Biochemical Pharmacology, Vol. 16, pp. 1071–1081, Pergamon Press Ltd. 1967. Printed in Great Britain

ON THE USE OF NICOTINAMIDE IN ASSAYS FOR MICROSOMAL MIXED-FUNCTION OXIDASE ACTIVITY*

JOHN B. SCHENKMAN,† JEFFREY A. BALL and RONALD W. ESTABROOK‡

Department of Biophysics and Physical Biochemistry,
Johnson Research Foundation,
University of Pennsylvania, Philadelphia, Pa., U.S.A.

(*Received* 29 *September* 1966; *accepted* 15 *December* 1966)

Abstract—Nicotinamide, an additive to almost all media used to study oxidative metabolism of substrates of hepatic microsomal mixed-function oxidase, is shown to inhibit the metabolism of known substrates of this system. Inhibition of aminopyrine demethylation appears to be competitive, while inhibition of aniline hydroxylation appears to be a mixture of competitive and noncompetitive inhibition. It is shown that an accurate assay of substrate metabolism can be obtained in the absence of nicotinamide if the assay time is kept short, NADPH concentration is maintained at a high level, and the concentration of microsomes in the medium is kept low.

EARLY studies *in vitro* with liver microsomal mixed-function oxidase indicated a requirement for nicotinamide[1–6] to inhibit the pyridine nucleotidase of liver reported by Mann and Quastel.[7] The length of incubation of the assay media in these early studies ranged from 30 min to an hour; the amount of nicotinamide employed by the various investigators varied widely. Nicotinamide has been used in concentrations between 8 mM and 50 mM[1–8] without an attempt to determine whether this compound has any effect on the enzyme system when NADPH is not a limiting factor.

In addition to the pyridine nucleotidase mentioned above, which destroys nicotinamide adenine dinucleotide phosphate (NADP), Gillette *et al.*[9] have recently demonstrated the presence in liver microsomes of an enzyme that destroys the reduced form of this cofactor, NADPH. This pyrophosphatase splits NADPH to the reduced nicotinamide ribotide, which has an extinction at 340 mμ similar to that of NADPH, but does not serve as a cofactor for the microsomal oxidase. This enzyme is not inhibited by nicotinamide.[10]

Recently[11–13] this laboratory has reported spectral evidence of interaction of substrates with the liver microsomal cytochrome P-450. Further studies have indicated that the substrate concentration necessary to obtain half-maximal enzyme activity is nearly the same as that required to evoke half-maximal spectral change for the different substrates.[14] These investigations have provided evidence that the spectral changes are indicative of formation of an enzyme–substrate complex. In the

* Supported in part by Research Grants GM 12202 of the U.S. Public Health Service and Grant GB 2451 of the National Science Foundation.

† Postdoctoral Fellow of the U.S. Public Health Service.

‡ This work was carried out during the tenure of a Research Career Development Award of the National Institutes of Health (GM-K3-4111).

course of these studies it was observed that nicotinamide likewise causes spectral change, similar to that caused by aniline, when added to a liver microsomal suspension. Consequently, the effect of nicotinamide on the substrate-induced spectral changes, and on the microsomal mixed-function oxidase, was investigated.

MATERIALS AND METHODS

Microsomes were prepared from 0·25 M sucrose homogenates of livers of male adult rats (ca. 180-230 g) as previously described.[11] Assay media contained 50 mM Tris, pH 7·5; 5 mM $MgCl_2$; 8 mM sodium isocitrate; 0·33 mM NADP; 15 μg Sigma type IV isocitric dehydrogenase (IDH)/ml (capable of reducing 3 μmoles NADP/min/mg at 37°); and saturating levels of substrate (either 8 mM aminopyrine or 7·4 mM aniline) unless otherwise stated, in a volume of 3 ml. Nicotinamide was not included in the assay medium unless otherwise stated. Assay media were preincubated at 37° in a Dubnoff shaker for 15 min prior to the addition of microsomes to ensure reduction of all of the NADP to NADPH. Formaldehyde (HCHO) and *p*-aminophenol (PAP) were measured as previously described.[14] Protein was determined by a biuret procedure,[15] and was 1 mg/ml in the reaction medium unless otherwise stated.

