Y-3642·HCl inhibited the potentiation of ADP-induced platelet aggregation by collagen at this concentration and completely inhibited at a low concentration of collagen (OD_{660} = 0·018), but hardly affected the initial velocity of platelet aggregation, as shown in Fig. 1. The effect of Y-3642. HCl may be due to the suppression of the release of ADP from platelets.

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REFERENCES

1. G. V. R. BORN, *J. Physiol.* **162**, 67p (1962).
2. M. NAKANISHI, H. IMAMURA, K. IKEGAMI, and K. GOTO, *Arzneimittel-Forsch.* **20**, 1004 (1970).
3. H. R. BAUMGARTNER and G. V. R. BORN, *Nature, Lond.* **281**, 137 (1968).
4. D. C. B. MILLS and G. C. K. ROBERTS, *J. Physiol.* **193**, 443 (1967).
5. M. NAKANISHI, H. IMAMURA and Y. MARUYAMA, *Arzneimittel-Forsch.* **20**, 998 (1970).
6. M. NAKANISHI, H. IMAMURA and K. GOTO, *Yakugaku Zasshi*, **90**, 564 (1970).

Biochemical Pharmacology, Vol. 20, pp. 2118–2121. Pergamon Press, 1971. Printed in Great Britain

Inhibition of microsomal mixed-function oxidase activity with nicotinamide*

(*Received* 15 *August* 1970; *accepted* 15 *January* 1971)

UNTIL quite recently, virtually all investigators of hepatic microsomal mixed-function oxidase systems used nicotinamide in their incubation media to inhibit pyridine nucleotidase in an attempt to preserve maximal levels of NADP. This practice was discontinued in most laboratories after Schenkman *et al.*[1] showed that nicotinamide not only failed to enhance, but actually inhibited, the oxidation of certain drugs, probably by competing for the type II binding site of cytochrome P-450. These studies were rigorously controlled with respect to enzyme content, NADPH concentration and incubation time. The authors suggested that the "determination of Michaelis constants (K_m) currently in the literature, for example, 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". We were particularly interested in these recommendations because we were responsible for the reporting of a number of these Michaelis constants.[2–5] In those studies we had been mindful of incubation times and microsomal content of the media and were satisfied that reaction rates were proceeding linearly throughout the incubation period. When there was doubt that this was the case, as for example when a relatively high percentage of substrate was oxidized during the period of observation, values were adjusted in the form of an integrated Michaelis constant.[2] Nevertheless, nicotinamide was employed in these studies and, even though the concentration of 4 mM was considerably below the 50 mM concentration used by Schenkman *et al.*, we were concerned as to whether or not nicotinamide might have distorted the kinetic constants we had reported.

The *N*-dealkylation of morphine, ethylmorphine, 3-methyl-4-methylaminoazobenzene (3-MMAB) and 2-diethylaminoethyl-2,2-diphenylvalerate (SKF 525-A) was studied using microsomes (105,000 *g* pellet) prepared as described previously[2] from the livers of male Holtzman strain rats (100–130 g). The incubation medium was the same as that described previously,[2] but it was varied to contain 0, 4 or 50 mM nicotinamide. The incubation mixture contained from 0·6 to 0·8 mg of microsomal protein per ml as determined by the method of Lowry *et al.*,[6] which is equivalent to 50 mg of fresh liver. Uniform reaction rates were observed throughout the various incubation periods, which were 7·5, 10, 15 and 30 min, respectively, when SKF 525-A, 3-MMAB, morphine and ethylmorphine were employed as substrates. Formaldehyde formed from the *N*-demethylation of morphine, ethylmorphine

* This research was supported by United States Public Health Service Grant GM 15477.

and 3-MMAB and acetaldehyde formed from the *N*-deethylation of SKF 525-A were determined as described previously.[2] Reciprocal velocities were plotted against reciprocal substrate concentrations and the line best fitting the points, as judged visually, was used for preliminary estimates of kinetic values. The data were then analyzed by the method of Wilkinson,[7] which weights the points and thereby removes the bias that is almost inevitable in drawing these plots. All calculations were performed with a digital computer using programs written by Cleland[8] as described previously.[2] These programs provide the K_m, $1/v$ intercepts, inhibition constant (K_i) and the standard errors of their estimates. The curves were replotted using this information (Fig. 1). Results are summarized in Table 1.

