However, enzymatic studies were not reported for those cats since only Formalin-fixed tissues were obtained. Very recently a poodle with NPD was reported (*10*).

The use of the drug AY-9944 to produce an animal model of NPD in rats has been reported (*11*). This drug produces pathological changes similar to those seen in NPD, and causes an increase in the sphingomyelin content of the liver and a reduction in sphingomyelinase activity, possibly because of impaired enzyme synthesis. Thus animal models of some human genetic diseases may be produced by specific chemicals, but genetic models are more useful for studying methods of treatment. The availability of Siamese cats with a severe neurovisceral lipidosis apparently identical to human NPD type A should facilitate studies of treatment for this fatal lipid storage disease of humans.

DAVID A. WENGER
MARTHA SATTLER
TOORU KUDOH
Department of Pediatrics, University of Colorado Health Sciences Center, Denver 80262
STANLEY P. SNYDER
RICHARD S. KINGSTON
Department of Pathology, College of Veterinary Medicine, Colorado State University, Fort Collins 80523

References and Notes

1. A. C. Crocker, *J. Neurochem.* **7**, 69 (1961); R. O. Brady, J. N. Kanfer, M. B. Mock, D. S. Fredrickson, *Proc. Natl. Acad. Sci. U.S.A.* **55**, 366 (1966).
2. E. G. Brunngraber, B. Berra, V. Zambotti, *Clin. Chim. Acta* **48**, 173 (1973).
3. D. A. Wenger, C. Wharton, M. Sattler, C. Clark, *Am. J. Med. Genet.* **2**, 345 (1978); S. Gatt, T. Dinur, J. Kopolovic, *J. Neurochem.* **31**, 547 (1978).
4. D. A. Wenger, M. Sattler, C. Clark, H. Tanaka, K. Suzuki, G. Dawson, *Science* **188**, 1310 (1975); D. A. Wenger, M. Sattler, C. Clark, C. Wharton, *Life Sci.* **19**, 413 (1976).
5. R. S. Kingston, S. P. Snyder, D. A. Wenger, in preparation.
6. D. A. Wenger, in *Practical Enzymology of the Sphingolipidoses*, R. H. Glew and S. P. Peters, Eds. (Liss, New York, 1977), p. 39.
7. B. G. Rao and M. W. Spence, *J. Lipid Res.* **17**, 506 (1976).
8. H. J. Baker, Jr., J. R. Lindsey, G. M. McKhann, D. F. Farrell, *Science* **174**, 838 (1971); L. C. Cork, J. F. Munnell, M. D. Lorenz, J. V. Murphy, H. J. Baker, M. C. Rattazzi, *ibid.* **196**, 1014 (1977); P. F. Jezyk, M. E. Haskins, D. F. Patterson, W. J. Mellman, M. Greenstein, *ibid.* **198**, 834 (1977).
9. C. E. Chrisp, D. H. Ringler, G. D. Abrams, N. S. Radin, A. Brenkert, *J. Am. Vet. Med. Assoc.* **156**, 616 (1970); D. H. Percy and B. S. Jortner, *Arch. Pathol.* **92**, 136 (1971).
10. A. Bundza, J. A. Lowden, K. M. Charlton, *Vet. Pathol.* **16**, 530 (1979).
11. N. Sakuragawa, M. Sakuragawa, T. Kuwabara, P. G. Pentchev, J. A. Barranger, R. O. Brady, *Science* **196**, 317 (1977).
12. We thank D. J. Brooks, B. Reinhardt, and Mr. and Mrs. Daniel Gilmore for their contributions to this research. This research was supported in part by NIH grants HD10494, NS10698, HD08315, and by a grant from the National Foundation-March of Dimes. D.A.W. is a recepient of a Research Career Development Award (1 K04 NS00108).

