9. Huntington's disease: J. F. Gusella *et al.*, *Nature (London)* **306**, 234 (1983); J. F. Gusella *et al.*, *Science* **225**, 1320 (1984). Polycystic kidney disease: S. T. Reeders *et al.*, *Nature (London)* **317**, 542 (1985). Cystic fibrosis: L. C. Tsui *et al.*, *Science* **230**, 1054 (1985); R. G. Knowlton *et al.*, *Nature (London)* **318**, 380 (1985); R. L. White *et al.*, *ibid.*, p. 382; B. Wainwright *et al.*, *ibid.*, p. 384. X-linked disorders: P. Goodfellow, P. K. Davies, H. H. Ropers, *Cytogenet. Cell Genet.* **40** (*Human Gene Mapping 8*), 296 (1985).
10. E. Neibuhr, *Humangenetik* **21**, 99 (1974); J. Williams, R. L. Summitt, P. R. Martens, R. A. Kimbrell, *Am. J. Hum. Genet.* **27**, 478 (1975); A. Hagemeijer and E. M. E. Smit, *Hum. Genet.* **38**, 15 (1977); R. A. Pfeiffer, E. K. Kessel, K.-H. Soer, *Clin. Genet.* **11**, 207 (1977); S. Kitsiou-Tzeli, J. Hallett, L. Atkins, S. Latt, L. B. Holmes, *Am. J. Med. Genet.* **18**, 725 (1984).
11. Informed consent was obtained from all family members based on a reviewed and approved protocol.
12. G. McKhann *et al.*, *Neurology* **34**, 939 (1984); Z. S. Khachaturian, *Arch. Neurol.* **42**, 1097 (1985).
13. C. Eisdorfer and D. Cohen, *J. Fam. Pract.* **11**, 553 (1980).
14. J. P. Pro, C. H. Smith, S. M. Sumi, *Neurology* **30**, 820 (1980).
15. W. A. Hauser, N. L. Morris, L. L. Heston, V. E. Anderson, *ibid.* **36**, 1226 (1986).
16. P. C. Watkins *et al.*, *Nucleic Acids Res.* **13**, 6075 (1985); G. D. Stewart, P. Harris, J. Galt, M. A. Ferguson-Smith, *ibid.*, p. 4125; P. C. Watkins, P. A. Watkins, N. Hoffman, P. Stanislovitis, *Cytogenet. Cell Genet.* **40** (*Human Gene Mapping 8*), 773 (1985).
17. R. E. Tanzi *et al.*, *Cytogenet. Cell Genet.* **40** (*Human Gene Mapping 8*), 760 (1985); R. E. Tanzi *et al.*, in preparation.
18. S. Kittur *et al.*, *EMBO J.* **4**, 2257 (1985).
19. M. L. Van Keuren *et al.*, *Am. J. Hum. Genet.* **38**, 793 (1986); R. L. Neve *et al.*, *Gene*, in press.
20. M. Munke *et al.*, *Cytogenet. Cell Genet.* **40** (*Human Gene Mapping 8*), 706 (1985).
21. J. Ott, *Am. J. Hum. Genet.* **26**, 588 (1974); *ibid.* **28**, 528 (1976).
22. S. E. Hodge, L. A. Morton, S. Tidemam, K. K. Kidd, M. A. Spence, *ibid.* **31**, 761 (1979).
23. G. M. Lathrop, J. M. Lalouel, C. Julier, J. Ott, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3443 (1984); G. M. Lathrop and J. M. Lalouel, *Am. J. Hum. Genet.* **36**, 460 (1984); ———, C. Julier, J. Ott, *ibid.* **37**, 482 (1985).
24. G. G. Glenner and C. W. Wong, *Biochem. Biophys. Res. Commun.* **120**, 885 (1984); C. L. Masters *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4245 (1985); C. L. Masters *et al.*, *EMBO J.* **4**, 2757 (1985).
25. R. E. Tanzi *et al.*, *Science* **235**, 880 (1987).
26. A. P. Monaco *et al.*, *Nature (London)* **323**, 646 (1986).
27. M. A. Anderson and J. F. Gusella, *In Vitro* **20**, 856 (1984).
28. A. P. Feinberg and B. Vogelstein, *Anal. Biochem.* **137**, 266 (1983).
29. Supported by NIH grants ADRC P50 AGO5134, NS22031, and NS20012, by the McKnight Foundation, by the Julieanne Dorn Fund for Neurological Research, and by a generous donation from J. Freudenberger. P.St G.-H. is a Medical Research Council of Canada Fellow. J.L.H. is supported by PHS postdoctoral training grant T32DE07043. A.C.B. is a John Douglas French Foundation Fellow. L.A., S.S., and S.P. are supported by the Italian National Research Council. G.D.S. received a fellowship from the Wills Foundation. J.-F.F. received support from CNRS ATP96031 and Inserm Reseau 486011. J.F.G. is a Searle Scholar of the Chicago Community Trust. We thank T. Bird and E. Bryant for supplying two additional cell lines from FAD3. and C. Altshuler, B. Williamson, and D. O'Sullivan for assistance in pedigree collection.

