophotometer equipped with a Gilford o.d. converter and recorder. Studies revealed that recovery of sulfanilamide from incubation mixtures (37°) was essentially complete (99 ± 3 per cent) over a concentration range of 3–40 μg/ml (final concentration). Presence of neoprontosil (0·8 to $3{\cdot}0 \times 10^{-3}$ M, final concentration) or of varying amounts of homogenate did not affect the percentage of sulfanilamide recovery. Results were expressed as μmoles of neoprontosil metabolized per incubation flask (or per gram of protein, where specific activities were required) per hour. Azo-linkage reduction could also be measured by following the decrease in optical density (at 494 mμ) of the neoprontosil in solution. Protein determinations were made according to the method of Lowry *et al.*[5]

RESULTS

Optimal pH for NADPH- and NADH-dependent azo-linkage reduction. To determine optimal pH for the rate of the apparent enzymic reduction of neoprontosil, it was necessary to determine maximal reaction rates in the presence and absence of placental enzymes. The optimal pH was determined by varying the ratios of 0·1 M monobasic potassium phosphate to 0·1 M dibasic potassium phosphate and is depicted in Figs. 1 and 2, respectively, for NADPH-dependent and NADH-dependent azo-reduction reactions. The total reaction appeared to proceed most rapidly at about pH 6·8, regardless whether NADPH or NADH was employed as co-factor. The rate of the nonenzymic NADH-dependent reaction decreased with increased pH, whereas the relationship of nonenzymic NADPH-dependent reduction to pH was

more complex. The difference between the rates of the total reaction and the non-enzymic reaction was greatest at a pH of approximately 6·8 to 7·0 for NADPH- as well as NADH-dependent reactions. The total reaction appeared to proceed somewhat more rapidly in phosphate buffer (0·1 M, pH 6·8) than in Tris buffer (0·1 M,

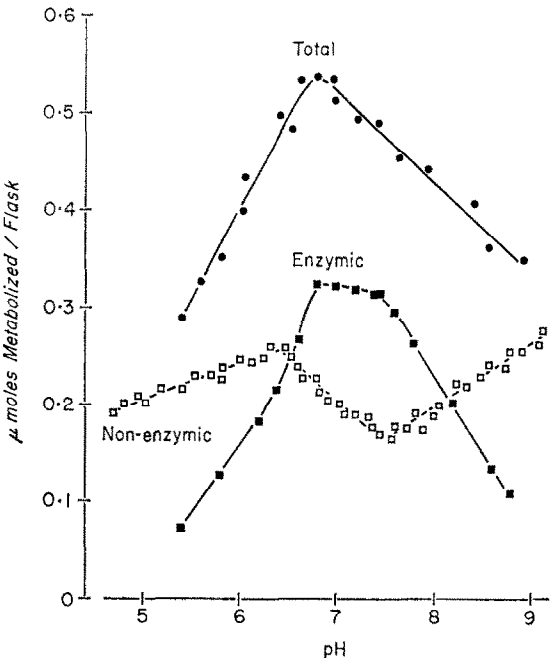

FIG. 1. The relationship of human placental NADPH-dependent azo-reductase activity to pH. pH was varied by altering the ratios of 0·1 M K_2HPO_4 and 0·1 M KH_2PO_4. Flasks were incubated 1 hr at 37·5°.

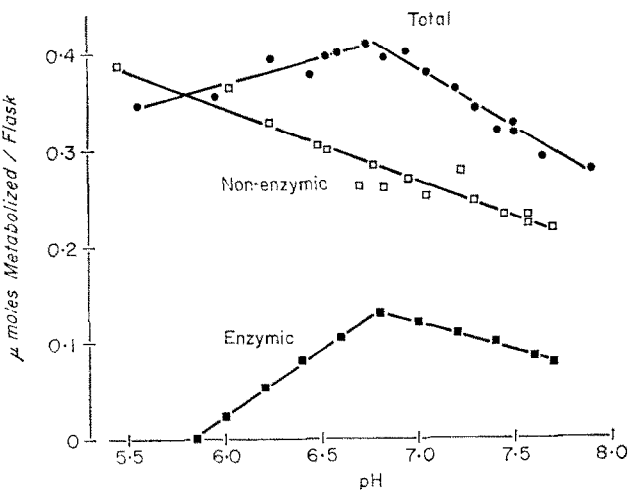

FIG. 2. The relationship of human placental NADH-dependent azo-reductase activity to pH. For other details see Fig. 1.

pH 6·8), but there was no apparent difference in the rates of the enzymically catalyzed reaction under these conditions. The pH of incubation mixtures changed only slightly during the course of the incubation. This was ordinarily observed as a slight (0·01 to 0·05 pH units) decrease and was observed even when buffering capacity was very low.

