Omura, T., & Sato, R. (1964) *J. Biol. Chem. 239,* 2379-2385.
Omura, T., & Takesue, S. (1970) *J. Biochem. (Tokyo) 67,* 249-257.
Peterson, J. A., White, R. E., Yasukochi, Y., Coomes, M. L., O'Keefe, D. H., Ebel, R. E., Masters, B. S. S., Ballou, D. P., & Coon, M. J. (1977) *J. Biol. Chem. 252,* 4431-4434.
Rein, H., Ristan, O., Friedrich, J., Janig, G.-R., & Ruckpaul, K. (1977) *FEBS Lett. 75,* 19-22.
Tsai, R., Yu, C. A., Gunsalus, I. C., Peisach, J., Blumberg, W., Orme-Johnson, W. H., & Beinert, H. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66,* 1157-1163.
Vermilion, J. L., & Coon, M. J. (1974) *Biochem. Biophys. Res. Commun. 60,* 1315-1322.
Vermilion, J. L., & Coon, M. J. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36,* 3632.
Yasukochi, Y., & Masters, B. S. S. (1976) *J. Biol. Chem. 251,* 5337-5344.
Yasukochi, Y., & Masters, B. S. S. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36,* 364.

Differential Effects of Prostaglandin Synthetase Stimulators on Inhibition of Cyclooxygenase†

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ABSTRACT: The different effects of prostaglandin synthetase stimulators on inhibition of the cyclooxygenase by structurally distinct classes of nonsteroidal antiinflammatory agents suggest that the enzyme is altered by interaction with these stimulators. Reversible stimulation of prostaglandin synthetase activity by phenols and some other compounds and the relative influence of these stimulators on inhibitors of the cyclooxygenase were determined quantitatively. Two distinct classes of inhibitors were established. The fenamates were relatively weak inhibitors alone but were much more potent in the presence of phenolic compounds. In contrast, ibuprofen, indomethacin, and flurbiprofen were more potent than the fenamates and were reduced in effectiveness by the stimulators, as expected on the basis of two opposing actions. The relative potency of the cyclooxygenase stimulators (phenol > norepinephrine > tryptophan > benzoquinone > anisole) paralleled their synergistic action on the fenamates and their antagonist action on the nonfenamates. This correlation suggests that an enzyme alteration which leads to cyclooxygenase stimulation may also result in increased sensitivity to fenamates and decreased sensitivity to the other inhibitors, possibly by altering their capacity to bind.

Several structurally distinct classes of nonsteroidal antiinflammatory agents are known to inhibit prostaglandin synthetase (EC 1.14.99.1) activity (Vane, 1971; Ham et al., 1972; Gryglewski, 1974). These agents vary from the structural simplicity of aspirin to the complexity of indomethacin and *N*-phenylanthranilic acid analogues. Most of these inhibitors elicit their effect on the cyclooxygenase (Smith & Lands, 1971; Lands et al., 1973; Egan et al., 1976a), the primary enzyme of the prostaglandin synthetase complex. Although their precise mechanism of action is not understood, several of these agents are time dependent (Rome & Lands, 1976) and aspirin acetylates the cyclooxygenase (Roth et al., 1975). Some enzymes other than prostaglandin synthetase are also inhibited by nonsteroidal antiinflammatory agents (Flower & Vane, 1974; Oyanagui, 1976) and in some instances the inhibitors show differential activity toward these enzymes (Zwarenstein et al., 1976).

In contrast, several compounds such as phenols, quinones, heme-containing proteins, and tryptophan have been shown to stimulate prostaglandin synthetase (Nugteren et al., 1966; Takeguchi et al., 1971; Yoshimoto et al., 1970; Miyamoto et al., 1974; Humes et al., 1976). A mechanism for the stimulatory action of phenols as radical scavengers has been proposed (Egan et al., 1976b). Previously, inhibition of the cyclooxygenase had been studied almost exclusively using an acetone-powder enzyme preparation which required the presence of these compounds for activity (Smith & Lands, 1972). Consequently, it was not possible to examine independently the effects of such stimulators on the action of the various prostaglandin synthetase inhibitors.

