Destruction of Cytochrome P-450 by Vinyl Fluoride, Fluroxene, and Acetylene. Evidence for a Radical Intermediate in Olefin Oxidation†

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ABSTRACT: Vinyl fluoride, vinyl bromide, fluroxene (2,2,2-trifluoroethyl vinyl ether), and acetylene alkylate the prosthetic heme group of cytochrome P-450 enzymes which catalyze their metabolism. The alkylated heme moiety has been identified in all four cases, after carboxyl group methylation and demetalation, as the dimethyl ester of *N*-(2-oxoethyl)protoporphyrin IX. The dimethyl acetal derivative of the aldehyde group in this structure is also isolated. The formation of the same prosthetic heme adduct with the four substrates requires introduction of an oxygen at the trifluoroethoxy or halide-substituted terminus of the π bond and reaction of the unsubstituted terminus with a heme nitrogen atom. This reaction orientation is consistent with a radical intermediate, possibly formed by way of an initial π-bond radical cation, but is difficult to reconcile with a cationic intermediate. The occurrence of a radical intermediate in the oxidation of olefins by cytochrome P-450 is thus suggested.

The catalytic action of cytochrome P-450 enzymes on olefinic π bonds results in the formation of epoxides. Examples of olefins known to undergo epoxidation are styrene (Leibman & Ortiz, 1969), cyclohexene (Leibman & Ortiz, 1978), ethylene (Ehrenberg et al., 1977), secobarbital (Harvey et al., 1977), alclofenac (Slack & Ford-Hutchinson, 1980), and vinyl chloride (Guengerich et al., 1979). Epoxides are in fact the only primary metabolites known to be formed in the enzymatic oxidation of olefinic bonds, although the isolated products often reflect secondary chemical or enzymatic transformations. In addition to epoxide formation, however, it is now known that catalytic turnover of olefins by cytochrome P-450 can result in irreversible inactivation of the enzyme (De Matteis, 1978; Levin et al., 1972; Ioannides & Parke, 1976; Ortiz de Montellano & Mico, 1980). This destruction of the monooxygenase by 2-isopropyl-4-pentenamide has been shown to be a suicidal process (Ortiz de Montellano & Mico, 1981) in which the substrate is oxidatively activated to a species which binds covalently to the prosthetic heme[1] of the enzyme (Ortiz de Montellano et al., 1979). Experiments with liver microsomes (Ortiz de Montellano & Mico, 1981) and purified reconstituted cytochrome P-450 isozymes (Ortiz de Montellano et al., 1981a) indicate that approximately 250 molecules of 2-isopropyl-4-pentenamide are turned over, presumably by epoxidation, for each enzyme molecule that is inactivated. The precise structure of the prosthetic heme adduct obtained with 2-isopropyl-4-pentenamide remains ambiguous (Ortiz de Montellano et al., 1979, 1981b), but that formed with ethylene has been identified as the iron complex of *N*-(2-hydroxyethyl)protoporphyrin IX (Ortiz de Montellano et al., 1981b,c). This corresponds *formally* to the alkylation of prosthetic heme by ethylene oxide.

Cytochrome P-450 is also inactivated during catalytic interaction with acetylenic (White & Muller-Eberhard, 1977; White, 1978; Ortiz de Montellano & Kunze, 1980a) and allenic (Ortiz de Montellano & Kunze, 1980b) π bonds. In the case of terminal acetylenes, enzyme inactivation also reflects covalent attachment of the substrate to the prosthetic heme moiety (Ortiz de Montellano & Kunze, 1980a). The porphyrin framework of propyne-alkylated heme has very recently been identified as *N*-(2-oxopropyl)protoporphyrin IX (Ortiz de Montellano & Kunze, 1981a). Using biphenylacetylene as a model substrate, we have demonstrated that the enzymatic oxidation of acetylenes results not only in enzyme inactivation but also in the formation of π-bond-oxidized metabolites (Ortiz de Montellano & Kunze, 1980c, 1981b).

The catalytic participation of the enzyme in its own destruction, the formation of epoxides from olefins, and the formal derivation of the ethylene adduct from reaction of ethylene oxide with heme naturally suggest that epoxide metabolites are responsible for enzyme inactivation. A solid body of evidence, however, stands against this interpretation. We have shown that cytochrome P-450 is not inactivated by the epoxides of three active olefins (Ortiz de Montellano & Mico, 1980; Ortiz de Montellano et al., 1979, 1981b), even though the epoxides have access to the active site of the enzyme and survive the incubation conditions. Chemical studies indicate that epoxides do not react with the nitrogen atoms in heme (P. R. Ortiz de Montellano and B. A. Mico, unpublished results). In fact, we have established in one instance that an epoxide competitively inhibits the destructive action of a closely related olefin (Ortiz de Montellano & Mico, 1981). An independent study has also provided evidence that the destruction of cytochrome P-450 by vinyl chloride is not mediated by the corresponding epoxide metabolite (Guengerich & Strickland, 1977). These results have led us to postulate that a transient intermediate is formed in the epoxidation sequence (or diverging from it) which can alkylate the prosthetic heme (Ortiz de Montellano et al., 1979, 1981b,c). A similar mechanism has been invoked to explain the destructive effect of acetylenes (Ortiz de Montellano & Kunze, 1980a, 1981a). The present investigation with oxygen- and halogen-substituted olefins was undertaken to explore the electronic properties of the postulated intermediates.[2]

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[1] Abbreviations: fluroxene, 2,2,2-trifluoroethyl vinyl ether; heme, iron protoporphyrin IX regardless of the iron oxidation state; EDTA, ethylenediaminetetraacetic acid.

[2] The results of this study have been briefly communicated (Kunze et al., 1981).

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Table I: In Vitro Loss of Hepatic Microsomal Cytochrome P-450

substrate	concn	NADPH (1 mM)	P-450 loss (%) (20 min)[c]	green pigment isolated
vinyl fluoride	1:1 with air	yes	27 ± 3	yes
		no	ND	
vinylidene fluoride	1:1 with air	yes	34 ± 1	yes
		no	ND	
vinyl bromide	1:1 with air	yes	ND	trace
		no	ND	
ethyl vinyl ether	10 mM	yes	ND	trace
		no	ND	
phenyl vinyl ether	10 mM	yes	6 ± 1	
		no	4 ± 1	
vinyl acetate	10 mM[a]	yes	ND	
		no	ND	
vinyl trifluoroacetate	10 mM[b]	yes	ND	
		no	ND	
2-chloroethyl vinyl ether	10 mM[b]	yes	ND	
		no	ND	
fluroxene	20 mM	yes	14 ± 1	yes
		no	ND	

[a] At 50 mM, vinyl acetate caused 8 ± 2% NADPH-dependent loss of cytochrome P-450. [b] At 50 mM, these agents caused NADPH-independent enzyme loss. [c] ND, not detectable.