RESULTS

Spectral effects

Previously we have described[11] the spectral changes accompanying the addition of a variety of substrates to rat liver microsomes. As illustrated in Fig. 1, A and B,

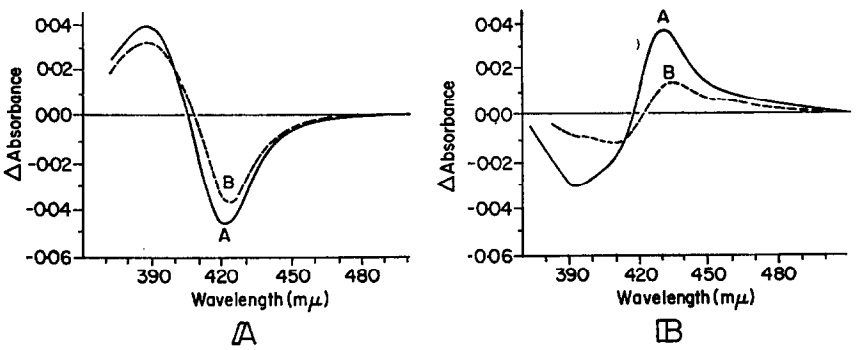

FIG. 1. The effect of nicotinamide on the formation of substrate-induced type I and type II spectral changes. Liver microsomes from phenobarbital-treated male rats were suspended in 50 mM tris buffer, pH 7·5 or in 50 mM tris buffer, pH 7·5, containing 50 mM nicotinamide to a protein concentration of 1 mg/ml. The difference in absorbance between an experimental cuvette and a reference cuvette, each containing 3 ml of the microsomal suspension, was obtained in a wavelength-scanning recording spectrophotometer. A: 3·3 mM hexobarbital was added to the experimental cuvette and the spectra recorded. B: 3·3 mM aniline was added to the experimental cuvette. The dashed line spectral curves were obtained with microsomes suspended in tris buffer containing nicotinamide; the solid curves were obtained in the absence of nicotinamide.

two types of spectral changes are obtained, dependent on the nature of the substrate used. The addition of hexobarbital, aminopyrine, chlorpromazine, phenobarbital, etc., results in the disappearance of absorbance at about 420 mμ with the concomitant increase in absorbance at about 390 mμ. This type of change in the spectral

properties of liver microsomes has been termed[13] type I spectral change, and is illustrated in Fig. 1A for the effect of hexobarbital addition to a rat liver microsomal suspension. A second type of spectral change, termed type II, is observed when nitrogenous base substances, such as aniline, nicotine, pyridine, etc., are added to liver microsomes. Figure 1B illustrates the effect of aniline addition to liver microsomes. The magnitude of the spectral change observed on addition of substrates was less intense when nicotinamide was present in the reaction medium. Figure 1 illustrates the influence of nicotinamide on the spectral changes observed on addition of hexobarbital or aniline to liver microsomes suspended in a medium without (solid-line curve) or with (dashed-line curve) 50 mM nictotinamide. As a consequence of this observation, the effect of nicotinamide on the enzymatic oxidation of two substrates of the microsomal mixed-function oxidase was investigated.

Enzymatic reactions

The extent to which pyridine nucleotidase and reduced pyridine nucleotide pyrophosphatase affect the microsomal mixed-function oxidase in a medium containing an adequate NADPH-generating system depends upon the amount of NADPH present at the initiation of the reaction, the amount of microsomal protein present, the length of the assay period, and the rate of NADPH-dependent substrate oxidation. Since nicotinamide is supposed to protect against a depletion of NADPH level, the requirements of the mixed-function oxidase for NADPH and nicotinamide were studied. Figure 2 illustrates the effect of microsome and NADPH concentrations on the rate of the NADPH-dependent oxidation of aminopyrine and aniline in the absence of nicotinamide. In Fig. 2A, curves A and B represent aminopyrine demethylation, measured as formaldehyde (HCHO) production in 10 min, in the presence of 1 and 2 mg microsomal protein/ml respectively. Curve C represents aniline hydroxylation, measured as *p*-aminophenol (PAP) production in 15 min, with 1 mg microsomal protein/ml. In Fig. 2B, reciprocal plots (Lineweaver-Burk) of the data in Fig 2A reveal a K_m value for NADPH of $2{\cdot}8 \times 10^{-5}$ M, with microsomal protein concentrations of 1 mg/ml, in excellent agreement with the value previously reported by Orrenius.[8] Aniline hydroxylation and aminopyrine demethylation therefore have the same NADPH dependency. Of interest is the observation that the requirement for NADPH increases at higher microsomal protein concentrations (curve B), presumably because of the increasing influence of other microsomal reactions which utilize NADPH, e.g. lipid peroxidation[16] and NADPH oxidase,[17] as well as the above-mentioned pyridine nucleotidases.[7, 9] The addition of 50 mM nicotinamide to the medium above does not alter the K_m obtained for NADPH.