This manuscript describes the influence of prostaglandin synthetase stimulators on inhibition of the cyclooxygenase by nonsteroidal antiinflammatory agents. The actions of enzyme stimulators on inhibition by the several structurally distinct classes of these inhibitory agents suggest stimulator-induced alterations of the cyclooxygenase molecule.

Experimental Procedure

Materials

Ram seminal vesicles were obtained from a local slaughterhouse and were stored at −70 °C. Arachidonic acid was purchased from P-L Biochemicals Inc. and [1-^{14}C]arachidonic acid (58 mCi/mmol) was obtained from Dhom Products Ltd. Indomethacin, flufenamic acid, meclofenamic acid, mefenamic acid, and niflumic acid were supplied by Merck & Co. Ibuprofen and flurbiprofen were obtained from the Upjohn Co. Silica gel GF thin-layer chromatography plates were purchased from Analtech Inc. New England Nuclear supplied the Liquifluor. All other chemicals and materials were purchased from standard suppliers.

Methods

Preparation of Microsomal Prostaglandin Synthetase. The prostaglandin synthetase enzyme complex from ram seminal

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TABLE I: Inhibition of Prostaglandin Synthetase and Cyclooxygenase by Nonsteroidal Antiinflammatory Agents.

Inhibitor	ID_{50} values (μM)			
	Cyclooxygenase[a]		PG synthesis[b]	
	− phenol	+ phenol	− phenol	+ phenol
Indomethacin	0.2	ND[c]	1	ND
Flurbiprofen	0.2	ND	2	ND
Ibuprofen	2.2	ND	100	ND
Meclofenamic acid	10	0.1	ND	ND
Mefenamic acid	150	6.3	130	50
Flufenamic acid	390	8.5	>200	6
Niflumic acid	605	18	>5000	80

[a] Protein at 0.6 mg/mL. [b] Protein at 1.9 mg/mL. [c] ND, not determined.

TABLE II: The Effect of Prostaglandin Synthetase Stimulators on Inhibition by Flufenamic Acid.

Stimulator[b]	Percentage change in rate[a]			
	Cyclooxygenase		Prostaglandin Synthesis	
	No inhibitor	Flufenamic[c] acid	No inhibitor	Flufenamic[d] acid
Phenol	+129	−96	+60	−77
Norepinephrine	+69	−76	+73	−71
Tryptophan	+33	−25	+48	−32
Hydroquinone	ND[e]	ND	+56	−97
Benzoquinone	+10	−28	+51	−97
Anisole	−11	+12	0	0

[a] Plus is increased rate and minus is decreased rate based on control values established with no additive. [b] Each stimulator was tested at 500 μM except for benzoquinone and anisole which were 2000 μM with the cyclooxygenase. [c] Flufenamic acid at a concentration of 33 μM. [d] Flufenamic acid at a concentration of 200 μM. [e] ND, not determined.

vesicles was prepared as a microsomal suspension in 100 mM potassium phosphate buffer (pH 7.5) as described previously (Egan et al., 1976b). The microsomal suspension at about 40 mg protein/mL was stored at −70 °C prior to use.

Oxygen Incorporation Measurements. The incorporation of oxygen into arachidonic acid was determined by measuring oxygen tension in solution with a calibrated Yellow Springs Instrument Co. Model 53 oxygen monitor and a Sargent-Welch Model DSRLG recorder (Egan et al., 1976b). Microsomes were added to the oxygen monitor chamber at 30 °C containing 3 mL of 100 mM potassium phosphate buffer (pH 8.5) and the compound to be tested. The reaction was initiated by the addition of 100 μM arachidonic acid. Initial reaction rates were determined from the linear portion of the oxygen monitor trace at the onset of reaction.