Materials and Methods

Chemicals. Fluroxene (Ohio Medical Products) was kindly provided by Dr. Y. J. Sohn. Ethyl vinyl ether was purchased from Aldrich Chemical Co. (Milwaukee, WI), 2-chloroethyl vinyl ether, vinyl acetate, phenyl vinyl ether, and vinyl trifluoroacetate were from Polysciences, Inc. (Warrington, PA), acetylene was from Matheson Chemical Co. (Newark, CA), and vinyl fluoride, vinylidene fluoride, and vinyl bromide were from Pfaltz & Bauer, Inc. (Stamford, CT). Unsaturated substrates were used as obtained. NADPH and other biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Solvents were of the highest quality and were glass distilled prior to use in chromatographic procedures.

In Vitro Destruction of Cytochrome P-450. Unstarved male Sprague-Dawley rats weighing 250 ± 25 g were injected once a day for 4 days with phenobarbital (80 mg/kg, as an aqueous solution). On the fifth day the rats were decapitated, and their livers, after perfusion in situ with ice-cold isotonic KCl solution, were removed. These were used to prepare a 33–50% (w/v) homogenate in isotonic KCl. The 10000*g* (20 min) supernatant obtained on centrifugation of the homogenate at 4 °C was recentrifuged at 96000*g* for 60 min (4 °C). The microsomal pellet was resuspended in isotonic KCl and was recentrifuged at 96000*g* for 60 min. The washed microsomal pellet was suspended in 0.1 M sodium/potassium phosphate buffer (pH 7.4) containing 150 mM KCl and 1.5 mM EDTA at a protein concentration of approximately 1 mg/mL. Protein concentrations were measured by the Lowry procedure (Lowry et al., 1951). Substrates (concentrations as given in Table I) were preincubated at 37 °C for 3 min with the microsomal suspension prior to the addition of NADPH (1 mM). The incubation vessels were capped in studies with volatile substrates. Gaseous substrates were mixed with air and were passed over the surface of the incubation mixture. Aliquots withdrawn from the incubation mixtures after 10, 20, and 30 min were placed in test tubes immersed in ice. The cytochrome P-450 content of the aliquots was measured as before (Ortiz de Montellano & Mico, 1981) on an Aminco DW-2 spectrophotometer. Control incubations were carried out without added substrates and, for each substrate, in the absence of NADPH. Enzyme losses in these controls, except where noted otherwise, were negligible (0–2%).

Isolation and Characterization of Abnormal Porphyrins. The green (red-fluorescent) porphyrins accumulated in the livers of phenobarbital pretreated Sprague-Dawley male rats injected with various unsaturated substrates were isolated by a modification of the procedure previously reported for the acetylene-derived pigment (Ortiz de Montellano & Kunze, 1980a). After purification of the zinc-complexed porphyrin by thin-layer chromatography on a 2000-μm plate, as reported (Ortiz de Montellano & Kunze, 1980a), the porphyrin band was rechromatographed on a 1000-μm silica gel plate (Analtech) using 3:1 (v/v) $CHCl_3$/acetone as the solvent. This step is necessary because the zinc complex of these porphyrin pigments is not well resolved from the dimethyl ester of heme by the thin-layer chromatographic system. The green porphyrin recovered in each instance from the second thin-layer plate was further purified by high-pressure liquid chromatography on a 4.6 × 250 mm Whatman Partisil 10-PAC column eluted with a 20-min 0–100% linear gradient of methanol into 1:1 (v/v) hexane/tetrahydrofuran. For mass spectrometric studies, the zinc ion was removed by treatment with 5% (v/v) H_2SO_4/methanol (Ortiz de Montellano et al., 1979; Ortiz de Montellano & Kunze, 1980a). Resolution of the porphyrin isomers, which can only be achieved by high-pressure liquid chromatography of the metal-free porphyrins (Ortiz de Montellano et al., 1981d,e), was not possible because the metal-free porphyrins isolated in this study are irreversibly absorbed on the chromatography column. We have been unable to circumvent this unusual behavior. It is very important that the hepatic porphyrins be purified as rapidly as possible because they are particularly unstable in the presence of heme.

Acetylene, vinyl fluoride, and vinyl bromide were administered by inhalation as reported already for acetylene (Ortiz de Montellano & Kunze, 1980a). Fluroxene and ethyl vinyl ether were injected intraperitoneally without solvent in two doses of 400 μL/kg, with the second dose being given 2 h after the first. The rats were decapitated 4 h after the first dose.

Spectroscopic Characterization of the Green Porphyrins. Electronic absorption spectra were obtained in methylene chloride on a Varian Cary Model 118 instrument. Field desorption mass spectra were determined on an AEI MS-902 instrument at the Berkeley Biomedical and Environmental Mass Spectrometry Resource (Berkeley, CA). The instrument conditions used for these studies have been reported (Ortiz de Montellano & Kunze, 1980a). NMR spectra were obtained in deuterated chloroform on a Nicolet NT-360 FT NMR spectrometer at the University of California (Davis) NMR Facility. A part of this work was carried out at the Stanford HXS-360 NMR Facility. The instrumental parameters used in these studies have been reported (Ortiz de Montellano et al., 1981c). The 99.96% deuterated chloroform used in the NMR work was washed with deuterated ammonium hydroxide to remove phosgene and acids and then with deuterated water until the water remained neutral. The washed chloroform solution was stored over anhydrous K_2CO_3 until used.

Results

The ability of a number of vinyl ethers and vinyl halides to destroy cytochrome P-450 in vitro and their ability to cause the accumulation of abnormal green porphyrins in vivo were examined in order to identify substrates suitable for a subsequent study of the heme alkylation event. Vinyl fluoride, vinylidene fluoride, and fluroxene (Table I) were found to cause major losses of cytochrome P-450 when incubated with

microsomes from phenobarbital-pretreated rats in the presence of NADPH. No enzyme was lost with the indicated concentrations of these agents in the absence of NADPH (Table I). Phenyl vinyl ether, on the other hand, caused a small amount of NADPH-independent loss when present at a 10 mM concentration. A slightly higher loss was observed with phenyl vinyl ether in the presence of NADPH. None of the other vinyl ethers was found to cause detectable cytochrome P-450 loss at a 10 mM concentration, although at a 50 mM concentration vinyl acetate caused significant NADPH-dependent loss and 2-chloroethyl vinyl ether caused measurable NADPH-independent loss of cytochrome P-450. Vinyl bromide and ethyl vinyl ether exhibited no activity in the in vitro assay (Table I) either in the presence or in the absence of NADPH. The present results are consistent with previous in vitro observations on fluroxene (Ivanetich et al., 1975) and vinyl acetate (Ivanetich et al., 1978) except for the fact that vinyl acetate did not cause NADPH-independent losses in our incubation system.

