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HEPATIC *N*-DEMETHYLATION OF AMINOPYRINE IN RAT AND TROUT*

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Abstract—Optimal conditions have been investigated for the *in vitro* assay of the hepatic *N*-demethylation of aminopyrine in rat (Wistar) and trout (*Salmo irideus*). In rat and trout *N*-demethylase shows differences in optimum temperature, stability and substrate affinity. The pH optimum is identical. Adverse actions of nicotinamide and semicarbazide, commonly used in the test, are discussed.

It is shown that under standardized (optimal) conditions per unit of liver weight the activity of the rat is about 15 times that of the trout. The significance of this difference is discussed.

A GROUP of enzymes dissimilating various compounds of exogenous origin, especially drugs, has been studied extensively during last years in mammalian livers. Up to a short time ago it was suggested[1, 2] that, contrary to terrestrial animals, aquatic animals (fishes, tadpoles of frogs and toads, and certain frogs) were not provided with oxidative capacity or the possibility of conjugation of various drugs, because of the lipoidal character of gills and/or skin through which excretion of lipid-soluble compounds could take place. Recently, however, evidence began to rise that also in the livers of some aquatic animals an although very slight capacity for drug metabolism is present.[3–11] Considering the *in vitro* assays for many drug-metabolizing enzyme activities the lack in uniformity in the methods reported by the various investigators is striking.[6, 7, 8, 18, 20, 21, 22] For this reason conclusions about quantitative aspects of the concerning enzymatic activities can be hardly drawn. Especially difficulties will arise in a comparative study of drug metabolism in several species. Among the enzymatic systems concerning drug metabolism, which are localized in the microsomal fraction of liver homogenates, the oxidative *N*-dealkylation has become recognized as a major pathway.[12] In the present study an effort has been made to determine some optimal conditions for the *in vitro* assay of the hepatic *N*-demethylation of aminopyrine in rat and trout in order to obtain comparable enzymatic activities.

MATERIALS AND METHODS

The animals used were adult male rats (Wistar) about 3–4 months old, weighing 200–300 g and rainbow trouts (*Salmo irideus*) about 1·5 yr old, weighing 150–180 g. The trout (bred by Kon. Ned. Heide Mij., Arnhem, The Netherlands) were kept in

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cooled aerated water. Both rat and trout were sacrificed by decapitation and 25% liver homogenates were prepared at 2–4° with a Potter–Elvehjem homogenizer in 0·25 M isotonic sucrose containing 10^{-3} M sodium ethylenediamine tetraacetate (EDTA-Na) and 5×10^{-2} M Tris(hydroxymethyl) aminomethane-HCl buffer (pH 7·4). The homogenates were centrifuged at 9000 *g* for 20 min at 2° and the supernatants were used for the enzyme assays. In experiments in which the cellular localization of the enzyme was studied the 9000 *g* supernatants were centrifuged at 105000 *g* during 60 min at 2° with a Christ preparative ultracentrifuge (Omega II).

Unless otherwise indicated the enzyme assay media contained 5×10^{-2} M tris-HCl (pH 8·0), 5×10^{-3} neutralized semicarbazide hydrochloride 8×10^{-4} M $MgCl_2$, 8×10^{-6} M $MnCl_2$, 5×10^{-3} M sodium isocitrate, 10 μg isocitric dehydrogenase per ml (Sigma type IV, capable of reducing 5·7 μmole NADP/min/mg) and saturating levels of aminopyrine and NADP. After preincubation of the assay media during 10 min, the reaction was started by adding the 9000 *g* supernatants. The total volume of the reaction mixture was 3 ml, containing about 3·0 mg/ml liver protein. After incubation the reaction was stopped by the addition of 0·5 ml 25% $ZnSO_4.7H_2O$ and 0·5 ml saturated $Ba(OH)_2$. After cooling the precipitated protein was removed by centrifugation at 18000 *g* for 10 min. The amount of formaldehyde in the supernatant was determined according to the method of Nash,[13] as modified by Cochin and Axelrod.[14] The protein was determined according to the method of Lowry *et al.*[15]

RESULTS

Influence of temperature, incubation time and pH on the enzyme activity

It has been demonstrated by some authors[3, 4, 7, 16] that the enzymes of poikilotherms and homoiotherms show differences in their optimum temperature. Since a temperature optimum is the resultant of two opposed factors—the rate of enzymatic conversion and the thermal denaturation of the enzyme—the optimum temperature measured depends on the length of the period during which the enzyme activity is determined.