Conversion of Arachidonic Acid to Prostaglandins. Compounds to be tested for their influence on the enzyme were incubated for 5 min at 25 °C with 0.1 mL of the diluted microsomal suspension (350–400 μg of protein). The reaction was then initiated by the addition of 22 nmol of arachidonic acid and 0.05 μCi of [1-^{14}C]arachidonic acid in 0.1 mL of 100 mM potassium phosphate buffer, pH 7.5. Following incubation of these mixtures for 1 min at 25 °C, the reactions were terminated by the addition of 0.5 mL of 30 mM citric acid containing 20 μg of PGE_2 and 10 μg of $PGF_{2\alpha}$. These mixtures were extracted with ethyl acetate and the organic phases reduced to dryness. The residues were dissolved in ethyl acetate and chromatogrammed on silica gel GF thin-layer plates with ethyl acetate/acetone/acetic acid (90/10/1). After exposure to iodine vapor, the PGE_2 zone (R_f 0.45) and the $PGF_{2\alpha}$ zone (R_f 0.2) were removed from the plate and added to a toluene/ethanol/Liquifluor (70/30/4.2) scintillation solution. The $PGF_{2\alpha}$ zone would also contain any 6-keto $PGF_{1\alpha}$. Radioactivity was determined in a Packard Model 3003 Tri-Carb liquid scintillation spectrometer with an efficiency of 75%.

Results

Properties of the Prostaglandin Synthetase Reaction. The microsomal prostaglandin synthetase enzyme utilized for these studies has been characterized in detail (Egan et al., 1976b). It generated the prostaglandins $PGF_{2\alpha}$ and/or 6-keto $PGF_{1\alpha}$ and PGE_2 by oxygenation of arachidonic acid (AA) as it underwent irreversible self-deactivation. As established by monitoring oxygen incorporation, the initial rate of arachidonic acid utilization was about 250 nmol of AA/(min mg of protein) at 30 °C. The background rate of oxygen uptake prior to arachidonic acid addition was less than 1% as rapid. At 25 °C, 6 nmol/(min mg of protein) of materials in the PGE_2 and $PGF_{2\alpha}$ zones were generated. Initial cyclooxygenase rates were much greater than the rate of formation of prostaglandins since the oxygenation of arachidonic acid to PGG_2 was more rapid than the subsequent metabolism to the stable prostaglandins. Furthermore, the reaction conditions differed between these two measurements and the initial reaction rate was not linear for a full minute.

Inhibition of Prostaglandin Cyclooxygenase and Prostaglandin Synthesis by Nonsteroidal Antiinflammatory Agents. The ID_{50} values for inhibition both of the cyclooxygenase and of prostaglandin synthesis ($PGF_{2\alpha}$ and/or 6-keto $PGF_{1\alpha}$ and PGE_2) by several nonsteroidal antiinflammatory agents are shown in Table I. The inhibitors are listed in order of their decreasing potency. Although prostaglandin synthesis was generally less sensitive than was the cyclooxygenase to each inhibitor, their order of relative potency was identical. To attain optimal effectiveness, the time-dependent inhibitors (indomethacin, flurbiprofen, and meclofenamic acid) were preincubated with enzyme for 2 min in the cyclooxygenase system and for 5 min in the assay for prostaglandin synthesis prior to initiating the reactions with arachidonic acid. The other inhibitors were fully active without an induction period (time independent). In the absence of preincubation, the ID_{50} values of the time-dependent inhibitors would have been significantly higher (Smith & Lands, 1971). The fenamates were generally weaker inhibitors than the nonfenamates and the time-dependent inhibitors were more potent than the time-independent inhibitors.

Stimulation of the Cyclooxygenase and of Prostaglandin Synthesis. The capacity of several compounds to increase both the cyclooxygenase activity and prostaglandin synthesis has been determined (Table II). These stimulators are listed in order of their decreasing potency on the cyclooxygenase as shown in the third column. Although most were evaluated at 500 μM, benzoquinone and anisole showed no effect on the cyclooxygenase at this concentration and, consequently, were also tested at 2000 μM. The order of stimulatory potency on the cyclooxygenase was phenol > norepinephrine > tryptophan > benzoquinone > anisole $\approx$ 0. Prostaglandin synthesis was stimulated with somewhat different relative potencies: norepinephrine > phenol $\approx$ hydroquinone > benzoquinone $\approx$ tryptophan > anisole $\approx$ 0. Despite these variations, the phenolic compounds were generally more effective than nonphenolics, while anisole was ineffective throughout.