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Cytochrome P-450–Catalyzed Formation of Δ^4-VPA, a Toxic Metabolite of Valproic Acid

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Liver damage induced by the antiepileptic drug valproic acid (VPA) is believed to be mediated by an unsaturated metabolite of the drug, Δ^4-VPA. In studies of the biological origin of this hepatotoxic compound, it was found that liver microsomes from phenobarbital-treated rats catalyzed the desaturation of VPA to Δ^4-VPA. Indirect evidence suggested that cytochrome P-450 was the responsible enzyme, a conclusion that was verified by studies with a purified and reconstituted form of the hemoprotein, which catalyzed the oxidation of VPA to 4- and 5-hydroxyvalproic acid and to Δ^4-VPA. Desaturation of a nonactivated alkyl substituent represents a novel metabolic function of cytochrome P-450 and probably proceeds via the conversion of substrate to a transient free radical intermediate, which partitions between recombination (alcohol formation) and elimination (olefin production) pathways. These findings have broad implications with respect to the metabolic generation of olefins and may explain the increased hepatotoxic potential of VPA when it is administered in combination with potent enzyme-inducing anticonvulsants such as phenobarbital.

VALPROIC ACID (VPA, FIG. 1) IS A simple, branched-chain fatty acid that has broad-spectrum anticonvulsant activity and is used in the treatment of multiple forms of epilepsy (*1*). Although VPA was initially considered to be relatively free from serious side effects, there is recent clinical evidence that small numbers of patients develop serious liver damage while maintained on VPA therapy (*2*). Indeed, this hepatic injury may prove fatal, and estimates of the frequency of VPA-related patient deaths have ranged from 1 in 37,000 to 1 in 500, depending on factors such as the age of the recipient and the nature of concomitant therapy (*3*). Although the mechanism of this liver toxicity remains unknown, animal studies have implicated 2-*n*-propyl-4-pentenoic acid (Δ^4-VPA), an unsaturated metabolite of the drug (Fig. 1), as a causative agent (*2*). Thus, Δ^4-VPA was found to be the most toxic metabolite of VPA in rat hepatocytes in vitro (*4*), to inhibit both hepatic cytochrome P-450 (*5*) and fatty acid β-oxidation activity (*6*) in vitro, and to be a potent inducer in vivo of hepatic microvesicular steatosis (the characteristic tissue lesion in VPA-induced liver injury) (*7*).

Typically, Δ^4-VPA is present at low concentrations in biological fluids from humans and animals given VPA (*8*), although very high levels of this metabolite were detected in a child who died from VPA-induced liver failure (*9*). Studies in the rhesus monkey have shown that the fraction of an intravenous dose of VPA that is metabolized to the Δ^4 olefin may be as high as 3 to 4% (*10*), and thus the quantitative significance of the desaturation pathway leading to this toxic product may be greater than suspected previously. Despite the interest in Δ^4-VPA, however, details of the metabolic process by which the parent drug is transformed to this unsaturated product are unclear. One early hypothesis was that Δ^4-VPA is formed by loss of the elements of water from 4-OH-VPA or 5-OH-VPA (*11*). This possibility is unlikely, since control experiments have shown that these alcohols are chemically (*8*) and metabolically (*12*) stable entities that do not undergo dehydration reactions. The metabolic origin of Δ^4-VPA, therefore, remains obscure.