17 December 1979; revised 4 March 1980

Immunofluorescence of NADPH–Cytochrome *c* (P-450) Reductase in Rat and Minipig Tissues Injected with Phenobarbital

Abstract. *The enzyme NADPH–cytochrome* c *(P-450) reductase was identified by indirect immunofluorescence in hepatocytes, bronchioles, and proximal tubules of liver, lung, and kidney, respectively, of rats and minipigs that had been injected with phenobarbital or saline. The distribution of this component of the cytochrome P-450–mediated microsomal system may be relevant to sites of drug toxicity and carcinogenesis.*

The enzyme NADPH-cytochrome *c* (P-450) reductase (E.C. 1.6.2.4), as well as cytochrome P-450, cytochrome b_5, and NADH-cytochrome b_5 reductase are components of the known microsomal electron transport systems that participate in the metabolism of endogenous substrates such as steroids and fatty acids, and in the metabolism of many exogenous substrates, including various drugs and carcinogens (*1*, *2*). Selected components of these systems are increased by the administration of drugs such as phenobarbital or certain polycyclic aromatic hydrocarbons (*1*). Paradoxically, the microsomal drug-metabolizing system may participate in the activation of many potential carcinogens to their proximate metabolites (*3*). Phenobarbital has been shown both to promote and to inhibit tumor formation in experimental models (*4*). Thus, the question of the relation of cytochrome P-450–mediated enzyme systems to carcinogenesis is a complicated one. It is logical to ask which cell types contain the mixed function oxidase enzymes, and if these cell types correspond to those which are frequent sites of tumor formation.

NADPH-cytochrome *c* (P-450) reductase has been shown biochemically to be present in liver, lung, and kidney microsomes (*5*). However, since microsomes are a product of tissue homogenates, microsomal data provide no insight into the cell type containing the enzyme, unless the tissue is very homogeneous. Baron *et al.* (*6*) have demonstrated NADPH-cytochrome *c* reductase in livers of control and phenobarbital-treated rats by immunohistochemical techniques. We have investigated by immunofluorescence the extrahepatic, as well as the hepatic, distribution of NADPH-cytochrome *c* (P-450) reductase in rats and minipigs (small pigs bred by Bastrop, College Station, Texas).

Male Sprague-Dawley rats were injected intraperitoneally with phenobarbital (80 mg/kg) or saline daily for 4 days, fasted, and then killed. The minipigs were given phenobarbital (15 mg/kg) in a bolus of food once daily for 6 days and then killed. Portions of liver, lung, and kidney were removed and frozen immediately in liquid nitrogen for immunocytochemistry. Biochemical assays were performed on microsomes prepared by standard methods from the remainder of the tissue from each organ; induction of the phenobarbital-treated rats was confirmed by a two- to threefold increase in NADPH-cytochrome *c* (P-450) reductase activity, and a three- to fivefold increase in cytochrome P-450 content when liver microsomes from phenobarbital- and saline-treated rats were compared. No change in activities was seen in kidney microsomes and only a slight increase was seen in lung microsomes from the phenobarbital-treated rats.

Sections of tissue (5 to 6 μm) prepared in a cryostat were stained by the indirect fluorescent antibody technique (*7*). For this purpose we used rabbit antibody to pig NADPH-cytochrome *c* reductase (0.025 mg/ml), prepared against the protease-solubilized form of the reductase and purified by affinity chromatography (*8*). Although there are differences in the reductase purified by proteolytic as opposed to detergent solubilization, antibody prepared against the proteolytically isolated form is also specific for the detergent-solubilized form as well as the intact, membrane-bound enzyme (*9*). Goat antibody to rabbit immunoglobulin G (IgG) conjugated to fluorescein isothiocyanate (FITC) was used as the secondary antibody. Controls included: unstained sections, sections stained with secondary antibody only, nonimmune IgG substituted for the primary antibody, and primary antibody that had been incubated with cytochrome P-450 reductase to block antibody binding sites. Although only the controls for liver are shown (Fig. 1), controls were performed on each tissue and consistently showed minimal or no fluorescence.

