COMPARATIVE BIOCHEMISTRY OF DRUG METABOLISM BY AZO AND NITRO REDUCTASE

By Richard H. Adamson, Robert L. Dixon, Frances L. Francis, and David P. Rall

NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND, AND THE LERNER MARINE LABORATORY, BIMINI, BAHAMAS

Communicated by Seymour S. Kety, September 16, 1965

The activity of enzyme systems catalyzing the metabolism of drugs and other chemicals foreign to the body varies with the species, sex, genetic background, and the physiologic state of the animal. A variety of drugs and chemicals which cause increases in the activity of microsomal drug-metabolizing enzymes have been reported.[1, 2] There is a decrease or lack of oxidative drug-metabolizing enzyme activity associated with the following conditions: (1) newborn state, (2) starvation, (3) alloxan-induced diabetes, (4) hepatic tumors or regeneration.[3–7] In some of these conditions azo and nitro reductase activity were not studied, and in others there were differences between the oxidative enzymes that metabolize drugs and azo and nitro reductase. Thus, in the starved animal there may be a stimulation of enzymes reducing azo and nitro groups in contrast to a depression of oxidative enzymes that metabolize drugs.[4] Moreover, the reductive enzymes are present, albeit to a small degree of the normal activity, in various liver tumors and in the fetus and newborn animals, whereas the oxidative enzymes are absent.[6, 8] These results suggest that the enzymes which reduce azo and nitro groups may develop before the oxidative drug-metabolizing enzymes and may be more primitive.

In a comparative study, it was demonstrated that fish and amphibians lacked oxidative microsomal drug enzymes. It was pointed out that these enzymes were not required by these water-dwelling species since the potentially toxic foreign lipid-soluble substances ingested could diffuse through the membranes of the gills or through the skin into the surrounding aqueous medium. However, the higher vertebrates having gone from water to land life in the process of evolution could no longer rid themselves of toxic lipid-soluble foreign compounds in this manner. As a prerequisite for life, land animals had to develop nonspecific enzyme systems to convert these compounds to less lipid-soluble derivatives which are more rapidly excreted by the kidney.[9]

The present problem was undertaken to follow the evolution of the metabolism of foreign compounds by azo and nitro reductase, since a comparative study of reductive enzymes that metabolize drugs has not been reported, and because these enzymes may be of a more primitive nature.

Materials and Methods.—Lemon sharks (*Negaprion brevirostris*), sting rays (*Dasyatis americana*), barracuda (*Sphyraena barracuda*), and yellow-tail snappers (*Ocyurus chrysurus*) were caught near Bimini, Bahamas. Frogs (*Rana pipiens* and *R. catesbeiana*), toads (*Bufo marinus*), turtles (*Pseudemys*), horned toads (*Phrynosoma*), and common domestic pigeons were obtained from the Lemberger Company, Oshkosh, Wisconsin. Mice, rats, and guinea pigs were obtained from the Animal Production Section, National Institutes of Health.

Preparation of tissue samples: Livers were homogenized in three parts of cold

isotonic KCl with a Potter homogenizer (plastic pestle) in the cold. Other cell fractions were prepared by spinning the homogenate in a high-speed angle centrifuge or in a Spinco model L ultracentrifuge.

Determination of enzyme activity: The metabolic pathways studied were the reduction of the aromatic nitro group of *p*-nitrobenzoic acid, and reductive cleavage of the azo linkage of neoprontosil. The metabolism of these two drugs was followed by methods previously reported.[10, 11] The homogenate or cell fractions were incubated for 1 hr under nitrogen. Incubations were carried out at 37°C and/or the environmental temperature of the animal studied.

Cofactors added were triphosphopyridine nucleotide (TPN), glucose-6-phosphate, nicotinamide, and magnesium sulfate. Final concentrations of cofactors were the same as previously reported.[12] The final concentration of *p*-nitrobenzoic acid and neoprontosil used in each incubation mixture was 5 μmoles and 6 μmoles, respectively, per final volume of 5.0 ml.