In studies with rats, Lewis *et al.* (*13*) reported that the incidence of VPA-induced liver injury was increased in animals treated with phenobarbital. Significantly, many of the human subjects who developed serious hepatotoxicity during VPA therapy had been treated concomitantly with enzyme-inducing drugs such as phenobarbital or phenytoin (*2*). Since these agents have a profound influence on microsomal enzymes in general, and on the cytochrome P-450 system in particular (*14*), we investigated whether Δ^4-VPA was formed in hepatic microsomes from phenobarbital-treated rats (*15*).

Metabolites of VPA formed in microsomal incubations were isolated, converted to

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trimethylsilyl (TMS) derivatives, and analyzed by selected ion-monitoring gas chromatography–mass spectrometry (GC-MS) (*16*). As shown in Fig. 2, Δ^4-VPA was indeed detected in such extracts, where it was accompanied by a second unsaturated metabolite of VPA, identified tentatively as the Δ^3 olefin. However, neither of these metabolites was formed in microsomal incubations that lacked nicotinamide adenine dinucleotide phosphate (NADPH), the necessary cofactor for mixed-function oxidase activity (Fig. 2B).

Having established that Δ^4-VPA was produced from VPA by rat liver microsomal preparations, we next determined the role of cytochrome P-450 in the desaturation reaction by performing experiments with inhibitors (and an inducer) of this enzyme. Three hydroxylated metabolites of VPA, namely, 3-, 4-, and 5-OH-VPA, were included in the analytical protocol in order to determine whether the formation of Δ^4-VPA in microsomes paralleled the production of these known (*17*) cytochrome P-450–derived products. The results of these studies (Table 1) showed that the formation of Δ^4-VPA (and each of the hydroxylated metabolites) was induced by phenobarbital, and was abolished either by denaturing the microsomal protein by heating or by omitting cofactors from the incubation media. Furthermore, generation of Δ^4-VPA was blocked by carbon monoxide and by metyrapone (an inhibitor of cytochrome P-450) and was also inhibited when air was excluded from the system (argon atmosphere). These results suggested that the formation of Δ^4-VPA from VPA was a consequence of cytochrome P-450 catalysis.

Although the cytochrome P-450–mixed-function oxidase system is known to mediate a wide variety of metabolic reactions (*18*), including dehydrogenation of heteroatomic substrates such as acetaminophen (*19*) and 4-aryl-1,4-dihydropyridine derivatives (*20*), only one report has appeared on P-450–catalyzed desaturation of an alkyl substituent to yield the corresponding alkene, namely, the conversion of testosterone to 17β-hydroxy-4,6-androstadien-3-one (*21*). In contrast to the situation with testosterone, however, desaturation of VPA takes place at a nonactivated position, which suggests that the reaction has wide applicability and may occur with a broad spectrum of aliphatic substrates. In view of the novel nature of this process, therefore, we conducted further metabolic experiments using a purified and reconstituted preparation of cytochrome P-450 (*22*). The major phenobarbital-inducible form of the enzyme from rat liver was used in these studies, and the products of incubation of VPA were analyzed, as before, by GC-MS. As indicated in Table 2, incubation of VPA with the purified enzyme confirmed that the formation of Δ^4-VPA was cytochrome P-450–dependent and showed that 4-OH- and 5-OH-VPA (but not 3-OH-VPA) were also produced by this isoenzyme. As expected, no metabolites were detected when incubations were performed in the absence of either NADPH–cytochrome P-450 reductase or the cytochrome P-450 itself.

In additional experiments, we incubated 4-OH- and 5-OH-VPA (at concentrations up to 1 m*M*) with hepatic microsomes from phenobarbital-treated rats to verify that Δ^4-VPA was not formed as an artifact by dehy-

Table 1. Effect of modifiers on the microsomal metabolism of VPA. Values cited are means ± SEM of the rates of formation of Δ^4-VPA, 3-OH-VPA, 4-OH-VPA, and 5-OH-VPA, expressed in picomoles of product per nanomole of cytochrome P-450 per 20 minutes. The specific contents of cytochrome P-450 in these preparations were 0.93 nmol per milligram of protein (control) and 2.0 nmol per milligram of protein (phenobarbital-treated). ND, not detected. The limits of detection for Δ^4-VPA, 3-OH-VPA, 4-OH-VPA, and 5-OH-VPA were 15, 75, 300, and 15 pmol of product per nanomole of P-450 per 20 minutes, respectively.