In the rat, specific fluorescence was seen throughout the liver (Fig. 2, a and d) in the hepatocyte cytoplasm but not in the nucleus. In both saline- and phenobarbital-treated rats, the fluorescence was more intense in centrilobular than in periportal zones; livers from phenobarbital-treated rats showed increased fluorescence compared to the livers from saline-treated animals. Fluorescence in the

0036-8075/80/0627-1473$00.50/0

vascular and connective tissues was weak to negative in both the phenobarbital- and saline-treated animals. The distribution of NADPH-cytochrome *c* (P-450) reductase in the livers of phenobarbital-treated minipigs (Fig. 2g) was similar to that in phenobarbital-treated rats.

In the rat kidney (Fig. 2, b and e) we found no differences in the distribution of the enzyme-specific fluorescence between phenobarbital- and saline-treated animals. Positive fluorescence was essentially confined to the cortex and outer stripe of the outer medulla. All segments (*10*) of the proximal tubules were posi-

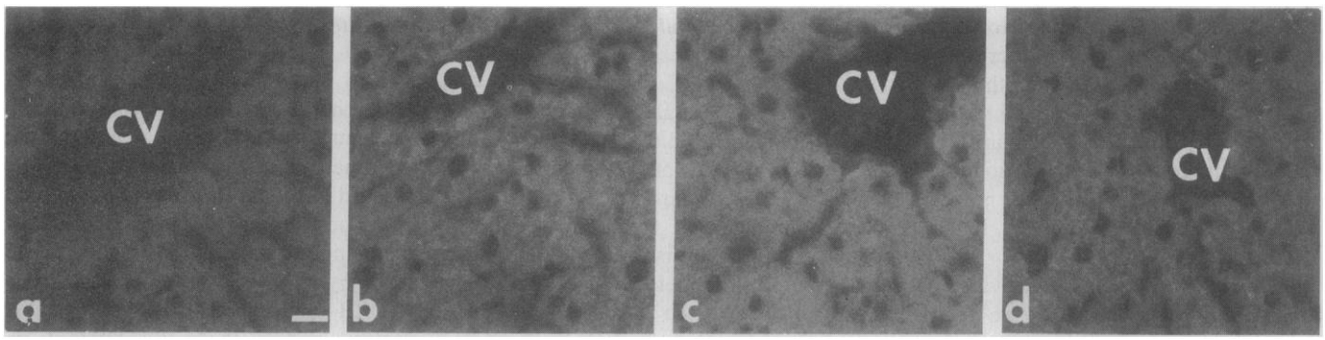

Fig. 1. Immunohistochemical controls. Sections of liver from rats injected with phenobarbital were treated as follows: (a) unstained; (b) fluorescein-conjugated secondary antibody only; (c) nonimmune rabbit IgG followed by FITC-conjugated goat antibody to rabbit IgG; (d) rabbit antibody to NADPH-cytochrome *c* (P-450) reductase that had been incubated first with purified NADPH-cytochrome *c* reductase to block primary antibody binding sites, and then with secondary antibody. Central vein, *CV*. Scale bar, 20 μm.