Results.—Results summarized in Table 1 show that both elasmobranch and teleost are able to reduce neoprontosil to sulfanilamide, but while the elasmobranchii (shark and rays) lack nitro reductase, the two teleost fish studied were able to reduce *p*-nitrobenzoic acid to *p*-aminobenzoic acid. The azo reductase activity in the fish livers is approximately one tenth of the activity found in that of the mouse. The nitro reductase activity of the teleosts studied was about one fourth of the activity of the mouse. Homogenates of sting ray or barracuda liver which were heated for 3 min (waterbath at 100°C) showed no enzyme activity. The addition of flavinadenine dinucleotide (FAD) 10^{-4} or 10^{-3} *M* to shark liver incubations stimulated the normal rate of reduction (two- and fourfold, respectively). The addition of riboflavin 10^{-3} *M* or riboflavin-5-phosphate (FMN) 10^{-3} *M* to sting ray liver incubations stimulated the basal level of reduction threefold. Addition of FAD or riboflavin 10^{-3} *M* to pigeon liver homogenate had a similar effect. The accelerating effect of flavins on mammalian azo reductase is known.[10] Since addition of various flavins to liver homogenates of different species similarly accelerated the normal rate of reduction, it is probable that differences in basal level of enzyme activity were not due to the availability of the flavoprotein(s). Azo reductase in shark liver homogenate was found to be present in both the microsomes and the soluble fraction of the cell (72,000 × *g* supernatant). This distribution is also similar to that of azo reductase in mammals.[10] In other experiments, in contrast to the 26°C results, incubation of shark liver homogenate or its subcellular fractions at 37°C resulted in a complete absence of reductase activity.

Aquatic amphibia are also able to metabolize neoprontosil, but the enzyme system which reduces nitro groups in the teleost is absent in both *R. pipiens* and *R. catesbeiana*. Enzyme activity was greater at the temperature of 37°C in the frog than at 21°C. Toads, the terrestrial ramification of the frog, had azo reductase activity similar to that of the frog, and also lacked the nitro reductase enzyme system. Aquatic amphibia are unable to oxidize lipid-soluble foreign compounds, whereas the toad can oxidize various compounds, but by a mechanism different from that in mammals.[9]

Both reptiles, the turtle and the horned toad, reduced the azo as well as the nitro compound. The nitro reductase enzyme in the turtle is 20 times as active at 21°C

TABLE 1

AZO AND NITRO REDUCTASE ACTIVITY IN LIVER OF VARIOUS SPECIES

Species	Sex	Temperature, °C	Azo reductase, μmoles sulfanilamide formed/(gm liver) (hr)	Nitro reductase, μmoles p-aminobenzoic acid formed/(gm liver) (hr)
Fish				
Lemon shark	M, F	26	0.605 ± 0.09 (5)*	0 (5)
Sting ray	M, F	26	0.757 ± 0.12 (3)	0 (3)
Barracuda	M, F	26	0.757 ± 0.05 (4)	0.495 ± 0.11 (4)
Yellow-tail snapper	M, F	26	0.781 ± 0.09 (3)	0.661 ± 0.11 (3)
Amphibia				
Frog (*R. pipiens*)	M	21	0 (4)	0 (4)
	M	37	1.34 ± 0.15 (4)	0 (4)
Frog (*R. catesbeiana*)	M	21	0.577 ± 0.06 (4)	0 (4)
	M	37	1.18 ± 0.12 (4)	0 (4)
Toad	M	26	0.680 ± 0.27 (4)	0 (4)
	M	37	1.60 ± 0.7 (4)	0 (4)
Reptile				
Turtle	M, F	21	0.496 ± 0.05 (4)	2.52 ± 0.06 (4)
	M, F	37	1.44 ± 0.20 (4)	0.134 ± 0.03 (4)
Horned toad	M	37	4.48 ± 0.40 (4)	0.438 ± 0.16 (4)
Bird				
Pigeon	M	37	7.05 ± 0.66 (4)	1.08 ± 0.14 (4)
	M	40	8.03 ± 1.78 (4)	1.65 ± 0.43 (4)
Mammals				
Mouse†	M	37	6.80 ± 0.55 (5)	2.72 ± 0.33 (5)
Rat‡	M	37	5.88 ± 1.34 (7)	2.08 ± 0.16 (7)
Guinea pig	M	37	8.97 ± 0.27 (5)	2.04 ± 0.16 (4)

* Values are averages ± standard deviations (duplicates in each determination). Number in parentheses is number of animals used.
† CDBA strain.
‡ Fischer strain.

as at 37°C. The azo reductase enzyme in the horned toad is almost as active as that found in the mammalian species studied.