Stimulation of both cyclooxygenase activity and prostaglandin synthesis by phenol was readily reversed by removing

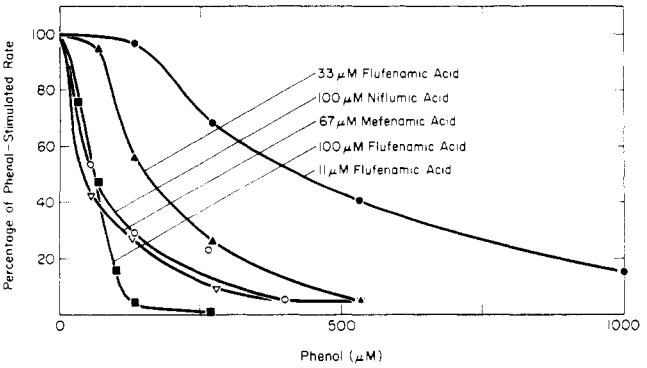

FIGURE 1: The effect of phenol on inhibition of prostaglandin cyclooxygenase by *N*-phenylanthranilic acids. Reaction rates for the cyclooxygenase were measured as described in the Methods section. The rate in the absence of additives was 360 nmol of O_2/(min mg of protein) and the 100% value on the ordinate reflects this rate (in the absence of phenol) attenuated only slightly by the inhibitors as described in Table I. The stimulation observed with phenol alone was 21, 53, 84, 115, and 90% at 53, 133, 267, 500, and 1000 μM, respectively. The rates shown are a percentage of the phenol stimulated rates.

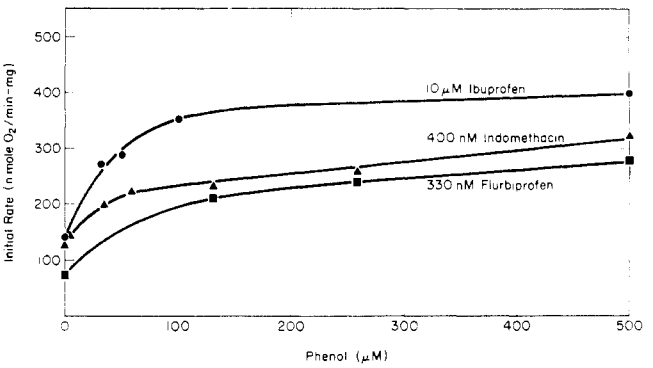

FIGURE 2: The effect of phenol on inhibition of the cyclooxygenase by non-fenamates. Reaction rates for the cyclooxygenase were determined as described in the Methods section. The initial rate of the control without any additive was 360 nmol of O_2/(min mg of protein). In the absence of phenol the initial rates were inhibited 65, 80, and 61% by 400 nM indomethacin, 330 nM flurbiprofen, and 10 mM ibuprofen, respectively.

the phenol. Upon centrifuging the microsomal suspension containing 100 μM phenol at 106 000*g* for 1 h and resuspending the pellet fraction in phenol-free buffer (at least at tenfold dilution of phenol), the enzyme was returned to its phenol-free rate. Furthermore, the activity of this resuspended enzyme was again susceptible to twofold stimulation by 100 μM phenol.