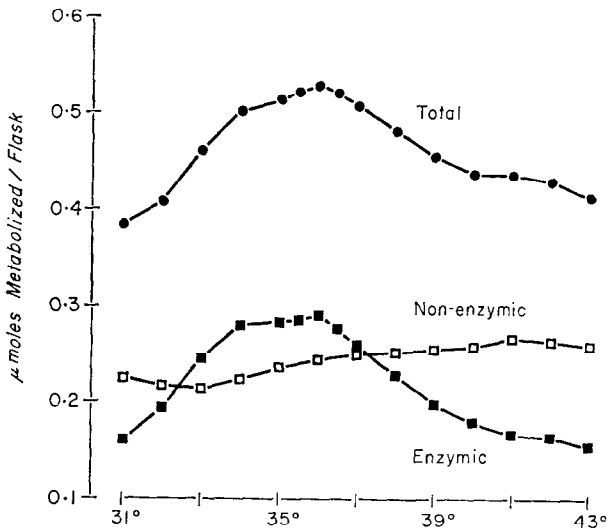

FIG. 3. The relationship of human placental NADPH-dependent azo-reductase activity to incubation temperature. Flasks were incubated for 1 hr; pH was 6·8.

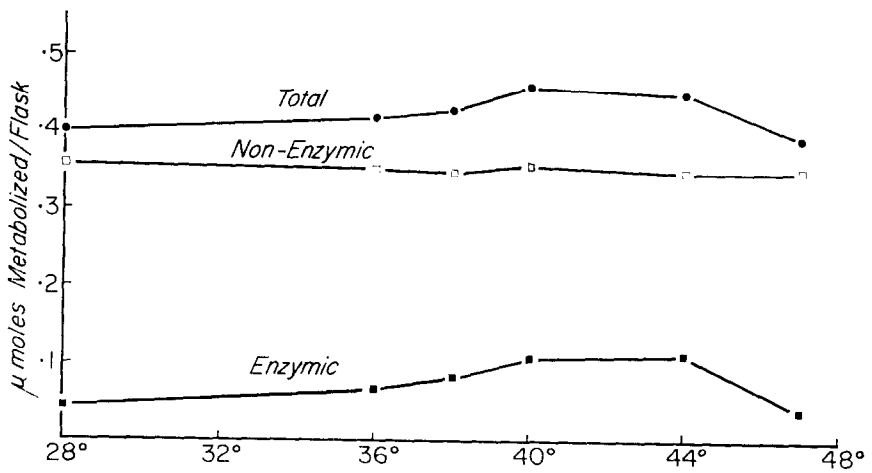

FIG. 4. The relationship of human placental NADH-dependent azo-reductase activity to incubation temperature. Flasks were incubated for 1 hr; pH was 6·8.

Optimal incubation temperatures for nucleotide-dependent reduction of neoprontosil. The NADPH-dependent placental reduction reaction proceeded most efficiently at 36° (Fig. 3). On the other hand, the NADH-dependent reaction seemed to be catalyzed most rapidly between 40 and 44° (Fig. 4). However, the very low activity of the NADH-dependent reaction prevented definite conclusions. The rate of nonenzymic NADPH reduction increased very slightly with increasing incubation temperatures, but the rate

of nonenzymic NADH-dependent reduction remained essentially constant over the temperature range studied.