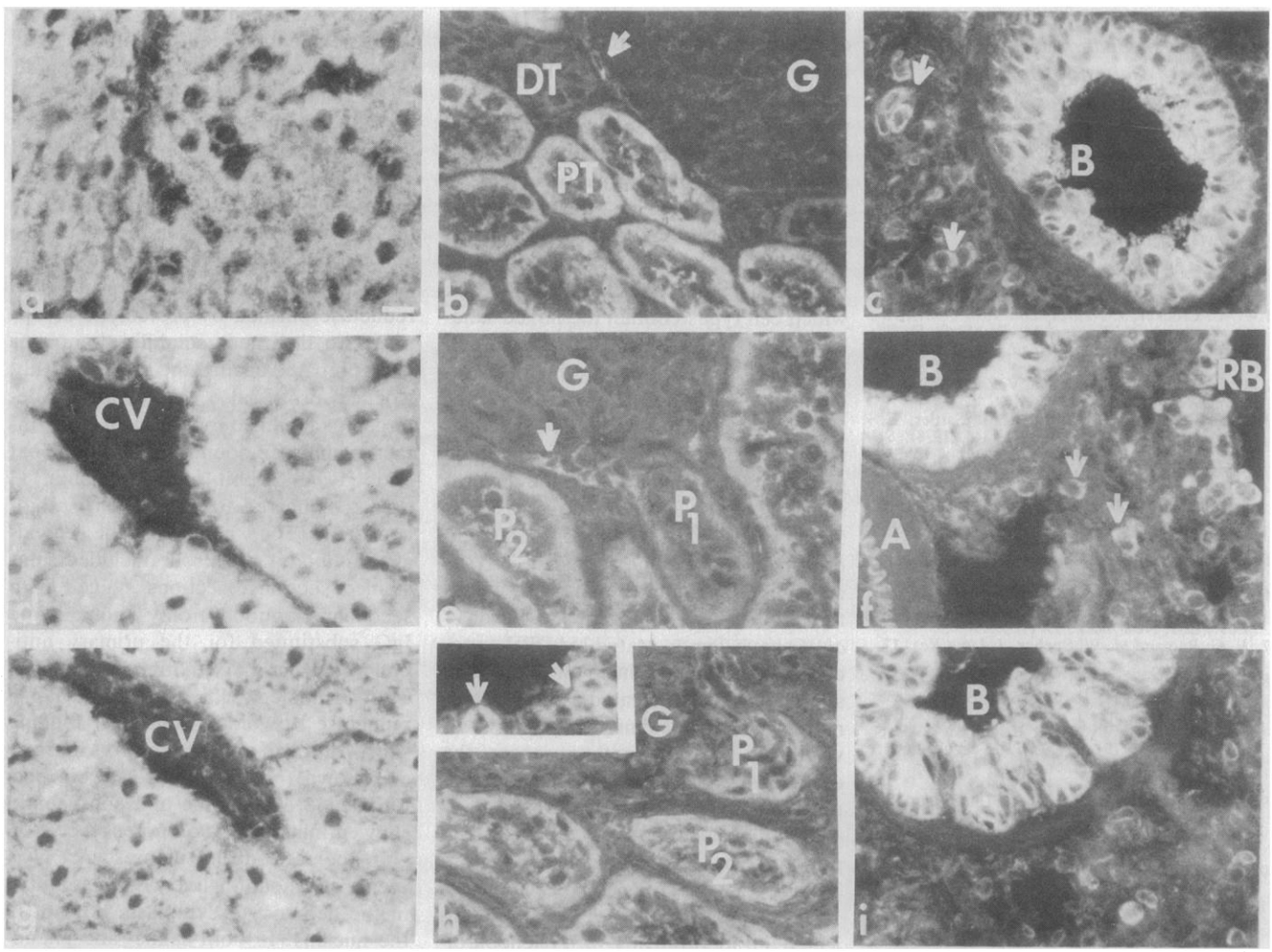

Fig. 2. Identification of NADPH-cytochrome *c* (P-450) reductase by specific immunofluorescence. Scale bar, 20 μm. (a to c) Rats injected with saline. (a) Liver: centrilobular zone; (b) kidney: proximal tubule (*PT*), distal tubule (*DT*), glomerulus (*G*), parietal layer of Bowman's capsule (arrow); (c) lung: bronchiole (*B*). (d to f) Rats injected with phenobarbital. (d) Liver: central vein (*CV*); (e) kidney cortex: glomerulus (*G*), parietal layer of Bowman's capsule (arrow), P_1 segment of proximal tubule (P_1), P_2 segment of proximal tubule (P_2); (f) lung: bronchiole (*B*), respiratory bronchiole (*RB*), artery (*A*), positively staining parenchymal cells (arrows). (g to i) Minipigs treated with phenobarbital. (g) Liver: central vein (*CV*); (h) kidney cortex: glomerulus (*G*), P_1 segment of the proximal tubule (P_1), P_2 segment of the proximal tubule (P_2); (inset) brightly staining cells (arrows) alternating with unstained cells in a cortical collecting duct; (i) lung: bronchiole (*B*).