A high yield of green pigment was isolated from the livers of phenobarbital-pretreated rats exposed to vinyl fluoride, vinylidene fluoride, and fluroxene. Despite their lack of measurable in vitro activity (Table I), ethyl vinyl ether and vinyl bromide were also evaluated in vivo. A green pigment was, in fact, obtained in very low yields from the livers of rats exposed to each of these two agents. The other vinyl ethers in Table I were not examined in vivo.

The green porphyrins resulting from the interaction of rat hepatic cytochrome P-450 with vinyl fluoride, fluroxene, and acetylene were extracted from the liver tissue with acidic methanol. This procedure esterifies porphyrin carboxyl groups and removes the metal ligand from N-alkylated heme derivatives (Ortiz de Montellano et al., 1981c,d). The isolated porphyrins were converted to their zinc complexes with zinc acetate before they were purifed by thin-layer chromatography. The three zinc-complexed porphyrins were found to have the same R_f value. All three porphyrins were found to be unusually unstable when compared, for example, to the porphyrin isolated from rats treated with ethylene (Ortiz de Montellano et al., 1981c). The pigments obtained with vinyl fluoride, fluroxene, and acetylene, after a second purification by thin-layer chromatography, were subjected to high-pressure liquid chromatography. The zinc-complexed porphyrin obtained with each of the three agents was found to migrate as a single asymmetric peak, with the retention times of the three samples being the same. Occasionally the zinc-complexed porphyrins were resolved on the column into more than one component. This fractionation of the porphyrin peak, however, was not reproducible and is most likely due to chemical decomposition of the porphyrins or to the presence of different counterions for the charged metal–porphyrin complex. The unusual properties of the porphyrins obtained with vinyl fluoride, fluroxene, and acetylene became most evident when attempts were made to purify them by high-pressure liquid chromatography after removal of the zinc ion. In sharp contrast to our results with other hepatic green porphyrins (Ortiz de Montellano et al., 1981c–e), the three porphyrins isolated here were found to be irreversibly retained by the chromatography column. It has consequently been necessary to carry out structural studies on porphyrins which have been purified by high-pressure liquid chromatography in the zinc-complexed, but not in the metal-free, form. This is a significant difference because we have found that the difficult separation of isomers due to alkylation of the different nitrogen atoms in protoporphyrin IX can only be accomplished by chromatography

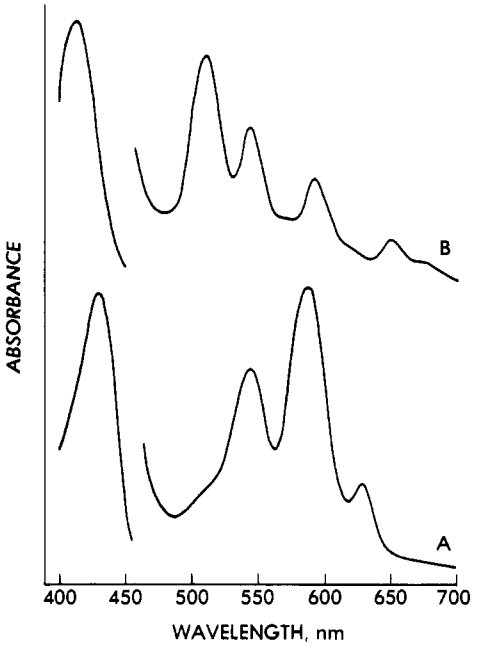

FIGURE 1: Electronic absorption spectra in CH_2Cl_2 of (A) the zinc complex of the green porphyrin isolated from fluroxene-treated rats and (B) the same porphyrin after removal of the zinc. The Soret bands are recorded at a 10-fold higher attenuation than the rest of the spectra. The same spectra are obtained with the porphyrins isolated from rats treated with vinyl fluoride, vinyl bromide, or acetylene.

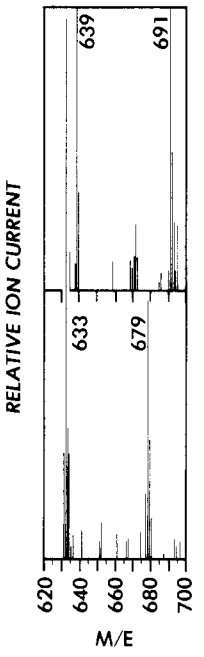

FIGURE 2: Field desorption mass spectrum of the metal-free green porphyrin isolated from fluroxene-treated rats. The upper spectrum is that obtained when the sample was treated overnight with 5% H_2SO_4 in trideuteriomethanol and was reisolated. Essentially the same mass spectrum is obtained with the porphyrins isolated from rats treated with vinyl fluoride, vinyl bromide, or acetylene. The ratio of the peaks at m/e 633 and 679, however, varies from spectrum to spectrum.

of the metal-free porphyrins (Ortiz de Montellano et al., 1981d,e).

The electronic absorption spectra of the purified green porphyrin obtained from fluroxene-treated rats, as the free base and as a zinc complex, are given in Figure 1. Exactly the same spectra are obtained with the porphyrins engendered by vinyl fluoride and acetylene (not shown). The spectra are very similar to those previously reported for *N*-alkylprotoporphyrin IX derivatives (Ortiz de Montellano et al., 1980, 1981c). The

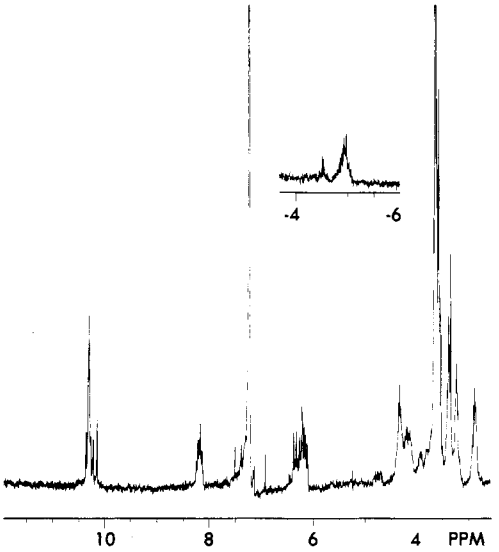

FIGURE 3: 360-MHz NMR spectrum of the zinc chloride complex of the green porphyrin isolated from rats treated with fluroxene. The spectrum was recorded in deuterated chloroform (the peak at 7.21 ppm is due to chloroform).