Time courses of NADPH- and NADH-dependent azo-linkage reduction. Time courses of the reduction of azo-linkage by NADPH are shown in Fig. 5. The nonenzymic reaction reached equilibrium by about 30 min, whereas the enzymic reaction

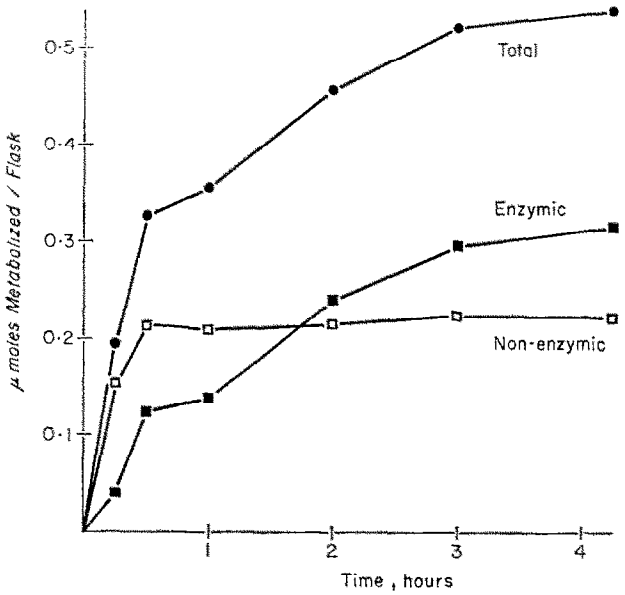

FIG. 5. Time course of NADPH-dependent azo-linkage reduction in homogenates (104,000 *g* supernatant) of human placentas. Incubation temperature was 37·5°; pH was 6·8.

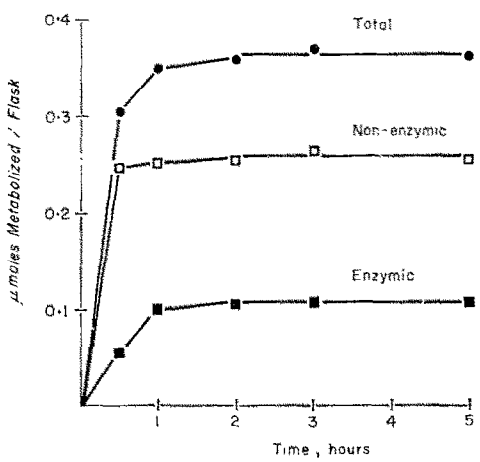

FIG. 6. Time course of NADH-dependent azo-linkage reduction in homogenates (104,000 *g* supernatant) of human placentas. Incubation temperature was 37·5°; pH was 6·8.

(total minus nonenzymic) proceeded at an essentially linear rate after 2 hr. The nonenzymic reduction by NADH was complete by 30 min (Fig. 6) and the reaction in the presence of placental homogenate reached equilibrium within 1 hr. Incubation conditions are given in the figure legends.

Activators and inhibitors of human placental azo-linkage reduction. In an attempt to partially characterize the NADPH-dependent reduction of neoprontosil by human placental soluble fraction, several potential activators and inhibitors were studied (Figs. 7 and 8). Reaction rates were studied under atmospheres of nitrogen, oxygen

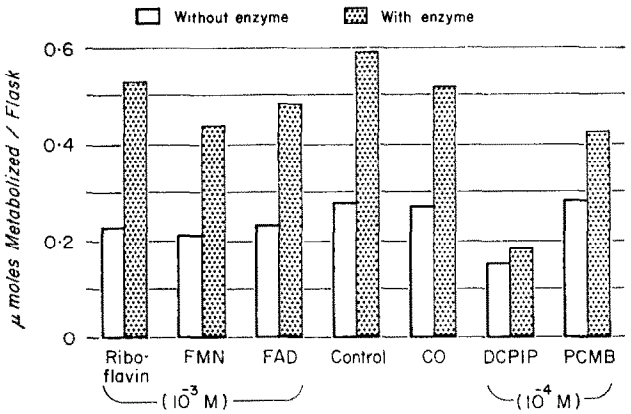

FIG. 7. Effects of several inhibitors of NADPH-dependent azo-reductase activity of homogenates (104,000 *g* supernatant) of human placentas. Flasks were incubated for 1 hr at 36°; pH was 6·8.