For routine assays of substrate oxidation by hepatic microsomal mixed-function oxidase, a NADPH concentration of about 0.3 mM should be employed, dependent on the length of the assay and concentration of microsomal protein used.

Enzyme stability

The effect of incubation time on formaldehyde production from aminopyrine and *p*-aminophenol production from aniline is illustrated in Fig. 3, A and B respectively. These experiments were carried out in a medium containing 0·34 mM NADPH and no nicotinamide. Although the media employed in both experiments were identical, except for the substrate added, the rate of aminopyrine oxidation begins to decrease

after 5 min, whereas no decrease in aniline oxidation rate at the same microsomal protein concentration is observed until after 15 min. The most probable explanation is that the more rapid onset of limiting conditions is related to the greater rate (fifteen fold) of aminopyrine oxidation than of aniline oxidation. Products of aminopyrine oxidation (HCHO, monomethylaminoantipyrine, and aminoantipyrine) do

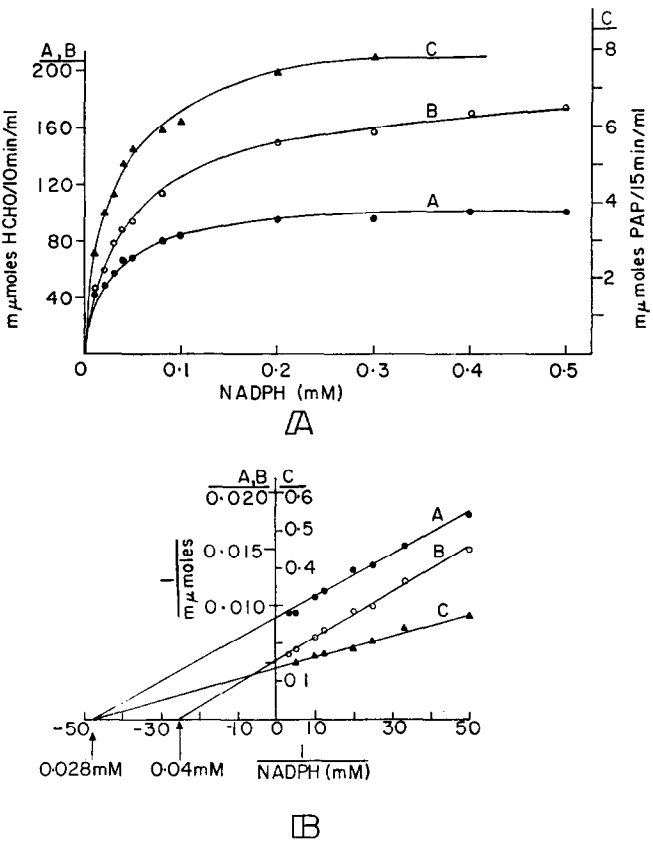

FIG. 2. The NADPH dependence of aminopyrine demethylase and aniline hydroxyalse.

A: Plots of NADPH dependence of aminopyrine demethylation rate (formaldehyde (HCHO) production) and aniline hydroxylation rate [*p*-aminophenol (PAP) production] in a medium described in Methods. Curves A and B are aminopyrine demethylase activities in media containing 1 and 2 mg microsomal protein/ml respectively. Curve C is aniline hydroxylase activities in a medium containing 1 mg microsomal protein/ml. Microsomes were obtained from livers of adult female rats, pretreated for 3 days prior to being sacrificed, with 80 mg phenobarbital sodium/kg. Assay times were 10 min for aminopyrine demethylase and 15 min for aniline hydroxylase.

B: Reciprocal plots of the data in 2A. The ordinate under C is the reciprocal of PAP production/15 min/ml assay medium, and that under A, B is the reciprocal of HCHO production/10 min/ml assay medium.

not inhibit aminopyrine demethylase at concentrations that could have been generated in 10 min in the experiments described. The decline in enzyme activity begins earlier in the incubation medium with higher concentrations of microsomal protein (Fig. 4). A plot of the different activities (determined from the initial rates of aminopyrine

oxidation in this figure) vs. the concentrations of microsomal protein in the medium (Fig. 5, curve A) shows that good assays can be obtained if the initial rates are used.