Fig. 1 illustrates the optimum temperature for the enzymatic *N*-demethylation of aminopyrine by the 9000 *g* supernatants from liver homogenates of trout and rat estimated at pH 8·0. After an incubation time of 10 min a clear difference in optimum temperature for the enzymatic activities becomes manifest. After much longer incubation periods—for instance 60 min—this distinction is less striking in view of the fact that the denaturation of the enzymes has been further progressed.

The decrease of the enzyme activities at different temperatures as a function of time is shown in Fig. 2. The decrease in enzyme activity of trout *N*-demethylase at 25° is rather slow. The *N*-demethylation is found to be unchanged for 20–30 min and then declines. On the contrary, at 37° the drop in activity starts much earlier and is more pronounced. The *N*-demethylating enzyme of the rat, too, appears to be more stable at 25°, but the maximal activity at this temperature is only a fraction of the maximal activity at 37°. At 37° the rat *N*-demethylase activity is found to be constant only for 5–10 min, and then rapidly declines. Also from these results it appears that there is a clear-cut difference in optimum temperature at which the demethylating enzymes of the two species show their maximal activity, which lasts only for a relatively short period.

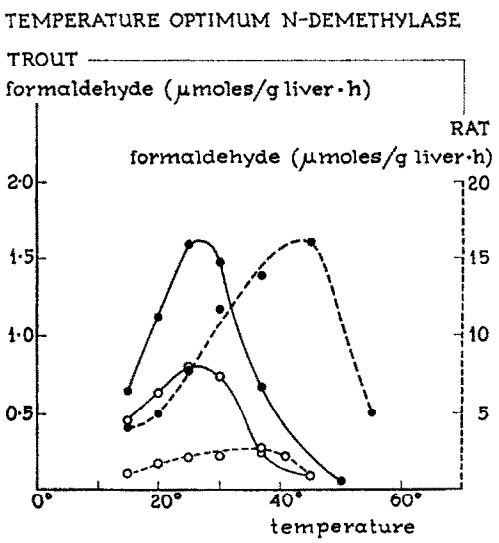

FIG. 1. Optimum temperatures for the *N*-demethylation of aminopyrine by 9000 *g* supernatants derived from liver homogenates of trout and rat. The enzyme activities are expressed as μmoles formaldehyde produced/hr/g fresh liver. The optima were measured during different incubation periods: ●——● trout, 10 min incubation; ○——○ trout, 60 min incubation; ●- - -● rat, 10 min incubation; ○- - -○ rat, 60 min incubation.

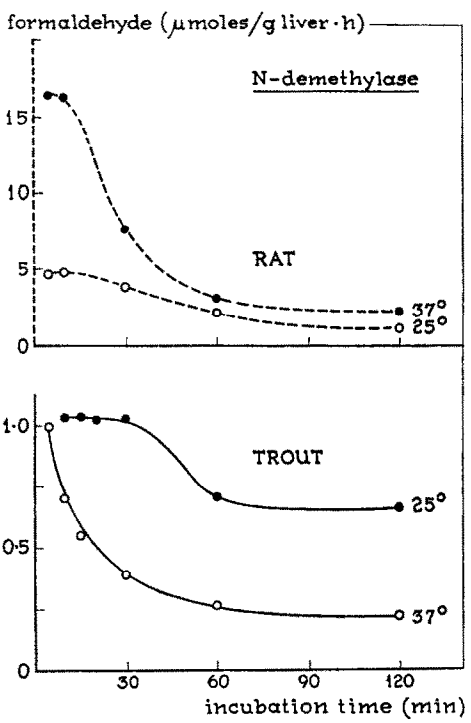

FIG. 2. The decrease of the *N*-demethylating enzyme activities in the 9000 *g* supernatants from liver homogenates of rat and trout at 37° and 25° as a function of time. Enzyme activities are expressed as μmoles formaldehyde produced/hr/g fresh liver.

The *N*-demethylase activities of rat and trout have also been compared using test systems of different pH, obtained by preparing incubation mixtures with tris-HCl buffer solutions of different pH. The stated pH-values have been determined during incubation. Fig. 3 represents these enzyme activities assayed at optimum temperatures, after incubation times of 10 and 20 min for rat and trout respectively and in the presence of saturating levels of aminopyrine and NADPH. It can be remarked, that the pH-optima for rat and trout liver enzyme are equal and approximaely 8.