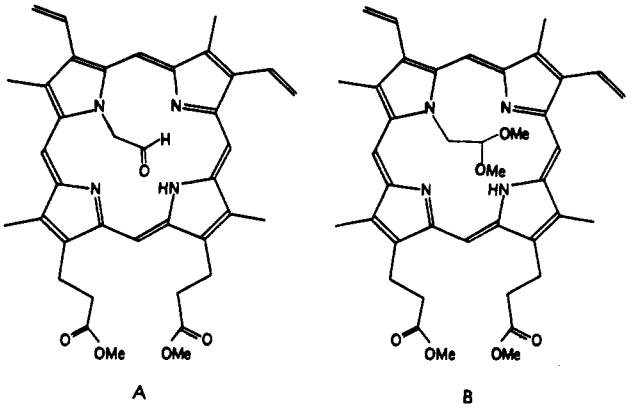

FIGURE 4: Structure of (A) one of the four possible isomers of dimethyl esterified *N*-(2-oxoethyl)protoporphyrin IX and (B) the corresponding dimethyl acetal derivative. The other three isomers are those with the alkyl group on each of the other three nitrogens of protoporphyrin IX.

field desorption mass spectrum of the green porphyrin obtained with fluroxene (Figure 2) exhibits a monoprotonated molecular ion at *m/e* 633 and a peak due to an impurity (the ratio of the two peaks is variable) at *m/e* 679. The mass spectra of the fluroxene- and acetylene-derived green porphyrins are indistinguishable from that reproduced in Figure 2 (not shown). The impurity with a peak at *m/e* 679 is also found in these latter spectra even though we have not observed its presence in the mass spectra of some two dozen related porphyrins (Ortiz de Montellano & Kunze, 1980a; Ortiz de Montellano et al., 1979, 1980, 1981d,e).

The identity in their thin-layer and high-pressure liquid chromatographic retention times, electronic absorption spectra, and mass spectrometric properties suggests that the porphyrins obtained with vinyl fluoride, fluroxene, and acetylene have the same structure. The unusual instability of the three porphyrins provides additional, if indirect, support for such a conclusion. The green porphyrin isolated from vinyl bromide treated rats, even if less extensively characterized, has also been shown to have the same electronic absorption spectra (see Figure 1), chromatographic properties, and mass spectrum (see Figure 2) as the other free porphyrins. The evidence thus suggests that vinyl bromide gives a green porphyrin with the same structure as that obtained with the other three agents.

The common structure of these porphyrins, elucidated by chemical exchange and NMR studies, has been identified as dimethyl esterified *N*-(2-oxoethyl)protoporphyrin IX. The NMR spectrum of the zinc-complexed porphyrin obtained with fluroxene (Figure 3) is essentially identical with that of the corresponding acetylene-derived pigment (not shown). The complexity of the individual signal patterns in the NMR spectrum (Figure 3) indicates that the sample is a mixture of at least two very closely related structural isomers. This is not unexpected in view of the omission, due to the instability of the porphyrin, of the chromatographic step in which isomers due to alkylation of different nitrogens are resolved. Slight differences between the spectrum of the fluroxene pigment (Figure 3) and that of the acetylene pigment (not shown) can be attributed to differences in isomer composition. The signals in the spectrum of the fluroxene adduct can be assigned to the following protons: 10.1–10.4 (4 meso protons), 8.1–8.3 (2 internal vinyl protons), 6.1–6.4 (4 terminal vinyl protons), 4.0–4.5 (4 methylene protons adjacent to the porphyrin ring), 3.4–4.0 (18 methyl group protons), multiplets at 3.4 and 2.9 (4 methylene protons adjacent to the carbonyl groups), and −5.0 ppm (2 methylene protons on an *N*-alkyl group). The peak at 7.21 ppm is due to chloroform. These assignments closely parallel the analogous assignments made in the case of the dimethyl ester of protoporphyrin IX bearing an *N*-methyl (Ortiz de Montellano et al., 1981d), *N*-(2-hydroxyethyl) (Ortiz de Montellano et al., 1981c), or *N*-(2-oxopropyl) (Ortiz de Montellano & Kunze, 1981a) group. These peak assignments account for all of the protons expected from incorporation of protoporphyrin IX (from heme) into the green porphyrin. Subtraction of the molecular weights of the parent porphyrin and of the *N*-methylene moiety indicated by the signal at −5.0 ppm from the molecular weight of the green porphyrin only leaves 29 mass units unaccounted for. This is equal to the molecular weight of an aldehyde group. The structure of the green porphyrin thus corresponds to *N*-(2-oxoethyl)protoporphyrin IX (as the dimethyl ester). One of the four possible structural isomers of this porphyrin is shown in Figure 4A, with the other possible isomers being those in which the other ring nitrogens are alkylated. Two lines of evidence substantiate this structural formulation. The treatment of aldehydes with acidic methanol results in their equilibration with dimethyl acetals (Schmitz & Eichorn, 1967). Conversion of the aldehyde group in the proposed structure to a dimethyl acetal moiety during the acidic methanol extraction would yield a porphyrin which would give a monoprotonated molecular ion in the mass spectrometer at *m/e* 679. The presence of a contaminating porphyrin with precisely this molecular ion (Figure 2) can thus be rationalized by conversion of the structure in Figure 4A to that in Figure 4B. The suggested relationship between the structures with monoprotonated molecular ions at *m/e* 633 and 679 is confirmed by the changes observed when the sample used for the spectrum in Figure 2 was allowed to stand overnight in 1,1,1-trideuteriomethanol containing 5% H_2SO_4. The reisolated sample gave a mass spectrum with two salient peaks, one at *m/e* 639 and the other at *m/e* 691 (Figure 2). The shift of the monoprotonated molecular ion of the parent green porphyrin from *m/e* 633 to *m/e* 639 is consistent with transesterification of the two carboxymethyl groups to the corresponding trideuterated structures. A similar shift has been observed in a related case (Ortiz de Montellano et al., 1979). More to the point is the shift of the peak at *m/e* 679 in the original sample to *m/e* 691. This requires the replacement