If the enzyme activity is determined from the concentration of product formed at a later time, as for example 10 min, the specific activity of the enzyme (amount of

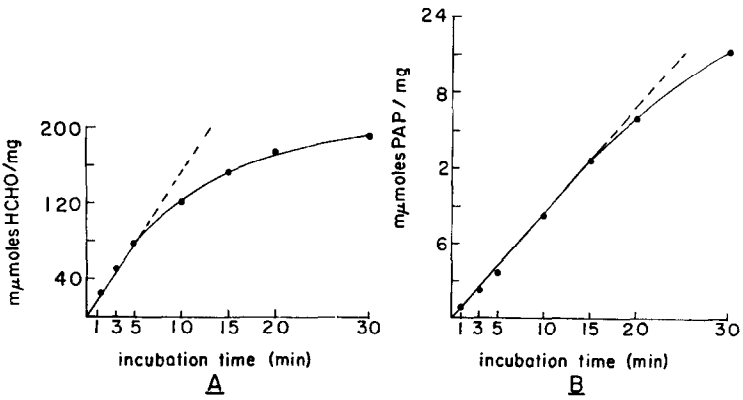

FIG. 3. The influence of length of incubation on the concentration of products formed.

A: The curve of aminopyrine demethylase activity for 1 mg microsomal protein/ml medium is presented. Enzyme activity is expressed as mμmoles of formaldehyde (HCHO) formed/ml medium.

B: Aniline hydroxylase activity was measured and is expressed as mμmoles *p*-aminophenol (PAP) formed/ml assay medium, in a medium containing 1 mg/microsomal protein/ml. Conditions are as described in Methods. Livers from normal adult male rats were used.

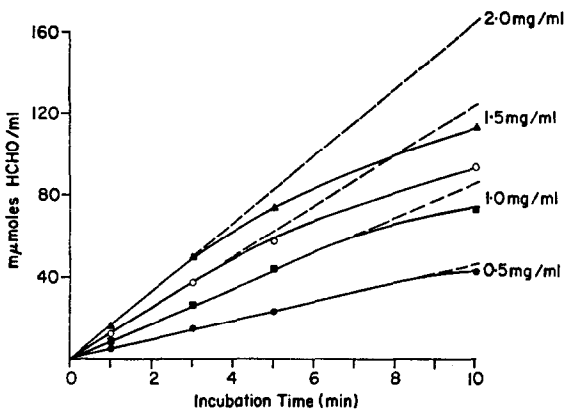

FIG. 4. The effect of microsome concentration on aminopyrine demethylase activity. Above is a family of curves showing the time course of formaldehyde production during aminopyrine demethylation in a medium containing microsomal protein concentrations ranging from 0·5 mg protein/ml to 2 mg/ml. The enzyme activity is expressed as mμmoles formaldehyde (HCHO) formed/ml medium. Livers from normal adult male rats were used.

product formed/min/mg protein) decreases with increasing protein concentration (Fig. 5, curve B). Since many workers have used 30- or 60-min reaction times, the divergence from linearity of enzyme activity as a function of protein concentration is sufficiently large to question the validity of many results determined with such long

incubation periods. Recently Gram and Fouts[18] and Leadbeater and Davies[19] have also observed a similar decrease in specific activity of rat liver microsomes during the time course of the reaction. Of interest is the failure to observe a similar decrease with preparations from the livers of rabbits or mice.

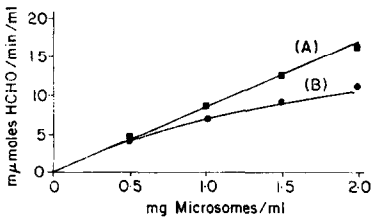

FIG. 5. The influence of time of incubation and protein concentration on the specific activity of aminopyrine demethylation. In curve A, the initial rate of formaldehyde production was determined; in Curve B the activity is based on the amount of formaldehyde actually formed in 10 min (data in Fig. 4).

The rate of NADPH oxidation in the presence of aminopyrine would not be expected to elevate the concentration of NADP in the medium in the presence of the more active NADPH-generating system; the system used was capable of generating NADPH at a rate three times that of its oxidation by aminopyrine demethylase, assuming that 1 mole of NADPH is consumed per mole of formaldehyde formed, and the reaction was initiated with all the NADP in the reduced form. Consequently, the decline in aminopyrine demethylase would not be expected to result from a reduction in the NADP–NADPH concentration by action of the Mann and Quastel enzyme, which attacks NADP. Furthermore, the presence of 50 mM nicotinamide in the medium does not prevent the decline in enzyme activity with incubation time (Fig. 6).