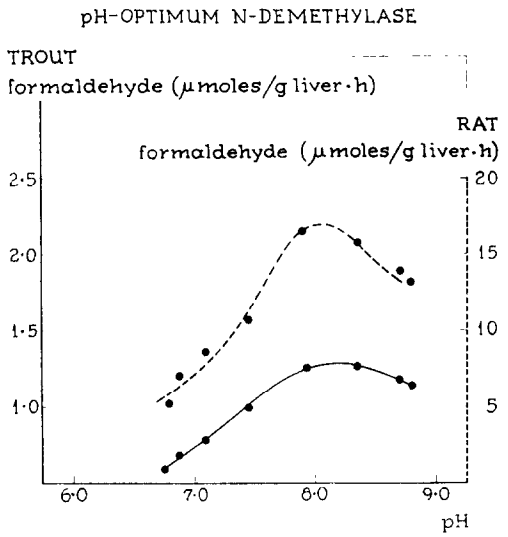

FIG. 3. Optimal pH for the *N*-demethylation of aminopyrine by 9000 *g* supernatants derived from liver homogenates of trout and rat. Enzyme activities are expressed as μmoles formaldehyde produced/hr/gram fresh liver. ●– – –● rat; ●——● trout.

Influence of substrate concentration

In the oxidative *N*-demethylation experiments in which aminopyrine was used as a substrate, for rat liver *N*-demethylase in 9000 *g* supernatant the K_m for aminopyrine is $6{\cdot}1 \times 10^{-4}$ M, assayed by 10 min incubation at 37°, pH 8·0. For trout liver *N*-demethylase in 9000 *g* supernatant the K_m for aminopyrine is $2{\cdot}1 \times 10^{-3}$ M, assayed by 20 min incubation at 25°, pH 8·0. For measurements of maximal *N*-demethylase activities both of rat and trout a saturating concentration of aminopyrine (16·7 10^{-3} M) was used. Higher levels of aminopyrine, f.i. $33{\cdot}3 \times 10^{-3}$ M, caused an inhibition of the enzyme activity. NADPH was supplied to the enzymatic system via generation from NADP by the isocitric dehydrogenase reaction. In order to ensure reduction of all NADP to NADPH the assay media were preincubated for 10 min prior to the addition of the 9000 *g* supernatants. The K_m-values for NADP were assayed by adding increasing amounts of NADP in the presence of sufficiently high concentrations of isocitric dehydrogenase and isocitrate. For rat and trout *N*-demethylase the K_m-values for NADP are $4{\cdot}0 \times 10^{-6}$ M and $2{\cdot}2 \times 10^{-5}$ M respectively. In measurements of maximal *N*-demethylase activities both of rat and trout the saturating concentration of NADP used was $8{\cdot}8 \times 10^{-5}$ M. Besides aminopyrine and

NADPH also a good supply of molecular oxygen is necessary for maximal *N*-demethylation. For this reason during the incubation the test media were saturated with air by shaking.

Influence of nicotinamide and semicarbazide

Requirements of semicarbazide and nicotinamide in measuring *N*-demethylase activities are reported by many investigators.[18–22] Semicarbazide is mentioned to function as a trapping agent for the formaldehyde produced in the enzymatic reaction.[22] Nicotinamide is supposed to inhibit the breakdown of NADP by liver pyridine nucleotidase.[20, 23] Recently an inhibition by nicotinamide of the aminopyrine *N*-demethylation has been reported by Schenkman *et al.*[24] We could confirm the decrease in *N*-demethylase activity applying nicotinamide in concentrations of 5 to 50 $\times$ 10^{-3} M. The inhibition, however, appears to be not simply competitive as suggested by Schenkman *et al.*,[24] but more complex, since we found not only the K_m-values for aminopyrine to be increased, but also the maximal rates of conversion (V_{max}) to be lowered by increasing amounts of nicotinamide, which implies a combination of competitive and non-competitive inhibition. The *N*-demethylase activity measurements mentioned in this study were carried out under optimal conditions for temperature, pH, incubation time and with saturating substrate concentrations. As can be seen from Table 1, the inhibiting action of nicotinamide is more pronounced in the *N*-demethylation by trout liver enzyme than by rat liver enzyme. However, if the *N*-demethylating activity is measured for the rat at 25° (10 min incubation time), the inhibition of the activity by nicotinamide is stronger than at 37°. For the trout at 37° (20 min incubation time), the inhibition by nicotinamide is also less than at 25°. If longer incubation times at 37° are involved (120 min), however, it is found that for the rat as well as for the trout nicotinamide gives a slight protection against the loss of activity caused by the prolonged incubation. That there is indeed a protection and not a re-activation follows from the observation that if the nicotinamide was added at the end of the incubation, the loss in activity remains unchanged.