Treatment	Rate of formation of				
	Δ^4-VPA	3-OH-VPA	4-OH-VPA	5-OH-VPA	*n*
Control microsomes	ND	221 ± 13	766 ± 30	146 ± 3	3
Phenobarbital microsomes					
Complete system	66 ± 8	1502 ± 175	4318 ± 179	497 ± 35	9
Minus cofactors	ND	ND	ND	ND	3
Plus argon*	ND	240 ± 47	500 ± 9	33 ± 2	3
Plus $CO:O_2$ (3:1)*	ND	508 ± 20	896 ± 323	79 ± 29	3
Plus metyrapone (0.5 m*M*)†	ND	412 ± 25	623 ± 20	97 ± 5	3
Plus metyrapone (1.0 m*M*)†	ND	144 ± 13	ND	64 ± 3	3
Denatured microsomes‡	ND	ND	ND	ND	3

*Experiments performed under argon, or a mixture of carbon monoxide and oxygen, were carried out in scintillation vials sealed with rubber septa. †Metyrapone was added to reaction vessels in phosphate buffer. ‡Denatured enzymes were prepared by immersion in a boiling water bath for 15 minutes.

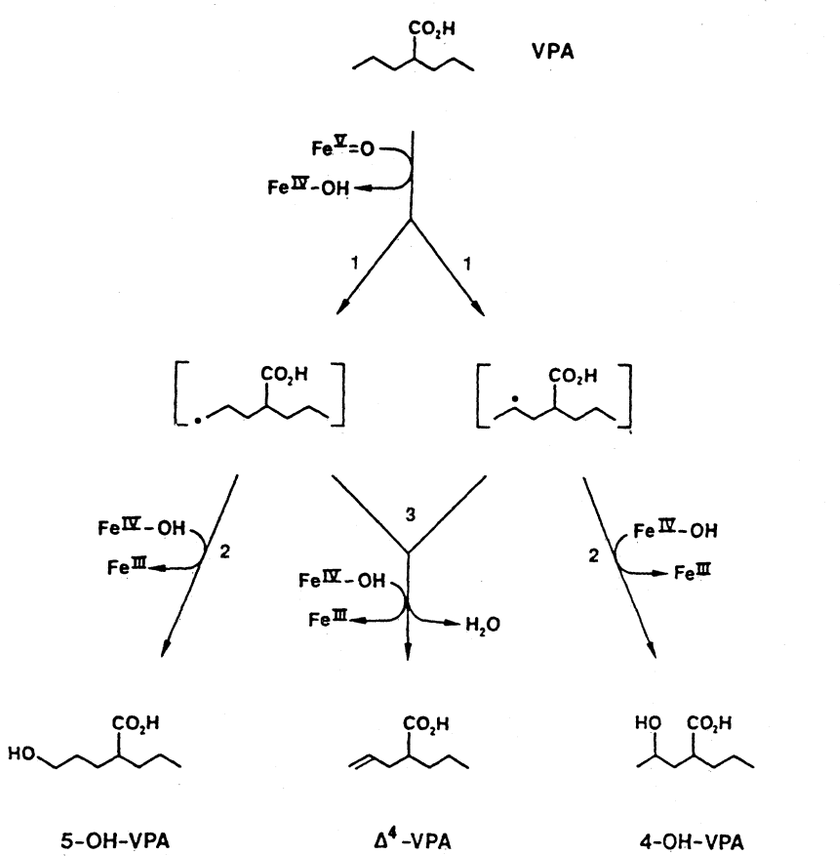

Fig. 1. Proposed scheme for the cytochrome P-450–dependent metabolism of VPA to 4-OH-, 5-OH-, and Δ^4-VPA. The oxidation states of the heme iron participating in hydrogen atom abstraction (pathways 1) and recombination (pathways 2) steps are as shown. Loss of a second hydrogen atom from either of the putative radical intermediates (depicted in brackets) would account for the formation of Δ^4-VPA (pathway 3).

Table 2. Metabolism of VPA by a purified and reconstituted cytochrome P-450 system. Values are expressed as means ± SEM of the rates of formation of VPA metabolites in picomoles of product per nanomole of cytochrome P-450 per 20 minutes. ND, not detected.