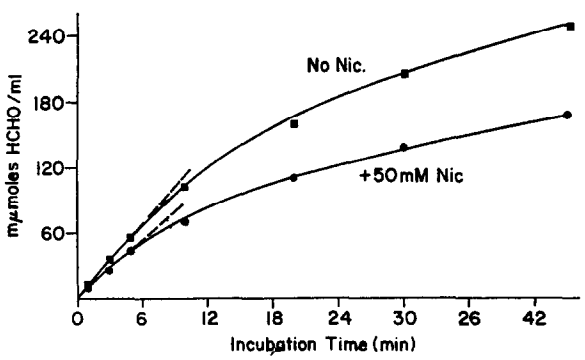

FIG. 6. The effect of 50 mM nicotinamide (NIC) on the time course of aminopyrine demethylase activity. One mg of microsomal protein was used/ml medium. Enzyme activity is expressed as mμmoles HCHO formed/ml medium. Other conditions are as described in Fig. 3A.

In agreement with Gram and Fouts,[18] increasing or decreasing the level of the NADP-reducing system (isocitric dehydrogenase) in the medium containing 0·34 mM NADP had no influence on the rate of the microsomal oxidase reaction. It was found that decreasing the IDH level to 7·5 μg/ml (half that used in Fig. 3), or increasing it

to 30, 60, 90, or 150 μg/ml, did not prevent the observed decrease in the rate of HCHO or PAP production. The inactivation of the enzyme system has been shown by Gram and Fouts[18] not to be due to lipid peroxidase activity, since the inactivation of the latter enzyme with the anti-oxidant α-tocopherol was without effect on the early decline in drug oxidase activity. In agreement with the work of Orrenius *et al.*,[16] they found that aniline and aminopyrine inhibited lipid peroxidation.

In a further attempt to determine the cause of early enzyme inactivation, and the possible influence of nicotinamide on the reaction, the concentration of NADP and NADPH was determined throughout a 45-min aminopyrine demethylase assay. It was observed that the concentration of enzymatically oxidizable NADPH rapidly declined during the assay (Fig. 7); the NADPH level decreased 30 per cent in the

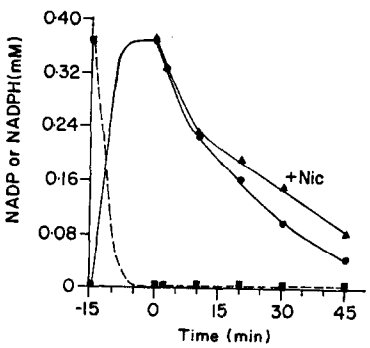

FIG. 7. The effect of incubation time on the NADP and NADPH concentration during the aminopyrine demethylase assay. Two 125-ml flasks containing 60 ml of a reaction medium composed of 50 mM Tris buffer, pH 7·5, 5 mM $MgCl_2$, 8 mM sodium isocitrate, 0·36 mM NADP, and 8 mM aminopyrine were prepared. The contents of one flask was supplemented by the addition of nicotinamide to give a final concentration of 50 mM. After temperature equilibration at 37°, 0·2 ml of an isocitric dehydrogenase solution (2 mg protein) was added (−15 min). The reaction mixture was further incubated for 15 min at 37°. At zero-time, 2·0-ml aliquots of rat liver microsomes (27 mg protein/ml) were added to each reaction flask. At the times indicated (−15, 0, 1, 10, 20, 30, and 45 min) 2-ml aliquots were removed for determination of NADP and NADPH, and 3-ml aliquots were removed for determination of formaldehyde concentration. Oxidized pyridine nucleotides were determined fluorometrically in neutralized acid extracts, with glucose 6-phosphate dehydrogenase as described by Estabrook and Maitra.[21] NADPH was determined fluorometrically, with neutralized alkaline extracts (2 ml of reaction medium plus 0·5 ml of 3 N KOH in 95% ethanol) together with oxidized glutathione and glutathione reductase. The concentration of NADPH (solid-line curves) in the presence (▲——▲) and absence (●——●) of 50 mM nicotinamide is indicated. The concentration of NADP is shown by the solid squares, dashed curve. Microsomes were prepared from livers obtained from normal adult male rats.

first 10 min after the addition of microsomes, and by 45 min was 10–20 per cent of the original level. Nicotinamide (50 mM) afforded only a minimal protection for maintenance of the NADPH. NADP did not accumulate in the assay medium, regardless of whether nicotinamide was present (50 mM) or absent. The assays for NADPH showed, however, the presence of a highly fluorescent compound similar to NADPH, but this was not oxidized in the presence of oxidized glutathione and glutathione reductase.