TABLE 1. EFFECT OF NICOTINAMIDE ON THE APPARENT KINETIC CONSTANTS OF THE MICROSOMAL *N*-DEALKYLATION OF DRUGS

Substrate	N	Concn of nicotinamide (mM)	Apparent K_m (mM ± S.E.)	Apparent V_{max} (μmoles HCHO or CH_3CHO formed/g of liver/hr ± S.E.)
Morphine	7	0	0·37 ± 0·05	3·25 ± 0·45
	7	4	0·37 ± 0·06 (N.S.)*	3·06 ± 0·45 (N.S.)
	7	50	0·85 ± 0·17 (S)*	2·72 ± 0·35 (S)
Ethylmorphine	4	0	0·35 ± 0·04	9·37 ± 0·50
	4	4	0·32 ± 0·06 (N.S.)	8·84 ± 0·45 (N.S.)
	4	50	1·04 ± 0·11 (S)	6·70 ± 0·40 (S)
3-MMAB	4	0	0·014 ± 0·01	5·53 ± 0·62
	4	4	0·017 ± 0·03 (N.S.)	5·86 ± 1·00 (N.S.)
	4	50	0·015 ± 0·01 (N.S.)	4·82 ± 0·37 (N.S.)
SKF 525-A	7	0	0·075 ± 0·01	6·10 ± 0·73
	7	4	0·045 ± 0·01 (S)	9·55 ± 0·61 (S)
	7	50	0·033 ± 0·00 (S)	12·60 ± 0·67 (S)

* S and N.S. indicate significance or nonsignificance ($P < 0·05$) of the difference between values obtained with and without nicotinamide in the incubation media. Statistical analysis was performed by randomized blocks.[9]

It is seen that whereas the *N*-dealkylation of morphine, ethylmorphine and SKF 525-A was inhibited by nicotinamide at a concentration of 50 mM, thus confirming the observation of Schenkman *et al.*[1] that nicotinamide inhibits the microsomal mixed-function oxidase system, the *N*-demethylation of morphine, ethylmorphine and 3-MMAB was not inhibited by nicotinamide when used at a concentration of 4 mM. We are therefore relieved that the kinetic values we reported previously for morphine, ethylmorphine and 3-MMAB were not invalid because nicotinamide was included in the medium. On the other hand, some inhibition of the *N*-deethylation of SKF 525-A occurred with the low concentration of nicotinamide. In a previous study, in which a 4 mM concentration of nicotinamide was employed, the K_i for the inhibition of ethylmorphine *N*-demethylation by SKF 525-A was compared with the K_m for the *N*-deethylation of SKF 525-A in an attempt to establish a case for substrate inhibition. In that study, the K_i differed significantly from the K_m. The presence of 4 mM nicotinamide undoubtedly contributed to that difference.

That nicotinamide, even in high concentration, did not inhibit the *N*-demethylation of 3-MMAB is of some interest because both compounds exhibit type II binding with microsomes;* substrate inhibition would have been predicted. However, predictions along this line of reasoning have not proved fruitful, as has been demonstrated in a recent study by Sasame and Gillette,[10] which revealed the vicissitude of the kinetics of the inhibition of drug metabolism by nicotinamide; the mechanism of inhibition of drug metabolism by nicotinamide, whether competitive, noncompetitive or mixed, depended upon the species, the substrate and the concentration of substrate.

The kinetic studies provide no immediate insight into the mechanism of inhibition by nicotinamide. The inhibition of morphine and ethylmorphine metabolism is neither competitive nor noncompetitive but mixed.[11] The kinetics of the inhibition of the *N*-deethylation of SKF 525-A by nicotinamide is

* The binding of 3-MMAB can be determined despite its intense color by filtering the reference beam of the dual beam spectrophotometer through a cuvette containing a concentration of 3-MMAB equal to that placed in the sample cuvette.