tive, although the specific fluorescence appeared to be stronger in the P_2 and P_3 segments. Distal tubules and collecting ducts were negative. The glomeruli, except for the parietal layer of Bowman's capsule, were negative. There was a marked reduction in fluorescence from the outer stripe to the inner stripe of the outer medulla (not shown). Low-lying cells, showing a faint fluorescence and probably representing thin limbs of the loop of Henle, were seen in the inner stripe, but all other segments of the nephron in the inner stripe and inner medulla were essentially negative. Minipig kidneys (Fig. 2h) resemble the rat kidneys with one exception: occasional brightly staining cells were seen among essentially negative cells in cortical collecting ducts; these cells may represent the "dark" cells of the pig collecting duct.

We found no differences in the distribution or intensity of specific fluorescence between the lungs of phenobarbital- and saline-treated rats (Fig. 2, c and f). Positive fluorescence was seen in bronchi and bronchioles and in an additional cell type in the lung parenchyma. Minipig lungs (Fig. 2i) showed a similar distribution of NADPH-cytochrome *c* (P-450) reductase.

Our results concerning the distribution of NADPH-cytochrome *c* (P-450) reductase in the liver agree with those of Baron *et al.* (*6*). To our knowledge, the immonohistochemical demonstration of NADPH-cytochrome *c* (P-450) reductase in kidney or lung has not been reported previously. Our results agree with morphologic and biochemical evidence suggesting that the proximal tubule is the site of drug metabolism in the kidney (*11*). Boyd (*12*) has shown by autoradiography that a metabolite of the pulmonary toxin, 4-ipomeanol, is localized in pulmonary nonciliated bronchiolar (Clara) cells, and has postulated that the Clara cell is the primary location of P-450-mediated mixed function oxidase enzymes in the lung. Our results are consistent with the presence of the enzyme in the bronchioles, but indicate that additional cell types in the bronchi and bronchioles, and possibly a parenchymal cell type, are positive for NADPH-cytochrome *c* (P-450) reductase. Although the Clara cell may be the predominant cell metabolizing ipomeanol, the more general method of determining the distribution of NADPH-cytochrome *c* (P-450) reductase, which (according to current knowledge) exists in only one form and is presumably present wherever any of the multiple forms of cytochrome P-450 occur, may be a more sensitive way to identify cells containing cytochrome P-450-specific mixed function oxidase enzymes.

JANE HOWARD DEES
LARRY DEAN COE
YUKIO YASUKOCHI
BETTIE SUE MASTERS

Department of Biochemistry, University of Texas Health Science Center at Dallas, Dallas 75235