The decreasing level of NADPH alone can not explain the loss in enzyme activity, because the further addition of 1 mM NADPH to the assay medium, 20 min after initiation of the aminopyrine demethylase reaction, did not restore linear enzyme activity with time. This lack of effect of a second addition of NADPH is in confirmation of an observation by Leadbeater and Davies.[19] Gram and Fouts[18] also found that the further addition of NADPH, 5 min after the reaction was initiated, did not prevent the decrease in enzyme activity with time. At present the reason for the early inactivation of the drug oxidase is unknown, but there is an indication that it may be due, in part, to the aerobic incubation of the microsomes. In confirmation of the report by Gram and Fouts,[20] incubation of microsomes in the absence of substrate, but with NADPH, resulted in a deterioration of enzyme activity similar to the inactivation that occurs in the presence of substrate. During such incubations, both in the presence and absence of substrate, it was noticed that after about 5 min clumps begin to appear in the previously homogeneous suspension. These increase both in size and number during the incubation period. Such agglutination of particles probably also contributes to the observed decline in drug oxidase activity.

Influence of nicotinamide

As shown above, the presence of nicotinamide in the assay medium does not prevent the observed decline in the NADPH level, and it causes a decrease in the observed enzyme activity. Since nicotinamide does not appear to be necessary for determining the initial linear rates of enzyme activity, what then is its effect on the mixed-function oxidase system? Figure 6 shows that, rather than protecting enzyme activity, nicotinamide addition actually decreased it. Figure 8A shows the inhibitory effect of 5, 25, and 50 mM nicotinamide on the substrate dependence curves of aminopyrine demethylase. The reciprocal plot of these data is illustrated in Fig. 8B, and suggests that the observed inhibition may be competitive.[22] The values under the abscissa (at arrows) indicate the extent to which the presence of nicotinamide in the assay medium can alter the graphically obtained K_m for a substrate. As much as a fivefold difference in the K_m can be obtained—dependent on the concentration of nicotinamide employed (Table 1). A plot of the reciprocal of aminopyrine demethylase activity vs. nicotinamide concentration[22] indicated that the inhibitor constant (K_i) for nicotinamide is about 20 mM with this system.

Nicotinamide also inhibits aniline hydroxylase activity. However, although also inhibitory, its effect here is more complex than its effect on aminopyrine demethylase. In the presence of 5 and 15 mM nicotinamide, the shapes of the substrate dependence curves (Fig. 9A) are also somewhat altered, as reflected in the Lineweaver–Burk plot (Fig. 9B). The intercept of the reciprocal plot appears to the left of the ordinate in a manner indicative of a mixed-type inhibition.[22]

DISCUSSION

Previous studies from this laboratory have indicated[11–14] that the addition of substrates of the microsomal mixed function oxidase to liver microsomes causes two types of changes in the spectral properties of microsomal hemoproteins. These changes have been interpreted[11] as being due to substrate interactions with cytochrome P-450, and to represent the formation of an enzyme–substrate complex with hepatic microsomal mixed-function oxidase.

The observation that nicotinamide causes spectral changes similar to those caused by aniline indicated that this chemical also interacts with the microsomal oxidase, possibly serving as a substrate. (Subsequent attempts to find products of nicotinamide metabolism by paper chromatography and electrophoresis were negative.)* Consequently it was suspected that nicotinamide might also inhibit the enzymatic oxidation