Semicarbazide applied in concentrations higher than 5 $\times$ 10^{-3} M also causes an inhibition (Table 1). However, in this case decrease in the amounts of formaldehyde

TABLE 1. INFLUENCE OF NICOTINAMIDE AND SEMICARBAZIDE ON THE *N*-DEMETHYLATION OF AMINOPYRINE

	Per cent determinated formaldehyde*	
Nicotinamide Concentration (10^{-3} M)	rat liver (37°, 10 min incubation)	trout liver (25°, 20 min incubation)
0	100	100
5	94	65
20	82	51
50	69	42
Semicarbazide Concentration (10^{-3} M)		
0	82	76
5	100	100
8	97	94
20	62	63
50	7	9

* The amounts of produced formaldehyde are expressed as percentages of the maximal values.

measured with the method of Nash[13] is found not to be due to an inhibition of enzymatic activity but to an interference of semicarbazide with the colorimetric determination. This could be demonstrated by *in vitro* assays of standard formaldehyde concentrations in the presence of ascending amounts of semicarbazide, which below 5×10^{-3} M had no influence, but in concentrations of 20×10^{-3} M and 50×10^{-3} M depressed color development to 80 per cent and 12 per cent respectively.

Comparison of the activities in rat and trout liver under optimal conditions

The assay of the *N*-demethylase activities with aminopyrine as a substrate in 9000 *g* supernatants from rat liver and trout liver under optimal conditions—i.e. at 37° during 10 min and at 25° during 20 min respectively, both at pH 8·0, without nicotinamide and in the presence of only 5×10^{-3} M semicarbazide—gives as comparable values a production of $15{\cdot}0 \pm 2{\cdot}6$ μmole formaldehyde/hr/g rat liver and $1{\cdot}1 \pm 0{\cdot}3$ μmole formaldehyde/hr/g trout liver.

It has to be noted that the above mentioned results hold for 9000 *g* supernatants. It is well-known from previous studies on localization of enzyme activity, that the microsomes are the principal site for *N*-demethylation of aminopyrine. Apart from enzyme assays in the 9000 *g* supernatants, also 105000 *g* sediments and 105000 *g* supernatants have been tested under the above-mentioned conditions. The activities per g liver assayed in the 105000 *g* sediments (microsomal fractions) were only 90 per cent of the activities measured in the 9000 *g* supernatants. The lower activities may be due to a loss in enzyme activity caused by the longer preparative procedure for the 105000 *g* sediment. No activity could be detected in the 105000 *g* supernatants. Considering the advantage of a rapid assay procedure all determinations have been done with 9000 *g* supernatants. It can be remarked that, using 9000 *g* supernatants both from rat and trout liver, the enzyme activities measured were directly proportional to increasing amounts of enzyme extract applied to the test system, calculated as mg 9000 *g* supernatant protein per ml reaction mixture, up to a protein concentration of 5·0 mg/ml.

DISCUSSION

In a survey about drug metabolism in marine vertebrates Adamson[17] emphasizes the differences in the nature of enzymes in the various species. In the present comparative study the optimal *in vitro* conditions of the *N*-demethylation of aminopyrine in the livers of rat and trout have been investigated. In accordance to previous results[3, 4, 7, 16] in this study, too, a difference in temperature optimum, 37° for the rat and 25° for the trout, is evident. Moreover, it appears that in this respect the stability of the enzyme at different temperatures plays an important role. The *N*-demethylase activity *in vitro* is constant only during a short period, especially if measured at 37°. The pH-optima are equal for rat and trout and are approximately 8. The K_m-values of aminopyrine and NADPH for the trout *N*-demethylase appear to be about 20 times higher than the values for the rat enzyme. Concentrations of semicarbazide higher than 5×10^{-3} M in the reaction mixture have to be avoided because of their interference with the colorimetric determination of formaldehyde.