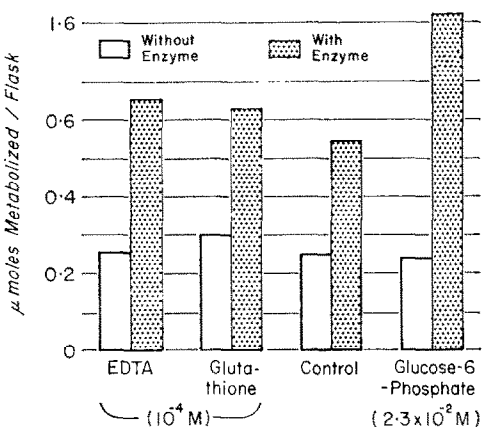

FIG. 8. Effects of several activators of NADPH-dependent azo-reductase activity of homogenates (104,000 *g* supernatant) of human placentas. For other details see Fig. 7.

air and carbon monoxide. The enzymic reaction appeared to proceed as rapidly under air as anaerobically under nitrogen, but was slightly inhibited (6 per cent) under atmospheres of pure oxygen or carbon monoxide. The degree of inhibition under atmospheres of the two latter gases appeared to be approximately equal, but subsequent studies revealed that inhibition by neither gas was statistically significant ($P > 0{\cdot}2$). Flavins appeared to inhibit the NADPH-dependent placental enzyme system. The mononucleotide (FMN) was more inhibitory than flavin adenine dinucleotide (FAD) or riboflavin in equimolar concentrations. Flavin inhibition was observed under atmospheres of air or nitrogen. Even at 10^{-3} M (final concentration), however, the degree of inhibition was not great (25 per cent). 2,6-Dichlorophenolindophenol

markedly inhibited the azo-reducing enzyme, probably by competing for the hydrogen donor. The inhibitory effect of DCPIP also was observed at 10^{-6} M, final concentration. Sodium fluoride and sodium cyanide failed to affect enzymic activity in concentrations of 10^{-5}, 10^{-4} and 10^{-3} M. Addition of the same concentrations of estrone sulfate did not alter enzymic activity.

Addition of EDTA (Fig. 8) to incubation flasks enhanced the metabolism of neoprontosil. This appeared to be an effect on the enzyme system *per se*, since the nonenzymic reaction was not appreciably affected. Addition of 10^{-4} M reduced glutathione (GSH), on the other hand, produced an increase in azo-linkage reduction, apparently by enhancing the nonenzymic reaction. Interaction of GSH with the enzyme system appeared to be complex since, at 10^{-3} M concentration, inhibition rather than activation was observed. Addition of glucose 6-phosphate very markedly accelerated the conversion of neoprontosil to sulfanilamide. This was due to the presence of glucose 6-phosphate dehydrogenase in placental homogenates and the resultant regeneration of NADPH from the oxidized form. Glucose 6-phosphate did not enhance the activity of the NADH-dependent reaction. Additions *in vitro* of NADP, NAD and nicotinamide, or of magnesium chloride at 10^{-3}, 10^{-4} or 10^{-5} M concentrations did not visibly affect the rate of the NADPH-dependent reduction reaction.

Comparison of early vs. term placentas. Preliminary studies indicated that a wide range of specific activities (μmoles neoprontosil metabolized/g protein/hr) could be observed both in 18 early (8–12 weeks' gestation) and in 8 term placentas. Early placental homogenates appeared to display somewhat higher (13·2 $\pm$ 4·6 S.D.) sp. act's (expressed as μmoles neoprontosil metabolized/g placental protein/hr) than term placenta homogenates (10·6 $\pm$ 5·3 S.D.), but the difference was not statistically significant. Studies of optimal incubation conditions, cofactor requirements and inhibitors and activators likewise revealed no statistically significant differences between activities of homogenates from term vs. 8–12 week placentas.

Studies on the contribution of placental soluble NADPH-generating system(s) to the increased reduction reaction. It was recognized that NADPH (oxidized during neoprontosil reduction) could be regenerated by NADP oxidoreductase enzymes present in placental soluble fractions if glucose 6-phosphate, 6-phospho-gluconolactone or other substrates were present in sufficient quantity in these fractions. We therefore decided to evaluate the possible contribution of these factors to the overall reaction. Dialysis of the soluble fraction (24 hr against 3 changes of 0·1 M phosphate buffer, pH 6·8) resulted in reduction of neoprontosil by incubation mixtures which was exactly equivalent to the nonenzymic conversion (Table 1). Addition of glucose 6-phosphate in varying concentrations to dialyzed preparations restored the activity. Incubation with similar amounts of 6-phosphogluconate likewise restored activity. Additions of NADP to placental soluble fractions revealed that sufficient endogenous glucose 6-phosphate or 6-phospho-gluconolactone or both was present in the homogenate to account for the increased conversion of neoprontosil; i.e. NADPH generated (measured at 340 mμ) was stoichiometrically equivalent to the amounts of glucose 6-phosphate and 6-phospho-gluconolactone calculated to be required for the increased conversion. Addition of NAD to placental homogenates also resulted in a slight increase in optical density (340 mμ) which was sufficient to account for the increased conversion of neoprontosil to sulfanilamide by placental homogenates in the presence of NADH.