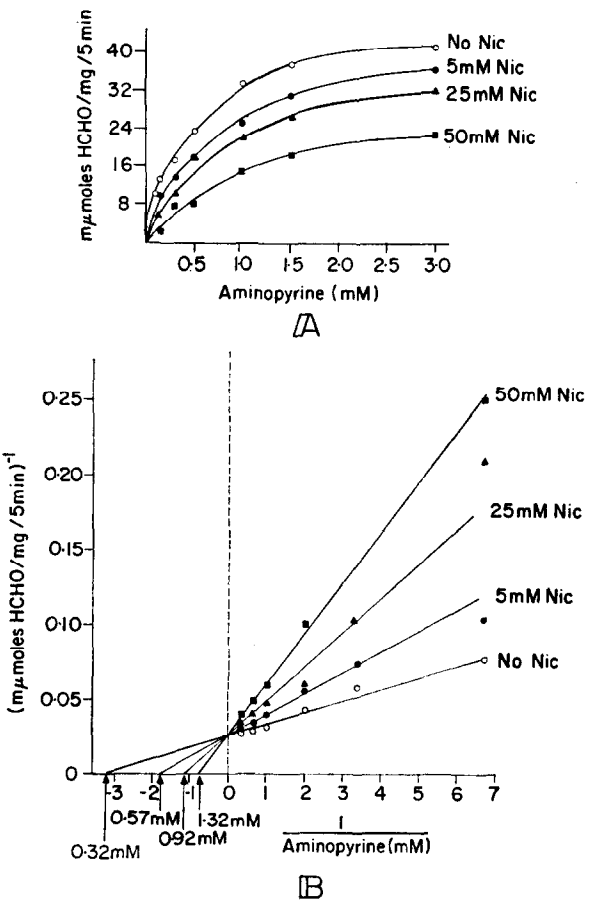

FIG. 8. The effect of nicotinamide on aminopyrine demethylase activity.

A: Substrate dependence plot of aminopyrine demethylase activity in the presence of 0, 5, 25, and 50 mM nicotinamide (NIC). Conditions are as described in Methods. Enzyme activity is expressed as mμmoles formaldehyde (HCHO) formed/15 min/mg microsomal protein.

B: Reciprocal plot of the data in Fig. 8A. Arrows on the abscissa indicate the concentration of aminopyrine at the intercept. Livers of normal adult male rats were used.

of substrates of the microsomal oxidase in a medium not limited by the NADPH concentration.

Data are presented indicating that nicotinamide is an inhibitor of microsomal oxidations, competitively inhibiting aminopyrine demethylation, and inhibiting

* We would like to acknowledge the help of Drs. B. Petrack, G. Quinn, and P. Greengard, and Mr. F. Sheppy of Geigy Laboratories, Ardsley, N.Y., in searching for possible products of ^{14}C-nicotinamide metabolism by rat liver microsomes.

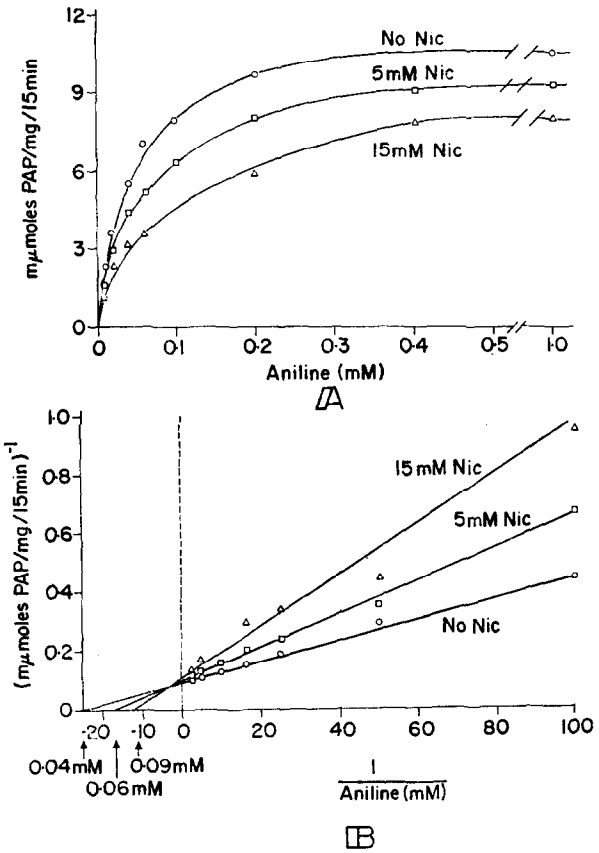

FIG. 9. The effect of nicotinamide on aniline hydroxylase activity.

A: Substrate dependence plot of aniline hydroxylase activity in the presence of 0, 5, and 15 mM nicotinamide. Conditions are as described in Methods. Enzyme activity is expressed as mμmoles PAP formed/15 min/mg microsomal protein. Livers of normal adult male rats were used.

B: Reciprocal plot of the data in Fig. 9A. Arrows on the abscissa indicate the concentration of aniline at the intercept. Nicotinamide is abbreviated as NIC.