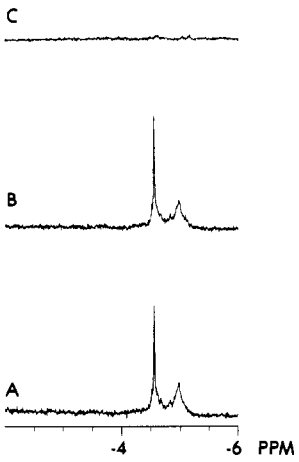

FIGURE 5: Exchange of the high-field protons in the spectrum of a sample of zinc-complexed green porphyrin isolated from acetylene-treated rats: (A) original 360-MHz NMR spectrum in deuterated chloroform; (B) spectrum after addition of 99.9% deuterated water; (C) spectrum after the further addition of a trace of deuterated trifluoroacetic acid.

of *four* methoxy groups by the corresponding trideuterated moieties. Two of the exchanged groups, as in the parent system, must be the methoxy groups in the esterified propionic acid side chains. The other two, however, can only be reasonably explained if a dimethyl acetal function in the derivatized porphyrin (Figure 4B) is converted by exchange to the analogous di(trideuteriomethyl) acetal.

A second exchange experiment was used to confirm the location of the carbonyl group on the *N*-alkyl substituent. This was considered necessary because the aldehyde proton signal, presumably located in the NMR in the congested region between 3 and 5 ppm, has not been specifically identified. A small signal is found, however, at approximately −4.5 ppm (Figure 3). This signal, which varies in intensity relative to that of the *N*-methylene group at −5 ppm, can be attributed to the *N*-methylene protons in the dimethyl acetal derivative. This is consistent with the finding, obtained by integration of the NMR signals, that the sum of the signals at −4.5 and −5.0 ppm is required to account for the two methylene protons. We have found in earlier studies that the *N*-alkyl protons in structures such as zinc-complexed *N*-methylprotoporphyrin IX do not exchange into deuterated water even in the presence of trifluoroacetic acid. Due to activation by the carbonyl group, however, the *N*-alkyl group protons in related *N*-(2-oxoalkyl)porphyrins do exchange in the presence of deuterated water and an acid catalyst (P. R. Ortiz de Montellano and H. S. Beilan, unpublished observations). An exchange experiment has therefore been carried out with a sample of the green porphyrin isolated from acetylene-treated rats (Figure 5). The difference in the ratio of the aldehyde to the dimethyl acetal in this sample compared to that in Figure 3, as revealed by the difference in the intensities of the peaks at −4.5 and −5.0 ppm, is to be noted. Addition of a small amount of deuterated water to the sample in the NMR tube causes no change in the spectrum (Figure 5A, B). However, the further addition of a trace of deuterated trifluoroacetic acid (Figure 5C) results in rapid disappearance of both high field signals. Since exchange of the *N*-methylene protons requires the presence of a vicinal carbonyl group, loss of the *N*-methylene protons in the acetal derivative must follow initial hydrolysis of the acetal moiety.

The combined structural and chemical results identify the porphyrin formed with fluroxene, vinyl fluoride, acetylene, and probably vinyl bromide as *N*-(2-oxoethyl)protoporphyrin IX (Figure 4A). Treatment with acidic methanol during the isolation procedure esterifies the carboxyl groups, presumably displaces the iron ligand, and results in partial conversion of the aldehyde group to a dimethyl acetal. The presence of an aldehyde group readily accounts for the unique instability of the porphyrin isomers. Although it has been possible to determine the structure of the porphyrin, we cannot, at this time, determine the proportion of the four possible isomers in the biological samples.

Discussion

Vinyl fluoride, like ethylene (Ortiz de Montellano & Mico, 1980; Ortiz de Montellano et al., 1981c), mediates the in vitro NADPH-dependent loss of cytochrome P-450 and the in vivo accumulation of a heme-derived green pigment (Table I). Vinyl bromide, in contrast, causes no detectable in vitro loss of cytochrome P-450 and gives only a very low yield of hepatic green pigment (Table I). Vinyl chloride, the analogue with the halogen which lies between fluorine and bromine in the periodic table, has been shown by others to destroy hepatic cytochrome P-450 (Guengerich et al., 1979; Guengerich & Strickland, 1977; Ivanetich et al., 1977). No evidence is available, however, on whether a green pigment is also formed with vinyl chloride. Studies of the rate of metabolism of vinyl halides in uninduced rats have shown that their zero-order rate (V_{max}) of elimination decreases in the sequence vinyl chloride > vinyl bromide > vinyl fluoride (Filser & Bolt, 1979). In fact, vinylidene fluoride, which is shown here to destroy cytochrome P-450 and to give in vivo a good yield of green pigment (Table I), is reported to be among the most slowly metabolized of the vinyl halides (Filser & Bolt, 1979). These results suggest the absence of a direct relationship between rate of metabolism, destruction of cytochrome P-450, and green pigment formation, although a formal correlation of these parameters is not possible because the former were determined in uninduced rats and the latter in phenobarbital-pretreated rats. Among the vinyl ethers, fluroxene, an anesthetic already known to destroy cytochrome P-450 (Ivanetich et al., 1975, 1976), was the only one which efficiently caused enzyme loss and green pigment formation (Table I). At higher (50 mM) concentrations, some of the ethers caused NADPH-independent loss of cytochrome P-450. This turnover-independent loss of the enzyme, analogous to that observed previously with 1-heptene and 1-heptane (Ortiz de Montellano & Mico, 1980), probably reflects disruption of the microsomal membrane by the elevated substrate concentrations. It is likely, however, that some of the vinyl ethers in Table I which appear to be inactive in vitro can in fact mediate a low level of NADPH-dependent enzyme inactivation in vivo. Indirect evidence for this is provided by the isolation of a small amount of green pigment from livers of rats treated with ethyl vinyl ether and vinyl bromide, neither of which gave measurable destruction of the enzyme in vitro (Table I). The results summarized in Table I led to the use of vinyl fluoride and fluroxene for the more detailed structural and mechanistic studies described in this paper.