The results described in this paper indicate that there is a clear *N*-demethylase activity in rainbow trout liver. This activity is about 500 times higher than the activities for this species measured by Buhler and Rasmusson[25] and even higher than the activities in rat reported by these authors. The only conversion of substrate

shown in the rat under our specified conditions is a mono-*N*-demethylation;[26] this has been confirmed by us by TLC in both rat and trout. This may explain why Buhler and Rasmusson[25] found little or no activity since they measured only the double *N*-demethylated product, which is formed only after much longer incubation times. The rate of *N*-demethylation of aminopyrine by the trout enzyme (*Salmo irideus*) as measured *in vitro* under the conditions outlined, is about 15 times lower than that in the rat (Wistar) liver. Such a comparison is only reasonable if the measurements are carried out under optimal assay conditions. If one tries to draw conclusions from these *in vitro* activities about the rate of *N*-demethylation of the drug *in vivo*—which is hazardous—it can be stated that since in the trout studied the liver weight is only about 1 per cent of the total body weight and in the rat about 4 per cent, the *N*-demethylation capacity in the liver of the rat per unit of body weight is approximately 60 times higher than for the trout. The question remains, whether—besides the possibility that other mechanisms for disposition are present—the apparently low drug-metabolizing capacity in fish will be nevertheless sufficient to dispose of compounds foreign to the body. Relating the different levels of drug-metabolizing capacity in two totally different species, like the homoiothermous rat and the poikilothermous fish to a basic parameter such as resting metabolism—which in its turn is related to the food intake and therewith to the degree of exposure to body-foreign compounds—might greatly reduce the difference in detoxificating capacity reported.

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REFERENCES

1. B. B. Brodie and R. P. Maickel, *Proc. 1st Int. Pharmac. Meet.* **6**, 299 (1962).
2. R. P. Maickel, W. R. Jondorf and B. B. Brodie, *Fedn Proc.* **17**, 390 (1958).
3. R. H. Adamson, R. L. Dixon, F. L. Francis and D. P. Rall, *Proc. natn. Acad. Sci. U.S.A.* **54**, 1386 (1965).
4. D. R. Buhler, *Fedn Proc.* **25**, 343 (1966).
5. T. M. Chan, J. W. Gillett and L. C. Terriere, *Comp. Biochem. Physiol.* **20**, 731 (1967).
6. P. J. Creaven, D. V. Parke and R. T. Williams, *Biochem. J.* **96**, 879 (1965).
7. P. J. Creaven, W. H. Davies and R. T. Williams, *Life Sci.* **6**, 105 (1967).
8. M. Hitchcock and S. D. Murphy, *Biochem. Pharmac.* **16**, 1801 (1967).
9. P. D. Lotlikar, E. C. Miller, J. A. Miller and J. E. Halver, *Proc. Soc. exp. Biol. Med.* **124**, 160 (1967).
10. S. D. Murphy, *Proc. Soc. exp. Biol. Med.* **123**, 392 (1966).
11. J. L. Potter and R. D. O'brien, *Science* **144**, 55 (1964).
12. R. E. McMahon, *J. pharm. Sci.* **55**, 457 (1966).
13. T. Nash, *Biochem. J.* **55**, 416 (1953).
14. J. Cochin and J. Axelrod, *J. Pharmac. exp. Ther.* **125**, 105 (1959).
15. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
16. G. J. Dutton and J. P. Montgomery, *Biochem. J.* **70**, 17P (1958).
17. R. H. Adamson, *Fedn Proc.* **26**, 1047 (1967).
18. M. W. Anders and G. J. Mannering, *Molec. Pharmac.* **2**, 319 (1966).
19. L. Ernster and S. Orrenius, *Fedn Proc.* **24**, 1190 (1965).
20. S. Orrenius, *J. cell Biol.* **26**, 713 (1965).
21. J. A. Smith, W. J. Waddell and T. C. Butler, *Life Sci.* **7**, 486 (1963).
22. J. T. Wilson and J. R. Fouts, *Biochem. Pharmac.* **16**, 215 (1967).
23. P. J. G. Mann and S. H. Quastel, *Biochem. J.* **35**, 502 (1941).
24. J. B. Schenkman, J. A. Ball and R. W. Estabrook, *Biochem. Pharmac.* **16**, 1071 (1967).
25. D. R. Buhler and M. R. Rasmusson, *Comp. Biochem. Physiol.* **25**, 223 (1968).
26. T. E. Gram, J. T. Wilson and J. R. Fouts, *J. Pharmac. exp. Ther.* **159**, 172 (1268).