TABLE 1. GRAPHICALLY OBTAINED MICHAELIS CONSTANTS IN THE PRESENCE (+) AND ABSENCE (−) OF 50 MM NICOTINAMIDE

Substrate	+ (mM)	− (mM)
Codeine	0·56	0·20
Morphine		0·25
Aminopyrine	1·60	0·33
Aniline	0·35	0·04

The incubation medium used was that described in Methods. The incubation time was 10 min. for all substrates except aniline, which was incubated for 15 min. Substrate levels ranged from 0·05 mM to 8 mM in each determination. Values for codeine and morphine were obtained at the National Heart Institute of the N.I.H. with the kind help of Dr. D. Davies. One mg of microsomal protein was used per ml of medium. Activity was determined from the amount of formaldehyde produced when codeine, morphine, or aminopyrine was the substrate. *p*-Aminophenol was measured when aniline was the substrate.

aniline hydroxylation by some mixed type of action. Examples of how nicotinamide can modify the apparent K_m of a substrate have been presented, and indicate that the substrate constants currently in the literature may be erroneous if determined in the presence of this compound.

When fractions of rat liver are used, it is suggested that assays of the oxidative metabolism of substrates by this enzyme system be as brief as possible, and employ low concentrations of microsomal protein (for example 1 mg/ml). When higher concentrations of microsomal protein are used, larger amounts of NADPH are necessary. The danger of using higher concentrations of microsomes is threefold: in addition to decreasing the effective NADPH concentration, and causing a more rapid decline in enzyme activity, the assay may be complicated by nonspecific binding of substrate, as shown by Gillette[23] with imipramine; higher concentrations of microsomes increased the apparent K_m for this drug. The determinations of Michaelis constants (K_m) currently in the literature, for example,[7, 23–25] for various substrates of the microsomal mixed function oxidase should be repeated in a more uniform manner, and in the absence of inhibitors like nicotinamide.

REFERENCES

1. J. Axelrod, *Biochem. J.* **63**, 634 (1956).
2. J. Axelrod, *J. Pharmac. exp. Ther.* **114**, 430 (1955).
3. B. N. LaDu, L. Gaudette, N. Trousof and B. B. Brodie, *J. biol. Chem.* **214**, 741 (1955).
4. B. B. Brodie, J. Axelrod, J. R. Cooper, L. Gaudette, B. N. LaDu, C. Mitoma and S. Udenfriend, *Science* **121**, 603 (1955).
5. A. H. Conney, E. C. Miller and J. A. Miller, *J. biol. Chem.* **228**, 753 (1957).
6. J. R. Cooper and B. B. Brodie, *J. Pharmac. exp. Ther.* **114**, 409 (1955).
7. P. J. G. Mann and S. H. Quastel, *Biochem. J.* **35**, 502 (1941).
8. S. Orrenius, *J. cell. Biol.* **25**, 713 (1965).
9. J. R. Gillette, W. Grieb and H. Sasame, *Fedn Proc.* **22**, 366 (1963).
10. J. R. Gillette, *Progr. Drug Res.* **6**, 13 (1963).
11. H. Remmer, J. B. Schenkman, R. W. Estabrook, H. Sasame, J. Gillette, S. Narasimhulu, D. Y. Cooper and O. Rosenthal, *Molec. Pharmac.* **2**, 187 (1966).
12. J. B. Schenkman and H. Remmer, *Fedn Proc.* **25**, 343 (1966).
13. W. Cammer, J. B. Schenkman and R. W. Estabrook, *Biochem. biophys. Res. Commun.* **23**, 264 (1966).
14. J. B. Schenkman, H. Remmer and R. W. Estabrook, *Molec. Pharmac.*, in press.
15. A. G. Gornall, C. J. Bardawill and M. M. David, *J. biol. Chem.* **177**, 751 (1949).
16. S. Orrenius, G. Dallner and L. Ernster, *Biochem. biophys. Res. Commun.* **14**, 329 (1964).
17. J. Gillette, B. B. Brodie and B. N. LaDu, *J. Pharmac. exp. Ther.* **119**, 532 (1957).
18. T. E. Gram and J. R. Fouts, *J. Pharmac. exp. Ther.* **152**, 363 (1966).
19. L. Leadbeater and D. R. Davies, *Biochem. Pharmac.* **13**, 1607 (1964).
20. T. E. Gram and J. R. Fouts, *Archs Biochem. Biophys* **114**, 331 (1966).
21. R. W. Estabrook and P. K. Maitra, *Analyt. Biochem.* **3**, 369 (1962).
22. M. Dixon and E. C. Webb, *Enzymes*, 2nd edn., p. 326 Academic Press, New York (1964).
23. J. R. Gillette, *Proc. 2nd Int. Pharm. Meeting* **4**, 9 (1965).
24. A. Rubin, T. R. Tephly and G. J. Mannering, *Biochem. Pharmac.* **13**, 1007 (1965).
25. T. C. Butler, W. C. Waddel and D. T. Poole, *Biochem. Pharmac.* **14**, 937 (1965).