The green pigments formed in the livers of rats treated with vinyl fluoride, vinyl bromide, fluroxene, and acetylene have been isolated and characterized. Acetylene was included in the study because an analysis of the probable structures of the green pigments obtainable with the three substituted olefins suggested that the same structure might be obtained with acetylene. This projection has, in fact, been confirmed. The green porphyrins obtained with vinyl fluoride, vinyl bromide, fluroxene, and acetylene have the same chromatographic, physical, electronic absorption (Figure 1), and mass spectro-

metric (Figure 2) properties. These include an unusual instability, irreversible retention of the free-base forms on the HPLC column, and the presence of a closely related contaminant with a protonated molecular ion at m/e 679. This contaminant has been shown by an exchange experiment in trideuterated methanol to be an artifact produced from the parent porphyrin during its extraction from the liver with acidic methanol (Figure 2). Large-scale experiments have led to the isolation of the green porphyrins obtained with fluroxene and acetylene in amounts sufficient for NMR spectra to be obtained (Figure 3). In addition to confirming the identity of the two isolated porphyrins, the NMR spectra have provided the information necessary to define their common structure. Because of the instability of the free-base form of the porphyrin to the chromatographic procedure, the NMR spectra obtained are those of a mixture of at least two closely related isomers. It has nevertheless been possible to locate all the protons expected from the incorporation of protoporphyrin IX into the structure and to identify the presence of an *N*-alkyl substituent. The *N*-alkyl group has been shown to be a 2-oxoethyl moiety by the presence of a high-field (−5.0 ppm) 2-proton signal which is lost due to acid-catalyzed exchange of the protons in the presence of deuterated water (Figure 5). This requires a carbonyl next to the methylene at high field. The conversion of the carbonyl to a dimethyl acetal in acidic methanol, yielding the derivative with a monoprotonated molecular ion at m/e 679, provides further confirmation of this formulation. The structure of the porphyrin isolated from fluroxene, vinyl fluoride, vinyl bromide, or acetylene treated rats is consequently shown to be *N*-(2-oxoethyl)protoporphyrin IX (as the dimethyl ester) (Figure 4A).

The reaction of prosthetic heme with catalytically activated acetylene to give *N*-(2-oxoethyl)heme, the presumed precursor of the isolated porphyrin, is consistent with the isolation of *N*-(2-oxopropyl)protoporphyrin IX from propyne-treated rats (Ortiz de Montellano & Kunze, 1981a) and with the mechanism we originally proposed for the destructive interaction of acetylenes with cytochrome P-450 (Ortiz de Montellano & Kunze, 1980a, 1981a). The central feature of this mechanism is that catalytic oxygen transfer to the acetylene π bond produces a transient species which can partition into two pathways, one leading to alkylation of the prosthetic heme moiety and the other to formation of acetylene group metabolites. The predicted outcome of such a reaction sequence in the case of acetylene is the formation of *N*-(2-oxoethyl)heme (Figure 6). The electronic nature of the reactive species which alkylates the heme group, however, cannot be inferred from the structure of the isolated adduct. If reaction of the π bond with the activated oxygen is a two-electron process, the alkylating species can be envisioned as an oxygen-substituted vinyl cation (Figure 6, path a) or as an α-oxocarbene (Figure 6, path b). The α-oxocarbene could also be coordinated to the iron through the carbene rather than the oxygen prior to reaction with the porphyrin nitrogen. If the initial reaction of the enzyme-activated oxygen with the π bond is a one electron process, the alkylating species is most reasonably represented by a vinyl radical (Figure 6, path c). Each of the three intermediates represented in Figure 6 not only is a plausible alkylating agent but also is a viable precursor of the ketene products which have recently been demonstrated to result from enzymatic oxidation of the acetylene function (Ortiz de Montellano & Kunze, 1980c, 1981b). This follows from the fact that the α-ketocarbene, the 2-oxovinyl cation, and the ketene are all part of a reaction manifold which also includes the unsaturated epoxide (oxirene) (Tanaka & Yoshimine, 1980; Strausz et al., 1976). Since the radical intermediate (path c) can close to give the unsaturated epoxide, it can also enter the same reaction manifold. The structure of the acetylene adduct thus outlines the features of the alkylation sequence without providing information on its detailed electronic properties.

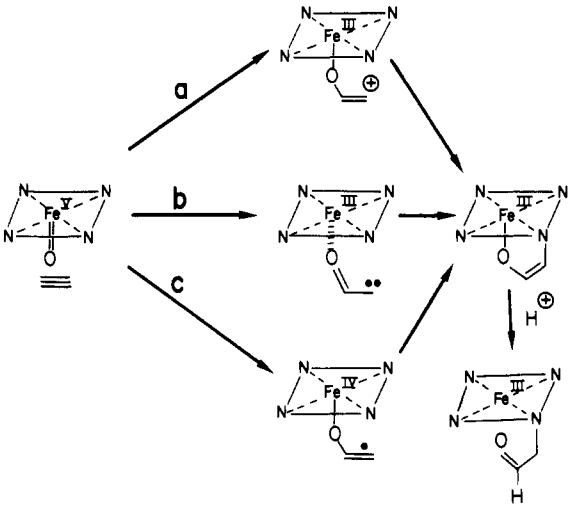

FIGURE 6: Alternative mechanisms for alkylation of the prosthetic heme during oxidation of acetylene by cytochrome P-450. A cytochrome P-450 iron–oxene complex is assumed as the activated oxygen species. The prosthetic heme is represented in the mechanisms by an iron and a nitrogen in a square. Formal iron oxidation states are given for electron counting purposes—actual oxidation states will depend on electron delocalization.

A much more sensitive probe of the electronic nature of the species which alkylates prosthetic heme is provided by the finding that the porphyrin formed in the catalytic reaction with fluroxene and vinyl fluoride is also *N*-(2-oxoethyl)protoporphyrin IX. In view of the formation of *N*-(2-hydroxyethyl)protoporphyrin IX in the interaction of the enzyme with ethylene (Ortiz de Montellano et al., 1981c), it is clear that the aldehyde is formed by elimination of a halide (from vinyl fluoride or bromide) or of a trifluoroethoxy (from fluroxene) moiety from an initial structure bearing both the enzymatically introduced oxygen and the substituent on the same carbon atom (Figure 7). The elimination of the fluoride or trifluoroethoxy group could occur in vivo or during the isolation procedure, although elimination in vivo would seem to be favored. The important point, however, is that the oxygen is added to the *substituted* end of the π bond. The unsubstituted carbon of the substrate, either as a radical or as a cation, is therefore responsible for the actual alkylation of the prosthetic heme. In order to explore the significance of this reaction orientation, it is first necessary to review, as with acetylene, the available alternatives.