The Effect of Prostaglandin Synthetase Stimulators on Inhibition of the Enzyme by N-Phenylanthranilic Acid Analogues. In the absence of any other additive, the substituted *N*-phenylanthranilic acids (flufenamic acid, meclofenamic acid, mefenamic acid, and niflumic acid) were relatively weak inhibitors both of the cyclooxygenase and of prostaglandin biosynthesis when compared with ibuprofen, indomethacin, and flurbiprofen (Table I). However, phenol dramatically potentiated the inhibitory capacity of these fenamates (Figure 1) to the extent that 500 μM phenol reduced the ID_{50} values for the cyclooxygenase of meclofenamic, mefenamic, flufenamic, and niflumic acids 100-, 24-, 46-, and 34-fold, respectively (Table I). As expected, this potentiating effect of phenol was also observed on prostaglandin synthesis. Inhibition of the cyclooxygenase was dependent upon both fenamate and phenol concentrations. A synergistic effect was observed between flufenamic acid at 11, 33, and 100 μM concentrations and phenol; the cyclooxygenase inhibition was increased by raising the concentration of either the flufenamic acid or the phenol.

The relative effectiveness of the prostaglandin synthetase stimulators on fenamate-type inhibition is shown in Table II. The capacity of each of these agents to promote inhibition of the cyclooxygenase by 33 μM flufenamic acid (fourth column) was directly proportional to its ability to stimulate the cyclooxygenase. Although this qualitative pattern also held for prostaglandin synthesis (sixth and seventh columns), the correlation was not as consistent as for the cyclooxygenase. For example, the most effective stimulator of prostaglandin synthesis, norepinephrine, was only a moderate synergistic promotor of fenamate inhibition, whereas hydroquinone and benzoquinone were less effective stimulators yet induced 97% inhibition by the 200 μM flufenamic acid.

The Effect of Phenol on Inhibition of Prostaglandin Biosynthesis by Ibuprofen, Flurbiprofen, and Indomethacin. The phenol-induced inhibition observed with fenamates was not detected with either ibuprofen, flurbiprofen, or indomethacin. In contrast, these nonfenamates were less effective inhibitors of the cyclooxygenase in the presence of phenol (Figure 2). This antagonistic action of the stimulators on nonfenamate inhibition would be expected from the conflicting actions of stimulation by phenol and retardation by the inhibitor. The effect was concentration dependent and, at 500 μM phenol, ibuprofen was 3.2-fold, flurbiprofen 3.7-fold, and indomethacin 2.6-fold less effective than in the absence of phenol. The relative effectiveness of the prostaglandin synthetase stimulators on the inhibition of the cyclooxygenase by the three nonfenamates was phenol > norepinephrine > tryptophan > benzoquinone > anisole ≈ 0. This antagonistic effect of the stimulators on ibuprofen, flurbiprofen, and indomethacin inhibition of prostaglandin synthesis was not detected.

Discussion

Using a microsomal preparation of prostaglandin synthetase, it has been possible to demonstrate significant stimulation of prostaglandin cyclooxygenase and overall prostaglandin biosynthesis by phenols, tryptophan, and quinones. Those compounds which stimulated prostaglandin synthetase also influenced the effectiveness of inhibitors of the cyclooxygenase. This influence has been examined for several classes of these inhibitors in order to better define the role of phenols on stimulation of prostaglandin cyclooxygenase.

An enzyme preparation which was highly active even without artificial stimulation provided a meaningful control against which to assess the effects of both stimulators and inhibitors and rendered kinetic measurements by oxygen monitoring accurate and reproducible. Although the inhibitors studied exerted their influence on the cyclooxygenase and the primary effect of the stimulators was likewise on this enzyme, prostaglandin biosynthesis was monitored to assure that the oxygen uptake measurements accurately reflected prostaglandin formation. Although qualitative agreement was observed between these measurements, the quantitative discrepancies probably resulted from effects of the compounds on enzymes other than the cyclooxygenase within the prostaglandin synthetase complex (Miyamoto et al., 1974).

Several acidic inhibitors (both fenamate and nonfenamate) of prostaglandin cyclooxygenase have been found to require preincubation with the enzyme in order to manifest their optimal effect (Rome & Lands, 1976). On the basis of their time dependence it has been assumed that these are irreversible inhibitors. In accord with this proposition, the similar ID_{50} values for the three time-dependent inhibitors (indomethacin,

flurbiprofen, and meclofenamic acid) suggest that the enzyme was titrated in an irreversible fashion.