Treatment	Rate of formation of				
	Δ^4-VPA	3-OH-VPA	4-OH-VPA	5-OH-VPA	*n*
Complete system	84 ± 8	ND	8746 ± 893	771 ± 36	3
Minus cytochrome P-450	ND	ND	ND	ND	3
Minus cytochrome P-450 reductase	ND	ND	ND	ND	3
Phenobarbital microsomes	66 ± 8	1502 ± 175	4318 ± 179	497 ± 35	9

dration of either of these alcohols. No Δ^4-VPA was found in any of these incubations, proving that the olefin is a genuine metabolite of VPA. These results together show that Δ^4-VPA is produced from the parent drug by the action of hepatic microsomal cytochrome P-450 functioning in the novel capacity of a desaturase enzyme.

This observation may explain earlier reports that certain drugs undergo desaturation reactions when incubated with liver microsomal preparations. The anticoagulant warfarin (*23*), for example, and the synthetic corticosteroid flunisolide (*24*) have been shown to yield unsaturated derivatives in microsomal incubations, although it was speculated that the underlying mechanism in these cases involves an initial cytochrome P-450–catalyzed hydroxylation step, followed by loss of the elements of water from a putatively unstable alcohol. In view of our findings with VPA as substrate, it is tempting to postulate that the desaturation of warfarin, flunisolide, and VPA may occur by a common mechanism, with cytochrome P-450 functioning as a desaturase enzyme attacking benzylic (warfarin), allylic (flunisolide), and alkyl (VPA) centers. A possible mechanism to account for the cytochrome P-450–dependent metabolism of VPA to 4-OH-, 5-OH-, and Δ^4-VPA is outlined in Fig. 1, and invokes the initial formation of a carbon-centered VPA free radical via hydrogen atom abstraction (from position 4 or 5) by the perferryl oxygen of the heme prosthetic group. This step is followed either by recombination of carbon radical–perferric hydroxide radical pairs to yield the isomeric alcohols or by elimination of a hydrogen atom from the carbon radicals to generate the Δ^4 olefin. In the latter case, the perferric hydroxide radical could serve as a convenient acceptor for the second hydrogen atom that is removed from the substrate (*25*). Although tentative at present, this scheme is nevertheless fully consistent with the emerging view of cytochrome P-450–catalyzed alkane hydroxylation reactions as proceeding via nonconcerted, free radical mechanisms (*18, 26*).