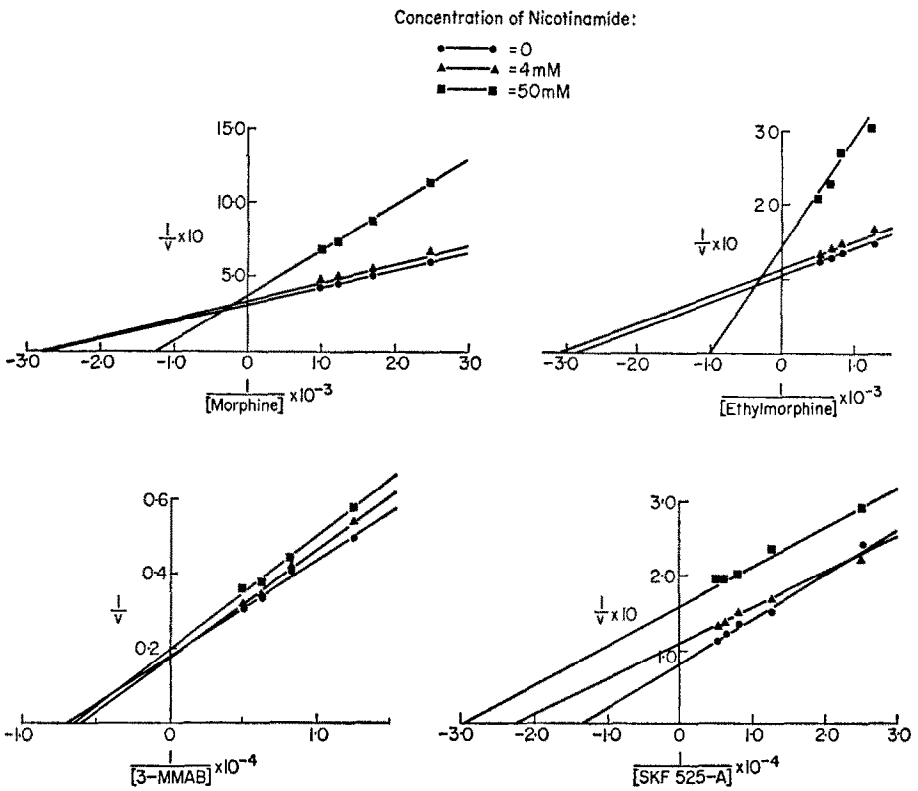

FIG. 1. Kinetics of inhibition of *N*-dealkylase reactions by nicotinamide. Substrate concentrations, moles/l; v = μmoles of HCHO or CH_3CHO formed/g of liver/hr. Points on the curves represent mean values obtained from several animals, the numbers of which are given in Table 1.

more difficult to interpret; when the concentration of nicotinamide was 50 mM, a plot was obtained which was nearly parallel to that seen when no nicotinamide was used. This is classified as coupling or uncompetitive inhibition, which has been interpreted to mean that the inhibitor increases the affinity of the enzyme for the substrate while decreasing the breakdown of the substrate–enzyme complex to form a product.[11] SKF 525-A binds irreversibly to microsomes.[12–14] Recent studies in our laboratory showed that SKF 525-A probably binds irreversibly to the type I binding site of cytochrome P-450.* Microsomes which had been suspended in a saturating solution of SKF 525-A, resedimented, washed, resedimented and dialyzed exhaustively were found to contain SKF 525-A. These microsomes gave type II binding spectra with type II drugs, but did not give type I spectra with type I drugs, presumably because the type I binding site was occupied by SKF 525-A. Kinetic studies of substrates that combine irreversibly with enzymes should be interpreted with care when Michaelis–Menten equations are employed.

Acknowledgement—The authors gratefully acknowledge the able technical assistance of Mrs. Janice Shoeman and Mrs. Kathleen Bidleman.