Based on their opposing actions, inhibitors and stimulators should normally be antagonistic when added together. However, the inhibitory capacity of the fenamates was augmented rather than diminished by phenol and the other stimulators. This synergism cannot be explained in terms of phenol acting only as an antioxidative enzyme stimulator (Egan et al., 1976b). Both the time-dependent and time-independent *N*-phenylanthranilic acids (fenamates) showed this phenol effect. The relative potency of the stimulators in promoting inhibition by the fenamates paralleled their effectiveness in stimulating the cyclooxygenase. Although this correlation was not borne out as precisely for prostaglandin biosynthesis, the results obtained with the cyclooxygenase are more meaningful since the compounds directly influence this enzyme without interference from other segments of the enzyme complex.

In contrast, inhibition of the enzyme by indomethacin, flurbiprofen, and ibuprofen was not augmented by phenol. In fact, phenol reduced the effectiveness of these inhibitors on the cyclooxygenase, as would be expected from their antagonistic effects. In each instance, the extent of stimulation was greater than expected from a twofold stimulation by the uninhibited fraction of the enzyme. Hence, phenol must interact with the enzyme to partially prevent inhibition, possibly by depressing inhibitor binding.

Since fenamates are physiologically active inhibitors of prostaglandin synthesis (Gryglewski, 1974), the cyclooxygenase in vivo must be in the "stimulated" conformation; the nature of the natural stimulator has not yet been established. In addition to increasing the rate of the cyclooxygenase reaction, stimulators such as phenol and tryptophan also promote the formation of PGH_2 at the expense of PGG_2 (Humes et al., 1976; Miyamoto et al., 1976; Egan et al., 1976b). Fenamates also inhibit mouse pancreas lipase while ibuprofen and other nonfenamates were not effective (Zwarenstein et al., 1976). Although the physiological effects of fenamates are complicated by their actions on several enzymes, their effects on prostaglandin synthetase reported herein are characteristically distinct from the other nonsteroidal antiinflammatory agents.

The purified cyclooxygenase has been reported to possess iron-containing prosthetic groups (Hemler et al., 1976). Although other investigators have failed to confirm this observation (Miyamoto et al., 1976; Van der Ouderaa et al., 1977), the heme requirement for this purified cyclooxygenase may represent reincorporation into the purified apoenzyme, of a transition metal containing moiety lost during purification. If such a prosthetic group is present in the cyclooxygenase, it could provide a locus for differentiation between fenamates and other nonsteroidal antiinflammatory agents because *N*-phenylanthranilic acids form stable bidentate complexes with transition metals (Decker & Frye, 1966), in a fashion not feasible with indomethacin, ibuprofen, or flurbiprofen. Consequently, the fenamates may bind to a transition metal on the cyclooxygenase either at a different site or at the same site in a different manner than the other inhibitors studied herein. Our data suggest that phenol may expose the site to binding by the *N*-phenylanthranilic acids while slightly depressing such binding by indomethacin, ibuprofen, and flurbiprofen.

On this basis, other inhibitors with the capacity to form complexes with transition metals should also be more effective in the presence of phenol. In accord with this concept, phenyl salicylates and phenylbutazone also inhibit the phenol-treated cyclooxygenase but do not inhibit the phenol-free system (Humes, J. L., unpublished experiments). The structural similarities between *N*-phenylanthranilic acids and phenylbutazone may not be readily apparent. However, the diketone on phenylbutazone could enolize and possibly form complexes with transition metals in a manner reminiscent of acetylacetone (Cotton & Wilkinson, 1972). Consequently, the phenomenon of phenol-augmented inhibition of the cyclooxygenase may extend to many other inhibitors which can form complexes with transition metals.