References and Notes

1. R. Sato and T. Omura, *Cytochrome P-450* (Academic Press, New York, 1978).
2. Abbreviations NADPH and NADH are for reduced nicotinamide adenine dinucleotide phosphate and the reduced dinucleotide, respectively.
3. P. L. Grover and P. Sims, *Biochem. J.* **110**, 159 (1968); E. S. Miller and J. A. Miller, in *The Molecular Biology of Cancer*, H. Busch, Ed. (Academic Press, New York, 1974); P. Sims and P. L. Grover, *Adv. Cancer Res.* **20**, 165 (1974).
4. L. W. Wattenberg, *Adv. Cancer Res.* **20**, 166 (1974); H. C. Pitot, *Am. J. Pathol.* **89**, 402 (1977).
5. C. H. Williams, Jr., and H. Kamin, *J. Biol. Chem.* **237**, 587 (1962); T. Matsubara and Y. Tochino, *J. Biochem. (Tokyo)* **60**, 981 (1971); S. V. Jakobsson, *Exp. Cell Res.* **84**, 319 (1974); G. W. R. Hook and J. R. Bend, *Life Sci.* **18**, 279 (1974); H. Remmer, in *Lung Metabolism*, A. F. Junod and R. de Haller, Eds. (Academic Press, New York, 1975).
6. J. Baron, J. A. Redick, P. Greenspan, Y. Taira, *Life Sci.* **22**, 1097 (1978).
7. T. H. Weller and A. H. Coons, *Proc. Soc. Exp. Biol. Med.* **86**, 789 (1954).
8. P. Cuatrecasas and C. B. Anfinsen, *Methods Enzymol.* **22**, 345 (1971); M. Noshiro and T. Omura, *J. Biochem. (Tokyo)* **83**, 61 (1978).
9. B. S. S. Masters, J. Baron, W. E. Taylor, E. Isaacson, J. LoSpalluto, *J. Biol. Chem.* **243**, 4143 (1971); B. S. S. Masters, *Methods Enzymol.* **52**, 240 (1978).
10. A. B. Maunsbach, *J. Ultrastruct. Res.* **16**, 39 (1966).
11. N. O. Jacobsen and F. Jorgensen, *Z. Zellforsch. Mikrosk. Anat.* **136**, 479 (1973); B. A. Fowler, G. E. R. Hook, G. W. Lucier, *J. Pharmacol. Exp. Ther.* **203**, 712 (1977); T. V. Zenser, M. B. Mattammal, B. B. Davis, *ibid.* **207**, 719 (1978).
12. M. R. Boyd, *Nature (London)* **269**, 713 (1977).
13. We thank Dr. James Gilliam, Department of Internal Medicine, for the use of his cryostat and fluorescence microscope. Supported by PHS HLBI fellowship F32HL05684 and, in part, by PHS grants NHLBI 13619 and GM 16488.

28 November 1979; revised 19 February 1980

Social Environment as a Factor in Diet-Induced Atherosclerosis

Abstract. *Rabbits on a 2 percent cholesterol diet were individually petted, held, talked to, and played with on a regular basis. Measurements of aortic affinity for a Sudan stain, serum cholesterol levels, heart rate, and blood pressure were made at the end of the experimental period. Compared to control groups, which were given the same diet and normal laboratory animal care, the experimental groups showed more than a 60 percent reduction in the percentage of aortic surface area exhibiting sudanophilic lesions, even though serum cholesterol levels, heart rate, and blood pressure were comparable.*

The apparent relation between stress and cardiovascular disease is based on a variety of evidence implicating physical, emotional, and behavioral factors (*1-6*). Data from animal studies link psychosocial disruption to pathological changes in the cardiovascular system (*7-14*). These studies include several in which states of severe emotional disturbance were produced with negative stressors. To our knowledge, however, there have not been any studies in which the effects of positive factors were investigated.

We designed a series of studies to investigate the influence of social environment on diet-induced atherosclerosis in rabbits. In the experimental groups (groups A, B, and D), the animals experienced social interaction with an experimenter (M.J.L.). In the control groups (groups C and E), the animals received normal laboratory animal care. Group A was studied during late 1977 without a concurrent control, although earlier control studies had been carried out. The results for group A, although initially considered anomalous, ultimately led us to conduct two additional studies. One was carried out in early 1978 and involved experimental group B and control group C. The other was carried out in late 1978 and involved experimental group D and control group E.

It should be noted that the essence of the experimental environment studied here was to establish a one-to-one relationship between each animal and the experimenter. This was achieved through an early morning, half-hour visit during which each animal was handled, stroked, talked to, and played with; an hour-long feeding period during which the animal was also touched and talked to; and a number of 5-minute visits during the day. Through this daily process, the animals quickly learned to recognize the experimenter, and when present, many even sought her personal attention. They were left alone for 10 hours each night.

The animals used in this study were young male New Zealand White rabbits. Upon being received, they were separated and subjected to a 2-week adaptation period during which they and the experimenter became acquainted. All experiments were carried out by the same experimenter with the same protocol.

After the adaptation period, the ani-

0036-8075/80/0627-1475$00.50/0