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REFERENCES

1. J. B. SCHENKMAN, J. A. BALL and R. W. ESTABROOK, *Biochem. Pharmac.* **16,** 1071 (1967).
2. A. RUBIN, T. R. TEPHLY and G. J. MANNERING, *Biochem. Pharmac.* **13,** 1007 (1965).
3. M. W. ANDERS and G. J. MANNERING, *Molec. Pharmac.* **2,** 319 (1966).
4. T. R. TEPHLY and G. J. MANNERING, *Molec. Pharmac.* **4,** 10 (1968).
5. N. E. SLADEK and G. J. MANNERING, *Molec. Pharmac.* **5,** 186 (1969).
6. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
7. G. N. WILKINSON, *Biochem. J.* **80,** 324 (1961).
8. W. W. CLELAND, *Nature, Lond.* **198,** 463 (1963).
9. C. C. LI, Ed., *Introduction to Experimental Statistics*, p. 174. McGraw-Hill, New York (1964).
10. H. A. SASAME and J. R. GILLETTE, *Biochem. Pharmac.* **19,** 1025 (1970).
11. J. L. WEBB, in *Enzyme and Metabolic Inhibitors*, p. 59. Academic Press, New York (1963).
12. L. A. ROGERS and J. R. FOUTS, *J. Pharmac. exp. Ther.* **146,** 286 (1964).
13. M. W. ANDERS, A. P. ALVARES and G. J. MANNERING, *Molec. Pharmac.* **2,** 328 (1966).
14. F. KRATZ and H. STAUDINGER, *Hoppe-Seyler's Z. physiol. Chem.* 349, 455 (1968).

Biochemical Pharmacology, Vol. 20, pp. 2121–2123. Pergamon Press, 1971. Printed in Great Britain

Iodoacetate inhibition of lactate production and lipid, protein, ribonucleic acid [RNA] and deoxyribonucleic acid [DNA] synthesis in newborn rat brain cortex slices

(*Received* 14 *November* 1970; *accepted* 22 *January* 1971)

IODOACETIC ACID (IAA) has long been considered a classical inhibitor of glycolysis, exerting its effect primarily on glyceraldehyde-3-phosphate dehydrogenase.[1] Early studies with pigeon and rat brains concluded that lactate production was inhibited by IAA at a concentration which did not affect cell respiration.[2,3] Although other metabolic processes such as RNA[4] and phospholipid[3] synthesis are inhibited to some extent by IAA, the concentration needed is higher than that which is required for inhibition of glycolysis.

During the course of our studies concerned with the regulation of rat brain metabolism during ontogeny,[5–7] it was discovered that DNA synthesis *in vitro* from glucose-6-^{3}H in brain slices was more sensitive than lactate production to the inhibitory effects of IAA. To further characterize this effect, IAA was used as a probe to examine the degree of interdependence of macromolecular synthesis on glycolysis.

Four-week-old male Wistar rats were housed in separate cages and fed Purina laboratory chow and tap water *ad lib.* Animals were decapitated and cerebral cortices rapidly removed and placed in a Petri dish containing ice-cold saline. Brain slices were prepared according to the method of McIlwain and Rodknight[8] and lactate production and glucose-6-^{3}H (Amersham/Searle, 2·3 c/m-mole) incorporation into lipid, protein, RNA and DNA were assayed as previously described.[6] Iodoacetate was purchased from the Sigma Chemical Co. and adjusted to pH 7·4 before being added to the medium.

Iodoacetate effected a dose-dependent separation of the relative interdependence of lactate production and lipid, protein, RNA and DNA synthesis (Table 1). The approximate ID_{50} (IAA concentration resulting in 50 per cent inhibition) for each of the five pathways may be categorized into three groups: (1) lactate production and DNA synthesis with an ID_{50} of $2–4 \times 10^{-5}$ M, (2) lipid and protein synthesis with an ID_{50} of 2×10^{-4} M, and (3) RNA synthesis with an ID_{50} of $1·5 \times 10^{-3}$ M. Thus a separation of RNA and DNA synthesis was achieved based on their respective sensitivities to IAA inhibition, despite their dependence on glucose as common precursor.

In agreement with previous results examining the effects *in vitro* of IAA on glyceraldehyde-3-phosphate dehydrogenase and pigeon and rat brain glycolysis,[1–3] lactate production was inhibited at 5×10^{-5} M IAA. This concentration was lower than that which affects brain respiration. However,