The different influences of phenol on the fenamate and the nonfenamate inhibitors suggest that phenol may alter the enzyme as well as acting as a nonspecific antioxidant to prevent enzyme deactivation (Egan et al., 1976b). The strong correlation between the potency of the antioxidants to stimulate prostaglandin synthetase and to influence inhibition by *N*-phenylanthranilic acids suggests that the effect which contributes to cyclooxygenase stimulation may also result in increased sensitivity to these agents. These phenomena could result from stimulator-induced changes in binding of the inhibitors to the cyclooxygenase, possibly resulting from a conformational change in the enzyme.

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References

Cotton, A. F., & Wilkinson, G. (1972) *Advanced Inorganic Chemistry,* 3rd ed, p 850, Interscience, New York, N.Y.

Decker, J. S., & Frye, H. (1966) *Z. Naturforsch. B 21,* 527.

Egan, R. W., Humes, J. L., Eckert, C. A., Galavage, M., & Kuehl, F. A., Jr. (1976a) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1652.

Egan, R. W., Paxton, J., & Kuehl, F. A., Jr. (1976b) *J. Biol. Chem. 251,* 7329.

Flower, R. J., & Vane, J. R. (1974) *Biochem. Pharmacol. 23,* 1439.

Gryglewski, R. J. (1974) in *Prostaglandin Synthetase Inhibitors* (Robinson, H. J., & Vane, J. R., Eds.) p 33, Raven Press, New York, N.Y.

Ham, E. A., Cirillo, V. J., Zanetti, M., Shen, T. Y., & Kuehl, F. A., Jr. (1972) in *Prostaglandins in Cellular Biology* (Ramwell, P. W., & Pharriss, B. B., Eds.) p 345, Plenum Press, New York, N.Y.

Hemler, M., Lands, W. E. M., & Smith, W. L. (1976) *J. Biol. Chem. 251,* 5575.

Humes, J. L., Egan, R. W., & Galavage, M. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1380.

Lands, W. E. M., LeTellier, P. R., Rome, L. H., & Vanderhock, J. Y. (1973) *Adv. Biosci. 9,* 15.

Miyamoto, T., Yamamoto, S., & Hayaishi, O. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 3645.

Miyamoto, T., Ogina, N., Yamamoto, S., & Hayaishi, O. (1976) *J. Biol. Chem. 251,* 2629.

Nugteren, D. H., Beerthuis, R. K., & VanDorp, D. A. (1966) *Recl. Trav. Chim. Pays-Bas 85,* 405.

Oyanagui, Y. (1976) *Biochem. Pharmacol. 25,* 1473.

Rome, L. H., & Lands, W. E. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 72,* 4863.

Roth, G. J., Stanford, N., & Majerus, P. W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 3073.

Smith, W. L., & Lands, W. E. M. (1971) *J. Biol. Chem. 246,* 6700.

Smith, W. L., & Lands, W. E. M. (1972) *Biochemistry 11,* 3276.

Takeguchi, C., Kohno, E., & Sih, C. J. (1971) *Biochemistry 10*, 2372.
Van der Ouderaa, F. J., Buytenhek, M., Nugteren, D. H., & VanDorp, D. A. (1977) *Biochim. Biophys. Acta 487*, 315.
Vane, J. R. (1971) *Nature (London), New Biol. 231*, 232.
Yoshimoto, A., Ito, H., & Tomita, K. (1970) *J. Biochem. (Tokyo) 68*, 487.
Zwarenstein, H., Sapeika, N., & Holmes, J. H. (1976) *Res. Commun. Chem. Pathol. Pharmacol. 13*, 563.

Reactions of Reduced Nicotinamide Adenine Dinucleotide in Acid: Studies by Reversed-Phase High-Pressure Liquid Chromatography†

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ABSTRACT: Reversed-phase high-pressure liquid chromatography was used to isolate acid breakdown products of reduced nicotinamide adenine dinucleotide (NADH) and products produced when NADH breakdown is catalyzed by glyceraldehyde-3-phosphate dehydrogenase (G-3-PD). Chromatographic and UV spectral data on these and related products support a mechanism for NADH acid degradation involving hydroxy addition at the nicotinamide C-6 followed by cyclization of the ring and the adjacent ribose moiety. G-3-PD is shown to catalyze a reaction in which two products are formed which are also intermediates in the acid degradation of NADH (α- and β-6-hydroxynicotinamide products). Formation of the major acid products fits a three-step, first-order mechanism curve, making it possible to calculate the rate constants k_2 and k_3 as well as the previously determined k_1.