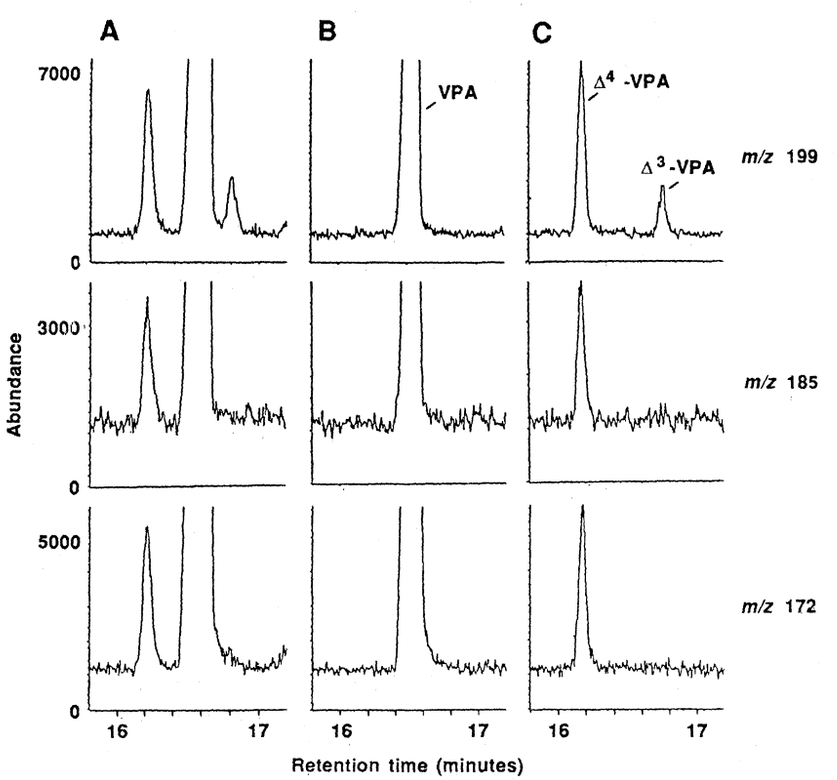

Fig. 2. Detection of Δ^4-VPA as a microsomal metabolite of VPA. (**A**) VPA was incubated with hepatic microsomes from phenobarbital-treated rats and metabolites were extracted and analyzed, as their TMS derivatives, by GC-MS. Identification of Δ^4-VPA in these extracts was based on the following criteria: (i) coincident responses were observed in the ion current chromatograms corresponding to three characteristic fragments in the mass spectrum of Δ^4-VPA TMS (namely, at *m/z* 199, 185, and 172); (ii) these responses occurred at the exact retention time (16.2 minutes) of the TMS derivative of Δ^4-VPA; and (iii) the ratios of responses in the three ion current chromatograms (2.19:1.00:1.74) were almost identical to those obtained from the authentic reference material (2.29:1.00:1.80). (**B**) Microsomal incubations were carried out under conditions identical to those of (A), except for the omission of reduced nicotinamide cofactors. (**C**) An authentic sample of Δ^4-VPA TMS was analyzed to determine accurately the retention time of the compound and also to establish the ratios of ion current intensities at *m/z* 199, 185, and 172. [Peaks in (A) and (C) are normalized with respect to Δ^4-VPA TMS. The ordinate for ion abundance, therefore, differs for each mass depicted.] The retention times of VPA TMS and Δ^3-VPA TMS under these conditions were 16.5 and 16.8 minutes, respectively.

Although the scope of cytochrome P-450–mediated desaturation reactions remains to be established, the metabolism of VPA by the "Δ^4-pathway" clearly has important clinical implications, especially with regard to the use of VPA in polytherapy. Thus, desaturation of VPA to yield the potent hepatotoxin Δ^4-VPA, catalyzed by isoenzymes of cytochrome P-450 that have been induced specifically by coadministration of phenobarbital or other anticonvulsants, may prove to be a pivotal step in the sequence of events leading to VPA-induced liver injury. Epileptic patients treated concomitantly with VPA and phenobarbital may be at especially high risk, therefore, and should be monitored carefully for early signs of hepatic dysfunction.

REFERENCES AND NOTES

1. R. H. Levy, *Epilepsia* **25** (suppl. 1), S1 (1984).
2. N. Gerber *et al.*, *J. Pediatr.* **95**, 142 (1979); H. J. Zimmerman and K. G. Ishak, *Hepatology (Baltimore)* **2**, 591 (1982).
3. P. M. Jeavons, *Epilepsia* **25** (suppl. 1), S50 (1984); F. E. Dreifuss and N. Santilli, *Neurology* **36** (suppl. 1), 175 (1986); D. Scheffner, *Lancet* **1986-II**, 511 (1986).
4. E. Kingsley, P. Gray, K. G. Tolman, R. Tweedale, *J. Clin. Pharmacol.* **23**, 178 (1983).
5. K. S. Prickett and T. A. Baillie, *Drug Metab. Dispos.* **14**, 221 (1986).
6. S. M. Bjorge and T. A. Baillie, *Biochem. Biophys. Res. Commun.* **132**, 245 (1985).
7. J. W. Kesterson, G. R. Granneman, J. M. Machinist, *Hepatology (Baltimore)* **4**, 1143 (1984).
8. W. Kochen and H. Scheffner, in *Antiepileptic Therapy: Advances in Drug Monitoring*, S. I. Johanessen *et al.*, Eds. (Raven, New York, 1980), pp. 111–120.
9. W. Kochen, A. Schneider, A. Ritz, *Eur. J. Pediatr.* **141**, 30 (1983).
10. A. W. Rettenmeier *et al.*, *Drug Metab. Dispos.* **14**, 443 (1986).