Since the reptiles have both azo and nitro reductase, it is not surprising that the bird (pigeon) has both azo and nitro reductase activity. This activity is higher than that found in either the turtle or the horned toad. Enzyme activity at 40°C was not significantly different from that of 37°C.

Of all classes studied, the activity of azo and nitro reductase was highest in the bird and mammal. The variation of azo and nitro reductase enzyme activity in mammals is known.[10, 11] Of particular interest are the strain differences in azo and nitro reductase. Results of ten strains of mice presented in Table 2 show differences in both azo and nitro reductase activity. An analysis of variance indicated significant differences between groups, and the Tukey test[13] indicated six different groups among the nitro reductase activities and four different groups among azo reductase values. It is likely that these differences are due to genetic background, since the various strains had access to the same food and were exposed to the same environmental conditions (excepting the axenic mice). It is of interest that the axenic mice had similar enzyme activity as nonaxenic mice of the same strain. Thus, the bacterial population or their products apparently do not trigger the synthesis or unmasking of the reductive enzymes.

The action of many drugs has been shown to persist longer in female rats than in male rats. An explanation of this sex variation is that the liver drug enzymes of male rats oxidize these drugs considerably faster than do those of females; such sex variation has not been seen in other mammalian species.[14] Table 3 summarizes

TABLE 2

AZO AND NITRO REDUCTASE ACTIVITY IN TEN STRAINS OF MICE

Strain	Azo reductase, μmoles sulfanilamide formed/(gm liver) (hr)	Nitro reductase, μmoles p-aminobenzoic acid formed/(gm liver) (hr)
$CALF_1$	6.66 ± 0.37*	2.84 ± 0.55
CDBA	6.80 ± 0.55	2.72 ± 0.33
C3H/HeN	7.12 ± 0.33	2.32 ± 0.19
CFW/N	8.07 ± 0.56	2.48 ± 0.33
C57BL	8.74 ± 0.28	2.92 ± 0.25
General purpose†	8.86 ± 0.30	3.24 ± 0.41
NIH‡	8.89 ± 1.02	2.14 ± 0.35
Axenic BALB/c	9.37 ± 0.70	2.44 ± 0.42
BALB/c	9.44 ± 0.40	2.61 ± 0.14
AKR/N	9.63 ± 1.31	3.13 ± 0.02

* Five male mice of each strain assayed for enzyme activity. All are inbred strains except for the general purpose strain. Values are averages ± standard deviations (duplicates in each determination). The four different groups for azo reductase ($P \leq 0.05$) are AKR/N; BALB/c, axenic BALB/c; NIH, general purpose, C57BL; and CFW/N, C3H/HeN, CDBA, $CALF_1$. The six different groups for nitro reductase ($P \leq 0.05$) are general purpose; AKR/N; C57BL; $CALF_1$, CDBA; BALB/c, CFW/N; and axenic BALB/c, C3H/HeN, NIH.

† Random-bred Swiss albino.

‡ Inbred Swiss albino, obtained from general purpose colony in 1936.

results obtained from assaying azo and nitro reductase in male and female rat liver. The male rat has significantly greater azo and nitro reductase activity although this difference is not twofold and is considerably less than the sex variation observed in oxidative drug-metabolizing enzymes.

Discussion.—Aromatic azo and nitro compounds are used industrially, as drugs, pesticides, and coloring for food additives. In addition, many azo compounds are potent carcinogens. The metabolic fate of these compounds in various species that might be exposed to them directly or inadvertently via waste products should be a matter of concern. A comparative study of azo and nitro reductase would also show whether these enzymes are more primitive than the oxidative drug-metabolizing enzymes which apparently are absent in fish and aquatic amphibia but are present in reptiles.