Reactions of reduced nicotinamide adenine dinucleotide (NADH[1]) in acid are difficult to study because a complex mixture of compounds is formed. Although these reactions have been studied using ultraviolet (UV), fluorescence, circular dichroism (CD), optical rotatory dispersion (ORD), and nuclear magnetic resonance (NMR) spectroscopy, there remains conflicting opinion about the nature and number of products and the mechanism of NADH breakdown. The inability to isolate intermediates and transient species and to separate breakdown products from reactants has been a deterrant in understanding the acidic breakdown of NADH. The use of high-pressure liquid chromatography (HPLC) with microparticle packing has greatly improved the separation of NADH from its breakdown products (Margolis et al., 1976; Miksic and Brown, 1977a,b). With this technique, NADH acid product intermediates can be observed and measured.

The acid reactions of NADH center primarily around the labile nicotinamide ring. Until recently, it was believed that the primary acid product of NADH was formed from the addition of water across the 5,6 double bond of the nicotinamide ring, (6HTN)AD (1) (Anderson and Berkelhammer, 1958; Stock et al., 1961; Diekman et al., 1964; Kim and Chaykin, 1968; Choi and Alivisatos, 1968). The mechanism of the reaction was thought to involve two steps; the first involves a slow and probably reversible step in which H^+ is added at the C-5 position, and the second step involves electrophilic addition of the anion (OH^-) at position C-6 (Anderson and Berkelhammer, 1958; Kim and Chaykin, 1968). No intermediates for these mechanisms were observed, although Stock et al. (1961) have interpreted a 3-nm shift in the isobestic point as indicative that an anionic intermediate is formed.

Recently, Oppenheimer (1973), Oppenheimer and Kaplan (1974a), and Williams et al. (1976) have provided evidence based on NMR data that the structure of the primary acid product is a cyclic structure, in which the C-6 position of the nicotinamide ring and the 2′-hydroxy of the adjacent ribose moiety are bound. Oppenheimer refers to this product as cyclic tetrahydronicotinamide adenine dinucleotide (C-THN)AD. Miles et al. (1968) obtained the same CD spectra for the acid products of the two stereoisomers, α- and β-NADH. Since α- and β-NADH have different CD spectra, these data indicate that epimerization takes place between the α and β forms of NADH prior to formation of the acid product. Two mechanisms were suggested by Oppenheimer and Kaplan (1974a); one includes epimerization of β-NADH to α-NADH with subsequent protonation at the C-5 and cyclization between the 2′-hydroxy and the C-6. The other mechanism involves initial addition of water across the 5,6 double bond of the nicotinamide ring, epimerization, and then dehydration to form the cyclic product (Figure 1). Williams et al. (1976) have used the increased sensitivity of ^{13}C NMR to support formation of the cyclic product. In addition, they have identified a second conformational isomer of the acid product not observed by Oppenheimer and Kaplan (1974a). Johnson and Tuazon (1977) have studied the stability of NADH and its analogues over a wide range of pH and have also concluded the first step in the acid-catalyzed breakdown of NADH to be proton addition at the C-5 position.

A reaction product similar to the primary acid product results from catalysis of β-NADH in a neutral or acidic solution

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[1] Abbreviations used: NADH, reduced nicotinamide adenine dinucleotide, β form unless specifically stated; (C-THN)AD, cyclotetrahydronicotinamide adenine dinucleotide; (6HTN)AD, 6-hydroxytetrahydronicotinamide adenine dinucleotide; G-3-PD, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; NADHX, product formed from catalysis of NADH by G-3-PD.

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