The possibility that the presence of blood in placental tissues might account for a significant fraction of NADPH regnerating capacity was investigated. The results (Table 2), however, indicated that only a very small fraction of NADPH generated by placental soluble fractions could be attributed to erythrocytic enzymes.

TABLE 1. REDUCTION OF NEOPRONTOSIL IN PLACENTAL SOLUBLE FRACTIONS*

Homogenate	Final concn of added G-6P (M)	Activity (μmoles metabolized/flask†/hr)
None	0	0·22
Placental supernatant	0	0·62
Placental supernatant (boiled)	0	0·23
Placental supernatant (dialyzed)	0	0·22
Placental supernatant (dialyzed)	10^{-5}	0·33
Placental supernatant (dialyzed)	10^{-4}	0·42
Placental supernatant (dialyzed)	10^{-3}	0·55
Placental supernatant (dialyzed)	10^{-2}	0·69
Placental supernatant (boiled)	10^{-2}	0·24

* The same pooled fraction was employed in each experiment.

† Each flask with placental supernatant contained approximately 20 mg protein. Incubations were in triplicate.

TABLE 2. CONTRIBUTION OF BLOOD TO AZO-LINKAGE REDUCTION IN PLACENTAL HOMOGENATES

Tissue*	Fraction	Activity (μmoles metabolized/g protein/hr)†
Hemolyzed fresh whole blood	104,000 *g* supernatant	1·8
Placental blood clot (term)	104,000 *g* supernatant	1·2
Placental tissue (term)	104,000 *g* supernatant	12·1

* Each tissue was homogenized and centrifuged in identical fashion.

† Corrected for nonenzymic azo-linkage reduction. Incubations were in triplicate.

DISCUSSION

The study of drug-metabolizing enzymes in placental tissues has far-reaching implications with regard to drug therapy in pregnant women and in fetal and newborn infants. A recent report by Welch and Harrison[6] indicates that placental tissues of rats will hydroxylate 3,4-benzpyrene. Activity appeared to be localized in the nuclear fraction of the cell. In a study of human term placentas (in which placental donors had received only meperidine), Hirsch and Van Petten very recently reported[7] that l-amphetamine and pentobarbital were metabolized by placental 9000 *g* supernatant fractions. The activity observed in human placental homogenates was approximately equal to that observed in 9000 *g* supernatant fractions of rat liver homogenates. In our own laboratories we have observed that rat placental homogenates readily catalyze the reduction of nitro radicals, but in homogenates of early or term human placentas the same reaction could not be detected.[8] Such observations, although preliminary, indicate that placental tissues are capable of enhancing the biotransformation of several xenobiotic substrates and may possess the potential for catalysis of the

metabolism of additional nonendogenous compounds. Drugs encountering the so-called "placental barrier" may therefore undergo metabolic transformation which could influence the pregnant mother, fetus or newborn infant in a number of possible ways: unusual metabolites could act *directly* upon fetal tissues to produce teratogenic or other abnormal effects; metabolites could *inhibit* the normal biochemical or physiological function of the placenta, resulting in pathological effects on mother or fetus; biotransformations of drugs in the placenta could *compete* for metabolism of endogenous substrates (such as hormones), thus upsetting the normal physiology of pregnancy; placental metabolism of xenobiotics could interfere with active or facilitated transport mechanisms known to be present in placental tissue; or (depending upon the type of biotransformation) metabolism of drugs by the placenta could result in the *concentration* of the drug in either maternal or fetal tissues. Still other possibilities may exist and likewise should be further explored.