An oxene–iron complex is now generally presumed, if not yet unequivocally proven, to be the reactive species produced by the catalytic action of cytochrome P-450 (White & Coon, 1980). The distribution of electrons between the oxygen, the iron atom, and the porphyrin ring in the catalytically formed species, however, is not known. Depending on the distribution of electrons, the oxygen can be expected to act as a one- or a two-electron acceptor. Two limiting mechanisms can be envisioned for heme alkylation by an olefin if two-electron processes are considered. One is a concerted sequence with no energy minima in the reaction trajectory, and the other is the formation of a cationic intermediate which, in a distinct step, reacts with the heme moiety (Figure 7, path a). Two one-electron mechanisms can also be formulated. In one of them, a carbon radical is formed by an unpaired electron

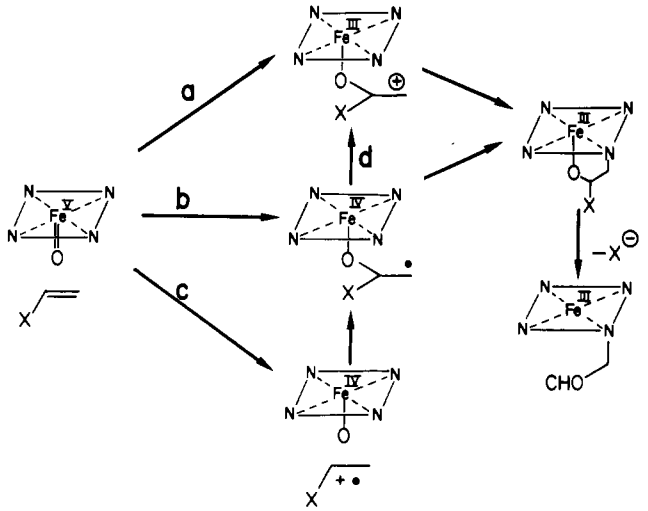

FIGURE 7: Alternative mechanisms for alkylation of the cytochrome P-450 prosthetic heme during catalytic turnover of olefinic substrates. The prosthetic heme is represented by an iron and a nitrogen in a square. The assumptions noted in the legend of Figure 6 also apply here. The orientation of the olefin shown in the addition reaction is that which leads to the isolated heme adduct and not that which is necessarily electronically favored.

reaction of the oxygen with the π bond (Figure 7, path b). In the second a similar radical is formed, except that it results from initial transfer of an electron from the π bond to the oxygen followed by collapse of the resulting radical cation–radical anion pair (Figure 7, path c). The possibility exists, of course, that a free radical produced via path b or c will be converted to the cation intermediate of path a by transfer of the electron from the carbon to the porphyrin (Figure 7, path d). It is also possible that the initially formed radical cation reacts with the porphyrin nitrogen before combining with the oxygen or that the two reactions are concerted. Most important in the present context is the fact that the favored orientation for the reaction of a substituted π bond with the heme depends on the electronic and steric properties of the substituent. The preferred orientation is not the same for the mechanistic alternatives in Figure 7.

Electrophilic reaction with a π bond substituted at one end by a π-electron donor strongly favors the reaction orientation which leads to localization of the positive charge on the carbon adjacent to the electron-donating substituent rather than on the distal (unstabilized) position. The chemistry of enol ethers is, in fact, dominated by the electron-donating interaction of the oxygen atom with the π bond (Fischer, 1980; Effenberger, 1969). One estimate of the energy difference between intermediates with the positive charge localized on the carbon vicinal or distal to the oxygen is provided by the relative stability of a methoxymethyl cation compared to an ethyl cation. Gas phase measurements have been used to determine that an ethyl cation is stabilized by 37 ± 3 kcal/mol relative to a methyl cation, whereas a methoxymethyl cation is stabilized by 66 ± 3 kcal/mol (Taft et al., 1965; Martin et al., 1966). The higher stabilization of the methoxymethyl cation by 29 kcal/mol cannot, of course, be directly translated into a solution energy difference. The gas phase data, however, provide some measure of the solution energy difference which lies behind the high reactivity and regiospecificity characteristic of enol ether reactions (Fischer, 1980). Fluorine, because of its higher electronegativity, is less effective than oxygen in the stabilization of vicinal cations. A fluoromethyl cation is nevertheless energetically more accessible in the gas phase than a methyl cation (Franklin, 1968). The regiospecificity of vinyl fluoride reactions with electrophiles confirms that, in solution, the localization of the positive charge on the fluorine-substituted carbon is also preferred (Chambers & Mobbs, 1965). Reaction of electrophilic enzymatically activated oxygen with fluroxene and vinyl fluoride is thus expected, on electronic grounds, to favor the reaction orientation *opposite* to that required (Figure 7, path a) for adduct formation. The difference between the two reaction orientations should be particularly overwhelming in the case of fluroxene, although specific binding of the trifluoroethoxy moiety could in principle attenuate the electronic preference for reaction of the activated oxygen with the terminal (unsubstituted) carbon of the vinyl ether function. A specific binding or steric effect cannot be invoked, however, in the case of vinyl fluoride due to the approximate parity in the size of a hydrogen and a fluorine (van der Waals radii of 1.10 and 1.35 Å, respectively). It is important to note that the reaction pathway which leads to adduct formation requires reaction of the oxygen with the more hindered as well as less electronically favored position. The present results thus provide no support for a two-electron mechanism such as that presented in path a of Figure 7. They also argue against a concerted reaction process since any transition state polarization in such a mechanism, assuming that reaction of the π bond with the activated oxygen drives the process, would also disfavor the observed alkylation regiospecificity.

Carbon free radicals are stabilized, like cations, by the presence of vicinal electron-donating substituents. This has been clearly established for vinyl fluoride by extensive studies of the orientation of addition of a variety of radicals (Tedder & Walton, 1976). All of the radicals preferentially reacted with the unsubstituted end of vinyl fluoride, with the ratio of addition to the unsubstituted vs. substituted carbons ranging from a minimum of 3.3 to a maximum of 50. The addition of perhaloalkyl radicals to fluroxene has also been shown to occur at the unsubstituted π-bond terminus due to stabilization of the ensuing radical by the oxygen atom (Tarrant & Stump, 1964). A radical pathway such as that in path b (Figure 7) thus also favors the reaction orientation which does not lead to the isolated adduct, although the preference for reaction with the unsubstituted end is expected to be much smaller for the one-electron than for the two-electron pathway. The magnitude of the reaction difference is important since a disfavored but still quantitatively significant reaction could account for the observed adduct. A lower limit on the fraction of catalytic events which must result in heme alkylation can be estimated from the molar ratio of metabolite formation to enzyme inactivation. A value of 296 has been reported for the ratio of trifluoroethanol liberated from fluroxene vs. cytochrome P-450 inactivated by using phenobarbital-pretreated microsomes (Marsh et al., 1977). This partition ratio is similar to that (230–300) established for the reaction of 2-isopropyl-4-pentenamide with cytochrome P-450 (Ortiz de Montellano & Mico, 1981; Ortiz de Montellano et al., 1981a). Partition ratios for enzyme inactivation by vinyl fluoride and acetylene are not available. It is nevertheless evident that a radical mechanism can account for the observed inactivation. If, for example, only every fiftieth substrate turned over resulted in a terminal radical, only approximately one of five or six such radicals would have to react with heme in order to satisfy the limit defined by the partition ratio.