The experiments presented suggest the evolution of azo and nitro reductase. Azo reductase was present in the most primitive of fish, elasmobranchii, and both azo and nitro reductase were present in the two teleost fish assayed. The frog and toad have azo reductase activity but lack the ability to reduce the nitro group of *p*-nitrobenzoic acid. The turtle and horned toad, reptiles, have both azo and nitro reductase, and the bird (pigeon) has enzyme activity comparable to that of the mouse. In doing comparative enzyme studies it is important to do assays at both the environmental temperature of the species studied as well as at 37°C. Thus, shark livers had no enzyme activity under our test conditions at 37°, while at 26°, the temperature of its environment in sea water, azo reductase enzyme activity was

TABLE 3

SEX DIFFERENCES IN FISCHER RATS IN AZO AND NITRO REDUCTASE ACTIVITY

Sex*	Azo reductase, μmoles sulfanilamide formed/gm liver	Nitro reductase, μmoles p-aminobenzoic acid formed/gm liver
Male†	5.88 ± 1.34	2.08 ± 0.16
Female	3.67 ± 0.2	1.43 ± 0.14

* Seven rats of each sex assayed for enzyme activity. Values are averages ± standard deviations (duplicates in each determination).

† A "t" test showed a significant difference ($P \leq 0.05$) between male and female rats for both azo and nitro reductase.

present. Azo reductase activity is greater at 37° in the turtle but nitro reductase activity is higher at 21°C. Thus, if temperature studies are not done, the enzyme activity present may be missed. In contrast to oxidative enzymes that metabolize drugs, the reductive enzymes, azo and nitro reductase, are present in fish with azo reductase being present both in elasmobranchii and teleosts. Therefore, these enzymes may have developed before and may be more primitive than the oxidative enzymes that metabolize drugs. Further support for this hypothesis is that an enzyme system that reduces the nitro group of *p*-nitrobenzoic acid and other aromatic nitro compounds has been found in *E. coli*.[15, 16] This enzyme system is different from the mammalian system in that it is DPN-dependent and in addition requires cysteine or glutathione, and Mn^{++} for its activation. The bacterial nitro reductase was also markedly inhibited by low concentrations of chlortetracycline and other chelating agents, whereas the mammalian system was insensitive to these. Moreover, in contrast to the mammalian system, which is strongly inhibited by air, the bacterial system is nearly as active in air as in nitrogen. Thus a similar function is being carried out by a different enzyme system. It may be that (1) nitro and azo reductase evolved in response to toxic compounds formed during the primeval atmosphere on earth, or (2) perhaps azo and aromatic nitro groups (and the intermediate compounds) serve as nonspecific acceptors of hydrogen from a number of flavoprotein systems. In regard to the second possibility, it is thought that under the reducing conditions of the primeval broth, the earliest type of enzyme system would be an oxidation-reduction reaction associated with the transfer of hydrogen or an electron.[17] The use of a bacterial DPNH oxidoreductase (flavin-containing) in an oxidation-reduction process has been proposed as a model experiment.[18]

The epigram "ontogeny recapitulates phylogeny" which has been applied to oxidative drug-metabolizing enzymes also applies to the reductive enzymes, azo and nitro reductase. In the newborn mammal the oxidative pathways are absent but nitro reductase is present in the newborn rabbit at about one half the adult level.[8] Azo and nitro reductase are also present in the newborn mouse, azo reductase being at about one third the level in the adult, rising to two thirds at one day and achieving adult activity at 2 days.[19]

Different strains of mice had varying amounts of azo and nitro reductase but the greatest differences were less than twofold, whereas fivefold differences have been shown to exist within the oxidative drug-metabolizing enzymes in various strains. Differences in azo and nitro reductase activity were seen in the male and female rat, but again these differences were small compared to the variation seen in male and female rats with respect to the oxidative enzymes.

Further studies with lower species and a detailed comparative study of the enzymes in the various species and organs may shed further light on the evolution of enzymes that metabolize drugs and the origin of enzymes in general.

Summary.—The ability of enzymes in the liver of fish, amphibia, reptile, bird, and mammals to reduce azo and nitro compounds was studied. Azo reductase was present in the elasmobranch and both enzyme systems were present in the teleost. Amphibia have only azo reductase, whereas both azo and nitro reductase are present in reptiles, birds, and mammals. These findings suggest that the reductive enzymes may be of a more primitive nature than the oxidative enzymes that metabolize drugs.