11. H. Nau *et al.*, *J. Chromatogr.* **226**, 69 (1981).
12. G. R. Granneman *et al.*, *Xenobiotica* **14**, 375 (1984).
13. J. H. Lewis, H. J. Zimmerman, C. T. Garret, E. Rosenberg, *Hepatology (Baltimore)* **2**, 870 (1982).
14. A. H. Conney, *Pharmacol. Rev.* **19**, 317 (1967).
15. Hepatic microsomes were prepared as described previously [P. A. Dean *et al.*, *Chem.-Biol. Interact.* **58**, 79 (1986)] from either untreated or phenobarbital-induced rats (80 mg/kg per day for 4 days, injected intraperitoneally). Microsomal incubations (1.0 ml final volume) contained 100 μmol of potassium phosphate, 1.5 μmol of NADPH, 1.5 μmol of NADH, 3 μmol of magnesium chloride, and 1 μmol of the sodium salt of VPA. (It was found in the course of these experiments that NADH was not a necessary cofactor for the desaturation of VPA, whereas the reaction was critically dependent on the presence of NADPH.) Incubations were carried out at 37°C and *p*H 7.4 for 20 minutes and were terminated by the addition of 1 ml of 10% HCl.
16. 2-*n*-Propylhexanoic acid (94 ng) and 2-(3-hydroxy-*n*-propyl)-hexanoic acid (72 ng) were added to acidified microsomal incubations as internal standards. Samples were extracted twice with 3 ml of ethyl acetate and the combined organic extracts were concentrated under a stream of dry nitrogen and derivatized with *bis*(trimethylsilyl)trifluoroacetamide. GC-MS analyses were carried out with a Hewlett-Packard model 5970 mass selective detector connected to an HP model 5890A gas chromatograph and interfaced to an HP model 59970C data system. The gas chromatograph was equipped with a fused silica capillary column (60 m by 0.32 mm internal diameter) coated with the bonded stationary phase DB-1 (J & W Scientific, Ventura, CA). Samples were injected in the splitless mode and "cold-trapped" on the column at 40°C, following which the oven temperature was raised rapidly to 80°C. A linear temperature gradient of 2°C per minute was then maintained to 120°C, after which the temperature was raised by 4°C per minute to 200°C. The temperatures of the ion source and the GC interface were maintained at 200° and 250°C, respectively. The mass spectrometer was operated in the selected ion-monitoring mode, and the following ions were used for quantitative analyses: mass-to-charge ratio (*m/z*) 199 (Δ^4-VPA TMS; $[M-CH_3]^+$), 100 (4-OH-VPA γ-lactone; $[M-C_3H_6]^{\cdot+}$), and 289 (3-OH- and 5-OH-VPA *bis*-TMS; $[M-CH_3]^+$). The retention times of the TMS derivatives of the various VPA metabolites were: Δ^4-VPA, 16.2 minutes; 3-OH-VPA, 29.3 minutes; 4-OH-VPA γ-lactone, 15.7 and 16.0 minutes (two diastereoisomers); and 5-OH-VPA, 32.9 minutes. (Δ^4-VPA was synthesized as described by A. W. Rettenmeier *et al.* [*Drug Metab. Dispos.* **13**, 81 (1985)], whereas Δ^3-VPA was obtained as described previously (*10*).
17. K. S. Prickett and T. A. Baillie, *Biochem. Biophys. Res. Commun.* **122**, 1166 (1984).
18. F. P. Guengerich and T. L. Macdonald, *Acc. Chem. Res.* **17**, 9 (1984).
19. D. C. Dahlin, G. T. Miwa, A. Y. H. Lu, S. D. Nelson, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1327 (1984).
20. O. Augusto, H. S. Beilan, P. R. Ortiz de Montellano, *J. Biol. Chem.* **257**, 11288 (1982); R. H. Böcker and F. P. Guengerich, *J. Med. Chem.* **29**, 1596 (1986).
21. K. Nagata, D. J. Liberato, J. R. Gillette, H. A. Sasame, *Drug Metab. Dispos.* **14**, 559 (1986).
22. Cytochrome P-450 (PB-4) and NADPH-dependent cytochrome P-450 reductase were purified from the livers of phenobarbital-treated male Sprague-Dawley rats, as described by D. J. Waxman and C. Walsh [*J. Biol. Chem.* **257**, 10446 (1982)]. Cytochrome P-450 was purified to a specific content of at least 13 nmol per milligram of protein, while cytochrome P-450 reductase was purified to a specific content of at least 10 nmol per milligram of protein. Incubations (1.0 ml final volume) with the purified enzymes contained 100 μmol of potassium phosphate, 2 nmol of cytochrome P-450, 2 nmol of cytochrome P-450 reductase, 60 mg of dilaurylphosphatidylcholine, 1.5 μmol of NADPH, 1.5 μmol of NADH, 3 μmol of magnesium chloride, and 1 μmol of the sodium salt of VPA. Reactions were carried out at 37°C and *p*H 7.4 and were terminated after 20 minutes by the addition of 1 ml of 10% HCl. Samples were analyzed as described in (*16*).
23. M. J. Fasco, P. P. Dymerski, J. D. Wos, L. S. Kaminsky, *J. Med. Chem.* **21**, 1054 (1978).
24. P. J. Teitelbaum *et al.*, *J. Pharmacol. Exp. Ther.* **16**, 2189 (1981).
25. L. D. Gorsky, D. R. Koop, M. J. Coon, *J. Biol. Chem.* **259**, 6812 (1984).
26. J. T. Groves, G. A. McClusky, R. E. White, M. J. Coon, *Biochem. Biophys. Res. Commun.* **81**, 154 (1978).
27. We thank W. F. Trager, R. H. Levy, and M. R. Juchau for their valuable suggestions and for critically reviewing this report and S. West for manuscript preparation. These studies were supported by NIH grants GM 32165, NS 17111, and DK 30699.