Carbon–oxygen bond formation can be envisioned to occur not by a direct reaction, as in path b (Figure 7), but by a process in which a π-bond electron is first transferred to the heme–oxygen complex (Figure 7, path c). The ability of enol ethers to form charge-transfer complexes (Kosower, 1965) is

consistent with such a mechanism. The particular advantage of such a mechanism, in which an initially formed radical ion pair collapses to a radical intermediate, is that it is the only process in which the orientation required to explain the observed heme adduct can actually be preferred. Localization of positive charge on the carbon adjacent to the heteroatom in the radical cation would, for example, favor reaction of this atom with the oxyheme radical anion. Insufficient data are available, however, to discriminate between reaction pathways b and c in Figure 7. The large difference in reactivity of the two ends of the π system in two-electron reactions, on the other hand, makes the process shown in path a (Figure 7) an unlikely one.

The probable intervention of a free-radical intermediate in the alkylation of prosthetic heme by olefinic substrates is of relevance to the catalytic mechanism of cytochrome P-450. The suicidal nature of cytochrome P-450 inactivation by olefins (Ortiz de Montellano & Mico, 1981) implies that a common catalytic pathway results in both heme alkylation and epoxide formation. The point at which the reaction process is committed to one of these two outcomes and the factors which govern the partition between them remain obscure. Although it is possible that epoxide formation diverges from heme alkylation at a very early stage and that a radical intervenes only in the latter, it is more likely that epoxide formation and heme alkylation are alternative fates for a common intermediate. If so, the evidence which suggests that heme alkylation is a free-radical process suggests that epoxidation is also a free-radical event.

One important ambiguity must be considered in order to place the present results in a balanced context. Although no evidence was found for the presence of a heme adduct other than that in Figure 4, the possibility remains that the adduct which would result from addition of oxygen to the unsubstituted end and of nitrogen to the substituted end of the vinyl substrate is not isolable by our procedures. The resulting adduct, in the case of vinyl fluoride, would bear an *N*-(2-hydroxy-1-fluoroethyl) substituent. The only porphyrin model known in which the *N*-alkyl group bears a halide on the nitrogen-bound carbon is cobalt complexed *N*-[chloro(ethoxycarbonyl)methyl]octaethylporphyrin (Johnson et al., 1975). On treatment with acidic ethanol, a condition which resembles our workup procedure, the chloride is displaced by a second nitrogen of the porphyrin. An N,N-bridged porphyrin is thus obtained. If a similar reaction occurred with an initially formed vinyl fluoride or fluroxene adduct, the resulting N,N-bridged product would probably have been detected. We have recently described the isolation of a two-carbon bridged N,N-dialkylated heme adduct by a procedure similar to that used in this work (Ortiz de Montellano & Mathews, 1981). Nevertheless, in the absence of an authentic sample, it is not possible to exclude the formation of an unstable (undetected) adduct. The fraction of heme alkylation events which yield the isolated adduct (Figure 4), even if clearly significant, cannot therefore be precisely defined.

Groves et al. (1978) have provided evidence that carbon hydroxylation by cytochrome P-450 is a nonconcerted, probably free-radical, process. The formation of radical intermediates in the nonphysiological, hydroperoxide-driven, reactions catalyzed by cytochrome P-450 has also received impressive support (Griffin et al., 1980; White et al., 1980). More closely related to the present study is the suggestion that polycyclic aromatic hydrocarbons may be metabolized in the presence of hydroperoxide by a radical pathway (Renneberg et al., 1981). The present results, however, provide the first evidence for the probable involvement of radical intermediates in the epoxidation of olefins by cytochrome P-450.

Acknowledgments

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Purification and Properties of a Neutral Endodeoxyribonuclease from Rat Small Intestinal Mucosa†

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ABSTRACT: An endodeoxyribonuclease has been purified to near homogeneity from rat small intestinal mucosa by a procedure involving Con A–Sepharose affinity chromatography. During the initial steps of purification, the presence of 5 mM $CaCl_2$ was essential for stability of the enzyme activity. The enzyme has a molecular weight of 32 000 and an isoelectric point of 4.7. NaCl, sulfhydryl reagents, and iodoacetate strongly inhibited the reaction, but tRNA did not. The enzyme required divalent cations for activity and had a pH optimum of pH 6.2 with Co^{2+} and pH 7.7 with Mn^{2+}. In both optimum conditions, the enzyme hydrolyzed native DNA more rapidly than denatured DNA, and the average chain lengths of limit digestion products of native and denatured DNA were 8 and 10, respectively, at pH 6.2 and 9 and 11, respectively, at pH 7.7. The enzyme activity to produce acid-soluble fractions from linear DNA substrate was similar in the two optimum conditions, but the activity to nick double-stranded, superhelical circular DNA substrate was significantly higher at pH 6.2 than at pH 7.7. The endonuclease formed single-strand breaks making 5′-phosphoryl and 3′-hydroxyl termini, and deoxythymidine was present at the 5′ termini with a frequency of about 50% in both optimum conditions. Bovine pancreatic DNase I antibody and G-actin inhibited the enzyme activity. Thus this endonuclease is classified as a DNase I.

Pancreatic DNase[1] I, which was first crystallized by Kunitz (1950), is one of the two representative mammalian DNases and has been widely investigated (Laskowski, 1967). Similar activity was found in guinea pig epidermis (Tabachinick, 1964), nuclei of liver cells (Ishida et al., 1974), and other organs (Tanigawa et al., 1974), but the activity does not seem so widely distributed as that of DNase II. The presence of relatively high DNase I like activity in the small intestinal mucosa of rats has been reported (Lee et al., 1972), but the structural identity of the enzyme responsible for this activity with pancreatic DNase I has not been established.

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[1] Abbreviations: DNase, deoxyribonuclease; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

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