[1] Conney, A. H., and J. J. Burns, *Advan. Pharmacol.*, **1**, 31 (1962).
[2] Remer, H., in *Proceedings of the First International Pharmacological Meeting*, vol. 6 (1962), p. 235.
[3] Fouts, J. R., and R. H. Adamson, *Science*, **129**, 897 (1959).
[4] Dixon, R. L., R. W. Shultice, and J. R. Fouts, *Proc. Soc. Exptl. Biol. Med.*, **103**, 333 (1960).
[5] Dixon, R. L., L. G. Hart, and J. R. Fouts, *J. Pharmacol. Exptl. Therap.*, **133**, 7 (1961).
[6] Adamson, R. H., and J. R. Fouts, *Cancer Res.*, **21**, 667 (1961).
[7] Fouts, J. R., R. L. Dixon, and R. W. Shultice, *Biochem. Pharmacol.*, **7**, 265 (1961).
[8] Hart, L. G., R. H. Adamson, R. L. Dixon, and J. R. Fouts, *J. Pharmacol. Exptl. Therap.*, **137**, 103 (1962).
[9] Brodie, B. B., and R. P. Maickel, in *Proceedings of the First International Pharmacological Meeting*, vol. 6 (1962), p. 299.
[10] Fouts, J. R., J. J. Kamm, and B. B. Brodie, *J. Pharmacol. Exptl. Therap.*, **120**, 291 (1957).
[11] Fouts, J. R., and B. B. Brodie, *J. Pharmacol. Exptl. Therap.*, **119**, 197 (1957).
[12] McLuen, E. F., and J. R. Fouts, *J. Pharmacol. Exptl. Therap.*, **131**, 7 (1961).
[13] Snedecor, G. W., *Statistical Methods* (Ames, Iowa: Iowa State College Press, 1956).
[14] Quinn, G. P., J. Axelrod, and B. B. Brodie, *Biochem. Pharmacol.*, **1**, 152 (1958).
[15] Saz, A. K., and R. B. Slie, *Arch. Biochem. Biophys.*, **51**, 5 (1954).
[16] Saz, A. K., and R. B. Slie, *J. Biol. Chem.*, **210**, 407 (1954).
[17] Oparin, A. I., *Advan. Enzymol.*, **27**, 347 (1965).
[18] Oparin, A. I., K. Serebrovskaya, and S. Pantskhava, *Dokl. Akad. Nauk SSSR*, **151**, 235 (1963).
[19] Adamson, R. H., unpublished observations.

---

*OPTICAL ROTATORY DISPERSION OF CYTOCHROME C**

BY YASH P. MYER AND HENRY A. HARBURY

DEPARTMENT OF BIOCHEMISTRY, YALE UNIVERSITY

Communicated by Joseph S. Fruton, September 24, 1965

Data on the optical rotatory dispersion (ORD)[1] of cytochrome *c* can in principle provide important information on relationships between the structure and function of this molecule. In conjunction with other studies on this topic, we have been performing rotation measurements over the wavelength range 190–660 mμ, beginning with some broad comparisons that might aid in the interpretation of subsequent, more detailed experiments. We summarize here the results for cytochrome *c* in the oxidized state. Data are given on the effects of change in pH, complex formation with added ligands, modification of functional groups, and species variation. Results for cytochrome *c* in the reduced state and for derivative heme peptide systems will be communicated separately. As these experiments were nearing completion, reports appeared of measurements partially paralleling this work.[2–4] There also have been two earlier ORD studies of cytochrome *c*.[5, 6]

Experimental.—Materials: Crystalline horse heart cytochrome *c* and human heart cytochrome *c* were generously given us by Dr. E. Margoliash. Horse heart cytochrome *c* was obtained also from Sigma Chemical Co. (Type III). Tuna heart cytochrome *c*,[7] chicken heart cytochrome *c*,[8] and modified forms of horse heart cytochrome *c* (TFA-cytochrome *c*,[9] guan-cytochrome *c*,[10] (ac·tyr)$_2$guan-cytochrome *c*[11]) were samples prepared in this laboratory.