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Substance P Activation of Rheumatoid Synoviocytes: Neural Pathway in Pathogenesis of Arthritis

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Several clinical features are consistent with nervous system involvement in the pathogenesis of rheumatoid arthritis. The neuropeptide substance P is one possible mediator of this interaction, since it can be released into joint tissues from primary sensory nerve fibers. The potential effects of the peptide on rheumatoid synoviocytes were examined. The results show that substance P stimulates prostaglandin E_2 and collagenase release from synoviocytes. Furthermore, synoviocyte proliferation was increased in the presence of the neuropeptide. Similar effects were observed with a truncated form of substance P. Synoviocytes were sensitive to very small doses of the neuropeptide ($10^{-9}M$), and its effects were inhibited by a specific antagonist. Thus, the specific stimulation of synoviocytes by the neuropeptide substance P represents a pathway by which the nervous system might be directly involved in the pathogenesis of rheumatoid arthritis.

MULTIPLE FACTORS APPEAR TO contribute to the pathogenesis of rheumatoid arthritis (RA), including genetic predisposition, impaired immunological control of virus infection, and autoantibody formation (*1*). In the synovium, activated lymphocytes and monocytes interact to generate interleukin-1 (IL-1). This lymphokine and monocyte-derived tumor necrosis factor–alpha (TNF-α) stimulate release of collagenase and prostaglandin E_2 (PGE_2) from synoviocytes (*2*).

Certain clinical findings are consistent with nervous system involvement in RA: (i) the distribution of synovitis is usually symmetric (*3*); (ii) joints in the paretic side of hemiplegic patients who later develop RA are spared from the inflammatory process (*4*); and (iii) onset and exacerbation of the disease are often preceded by psychological trauma (*5*). The basis for such effects in RA is unknown. Joints are innervated by corpuscular mechanoreceptors and plexus, as well as by free endings of small unmyelinated sensory afferent nerve fibers (*6*). These sensory fibers contain the neuropeptide substance P, which is involved in transmission of pain signals (*7*). Substance P is released in the spinal tract upon orthodromic stimulation, but is also released antidromically from the peripheral nerve terminals (*8*). This neuropeptide has multiple proinflammatory properties (*9*) and has been shown to increase the severity of adjuvant-induced arthritis in rats (*10*). The relative susceptibilities of knee and ankle joints to adjuvant arthritis are correlated with the densities of their sensory afferent nerve fibers (*10*). The nonneural target cell for the effects of substance P in joints is unknown, and the role of the peptide in RA has not been explored. We now report that in RA synoviocytes are stimulated by substance P.

The effect of substance P on the function of synoviocytes in RA was tested by measuring PGE_2 and collagenase production (*11*). Synoviocytes were isolated by collagenase-deoxyribonuclease treatment of synovial tissues obtained from RA patients undergoing joint replacement and were passaged once in culture before use in the experiments. We quantified by radioimmunoassay the levels of PGE_2 secreted during 48 hours of culture. Substance P, truncated substance P containing amino acids 4 to 11, and the antagonist [D-Pro^2, D-Phe^7, D-Trp^9]substance P (D–substance P) were added at start of culture.

Synoviocytes spontaneously released PGE_2 at 92 ± 14 ng/ml during 48 hours of culture (Fig. 1A). The addition of substance P increased PGE_2 release in a dose-dependent manner, with maximal stimulation occurring at $10^{-8}M$. A similar dose-response curve was obtained with substance P frag-

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