The results obtained in this study indicate that the hepatic enzyme system which catalyzes azo-linkage reduction is strikingly different from the azo-reductase of placental tissue. The hepatic enzyme(s) appears to be associated primarily with the microsomal fraction,[9] is enhanced markedly by additions *in vitro* of flavins,[10] and is more sensitive to the effects of carbon monoxide.[11] Our experiments indicate that the placental system is mostly in the soluble fraction, is inhibited by flavins and is only slightly sensitive to carbon monoxide. These differences can be nicely accounted for when it is realized that the reaction which occurs in placental soluble fraction is an enzymic–nonenzymic coupled reaction that involves activities of enzymes of the placental pentose phosphate pathway. Since we observed some activity (approximately 20 per cent of the whole homogenate) in the "microsomal" fraction,[3] we considered the possibility that flavins might enhance azo-reductase activity of this placental homogenate subfraction. Preliminary experiments, however, indicate that flavins affect activities of the "microsomal" system and soluble system in much the same fashion, i.e. similar inhibition was observed. Since our microsomal preparations exhibited no NADPH-generating capacity (with glucose 6-phosphate as substrate), another mechanism for the increased azo-linkage reduction in the presence of this subfraction must exist and should be investigated. It is possible (though studies with flavins render it less likely) that, since cytochrome P-450 has been reported[12] to be present in placental microsomal mitochondrial preparations, reduction of azo-linkages by microsomal preparations could occur via the classical P-450 pathway. Studies of this possibility are currently being carried out in our laboratory. Very recent studies from the laboratory of Gillette *et al.*[13,14] suggested that the reduction of azo-linkage by mammalian hepatic enzymes involved a P-450 pathway, microsomal NADPH cytochrome C reductase and a 3-methylcholanthrene inducible pathway. Our studies indicate that none of these pathways is involved in azo-linkage reduction in placental soluble fractions. It can be observed from our data, however, that the contribution of the nonenzymic–enzymic system (involving the pentose phosphate shunt) to azo-linkage reduction can be highly significant and may be a most important factor *in vivo*.

The pH dependence curve of the nonenzymic reaction (in which NADPH was utilized as the electron donor) was clearly biphasic in nature. The experiment was run several times in order to verify this point, and once again may illustrate differences between NADH and NADPH with regard to their interaction with substrates.

The wide variations in specific activities of azo-reductase from different placental donors are reminiscent of similar variations in the metabolism of other drug substrates by humans. Such differences could be the result of genetic variation (genetic variations in glucose 6-phosphate dehydrogenase activities are well recognized) or of environmental influences. These possibilities are also being investigated in our laboratory.

Acknowledgements—The authors wish to acknowledge the valuable technical assistance of Mrs. Carolyn Tocha and Miss Mary Morrisson in these investigations. Neoprontosil was a gift of the Sterling-Winthrop Research Institute, Rensselaer, N.Y.

REFERENCES

1. D. D. HAGERMAN, *Fedn Proc.* **23**, 785 (1964).
2. J. DANCIS, *Pediat. Clins N. Am.* **12**, 477 (1965).
3. M. R. JUCHAU, K. B. NISWANDER and S. J. YAFFE, *Am. J. Obstet. Gynec.* in press.
4. A. C. BRATTON and E. K. MARSHALL, *J. biol. Chem.* **128**, 537 (1939).
5. O. H. LOWRY, N. J. ROSENBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1961).
6. R. M. WELCH and Y. HARRISON, *Pharmacologist* **9**, 202 (1967).
7. G. H. HIRSCH and G. R. VAN PETTEN, *Proc. Can. Fedn biol. Soc.* (10th Ann. Mtg), p. 98 (1967).
8. M. R. JUCHAU and S. J. YAFFE, *Pharmacologist* **9**, 190 (1967).
9. J. R. FOUTS, L. A. ROGERS and T. E. GRAM, *Exp. molec. Path.* **5**, 475 (1966).
10. J. R. FOUTS, J. J. KAMM and B. B. BRODIE, *J. Pharmac. exp. Ther.* **120**, 291 (1957).
11. J. R. GILLETTE, in *Advances in Pharmacology* (Eds. S. GARRATINI and P. A. SHORE), vol. 4, p. 219. Academic Press, New York (1965).
12. R. A. MEIGS and K. J. RYAN, *Science* **158**, 532 (1967).
13. P. H. HERNANDEZ, P. MAZEL and J. R. GILLETTE, *Biochem. Pharmac.* **16**, 1859 (1967).
14. P. H. HERNANDEZ, P. MAZEL and J. R. GILLETTE, *Biochem. Pharmac.* **16**, 1877 (1967).