*Ikuo Ishiyama,*[1] *M.D.; Masahiro Mukaida,*[1] *M.D.;*
*Ryosuke Tanabe,*[1] *M.D.; Masayoshi Kaiho,*[1] *Ph.D.; and*
*Masaru Ueyama,*[1] *Ph.D.*

# Histochemical Demonstration of Phenobarbital by Immunocytochemistry

**REFERENCE:** Ishiyama, I., Mukaida, M., Tanabe, R., Kaiho, M., and Ueyama, M., **"Histochemical Demonstration of Phenobarbital by Immunocytochemistry,"** *Journal of Forensic Sciences,* JFSCA, Vol. 32, No. 5, Sept. 1987, pp. 1221-1234.

**ABSTRACT:** A method for the demonstration of the topographical distribution of phenobarbital at the cellular level in various tissues was established. Mice that had been exposed to various doses of phenobarbital by intraperitoneal injection were killed, and their tissues were fixed with 0.1*M* phosphate buffer solution (pH 7.4) containing paraformaldehyde and glutaraldehyde. Thereafter, paraffin and frozen sections were made and stained by the indirect immunoperoxidase method using antisera obtained from commercial sources and used for the immunochemical assay of the blood level of phenobarbital in clinical medicine. A specific positive reaction was observed solely in testing the intoxicated tissues, and this reaction was inhibited when phenobarbital was added to the antisera. The minimal sensitivity of the positive reaction, which can be discerned by observing the stained slides macroscopically, was in the range of 10 mg/kg. Thus, the diagnosis of phenobarbital intoxication in the forensic autopsy can be made by immunohistochemistry. A positive reaction was found in various tissue cells, including nerve cells, myelin sheaths, glia cells, hepatocytes, cells of the alveolar and bronchial wall, epithelial cells of the distal part of the renal tubules, and so forth. Endothelial cells of the capillaries in all tissues gave a strong positive reaction. The immunocytochemical electron microscopy of the hepatocytes revealed that the positive reaction in the cytoplasm was located solely in the intraluminal space of the smooth endoplasmic reticulum. These results indicate some interesting aspects of the pharmacokinetics of phenobarbital in vivo. It is expected that the antisera, which are used widely for the assay of the blood concentration of various drugs (phenobarbital, amphetamines, morphine, and so forth), may be regarded as excellent reagents for immunocytochemistry. This clearly indicates that morphological evidence in toxicology, which had so far remained obscure, can be easily obtained by applying these antisera against various drugs.

**KEYWORDS:** pathology and biology, phenobarbital, cytochemistry, nerve cells, brain, lung, liver, endothelium, smooth endoplasmic reticulum

Recent advances in chemical toxicology, especially in the techniques of gas chromatography/mass spectrometry and enzyme-linked immunosorbent assay (ELISA), have shown that the detection level of various toxic substances in biological fluids can be reached to obtain reliable data for the decisive diagnosis of drug intoxication and abuse. On the other hand, no concrete information concerning the histopathology of acute drug intoxication has been obtained, and no attempt to establish a histochemical determination of the topographic locali-

Supported in part by a Grant of the Ministry of Education, Science, and Culture Japan (A-59440037, C-61570286). Received for publication 2 Oct. 1986; revised manuscript received 7 Jan. 1987; accepted for publication 8 Jan. 1987.

[1]Professor, lecturer, postgraduate student, assistant, and research fellow, respectively, Department of Forensic Medicine, University of Tokyo, Tokyo, Japan.

zation of drugs—except for several reports on autoradiography [*1-5*]—has been encountered in the literature.

Recently, we have succeeded, using immunohistochemistry, in determining the topographic localization of amphetamines in various cells of mice that were exposed to these drugs [*6*]. Furthermore, we found that the immunohistochemical demonstration of phenobarbital can also be performed using certain antisera obtained from commercial sources. Our experiments distinctly indicate that the topographic localization of various drugs, which is the background for the investigation of their pharmacological activities, can be determined without any difficulty. These findings will be reported in this paper.

## Materials and Methods

### *Phenobarbital*

Phenobarbital sodium (Wako-Junyaku Co. Ltd., Osaka) was dissolved in 0.01*M* phosphate-buffered saline at the concentration of 10% and preserved at 4°C.

### *Animals*

Male ddY mice weighing 25 to 30 g were used. They were administered various doses of phenobarbital (0.01 to 10 mg) intraperitoneally or subcutaneously. After 30 min they were killed and the tissues were removed, cut to a thickness of 2 mm, and immersed in fixative at 4°C.

### *Fixation of Tissues*

The tissues were fixed in the 0.1*M* phosphate buffer solution (pH 7.4) containing 2% glutaraldehyde and 4% paraformaldehyde for 3 h. After fixation, they were washed with the phosphate-buffered saline containing various amounts of sucrose (5, 10, 15, and 20% sucrose with ascending series) at 4°C, successively, for 24 h.

### *Preparation of Histological Sections*

Two kinds of sections, that is, frozen and paraffin slices, were prepared from the same tissues. At the same time, the control animals were killed, and the histological preparations were made in the same way. The slices from the test and control animals were mounted on the same microscope slide glass for simultaneous staining.

### *Antisera Against Phenobarbital*

Two kinds of commercially available antisera were used. The antiserum (goat) contained in the Abbott TDX assay system kit (phenobarbital in the blood, Abbott, North Chicago, IL) was used. In a preliminary experiment, we confirmed that the serum diluted 1 : 1 to 1 : 4 gave the same staining, while the dilution of 1 : 8 gave a faint reaction. Thus, the dilution of 1 : 4 was applied throughout this experiment. The other serum was that of Scantibodies Laboratory, Lot R 5133E (goat, Scantibodies Lab., Lakeside, CA). This serum was diluted to 1 : 100 before use, giving the same staining image obtained by Abbott antiserum.

### *Immunoperoxidase Staining*

The indirect immunoperoxidase technique of Nakane and Pierce was applied [*7*]. The sections ready for staining were washed several times thoroughly with 0.1% Triton X-100

phosphate-buffered saline and coated with normal rabbit serum at the dilution of 1 : 10 for 30 min. After this, the rabbit serum was discarded and the sections were coated with anti-phenobarbital serum for 1 h at room temperature.

The samples were then thoroughly washed with Triton X-100-phosphate-buffered saline and sensitized with 1 : 100 diluted peroxidase conjugated rabbit anti-goat IgG (Cappel Laboratories Inc., U.S.) for 15 min at room temperature. The samples were washed with Triton X-100 containing phosphate-buffered saline and then stained with a 3-3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co. Ltd., U.S.) hydrogen peroxide mixture. Inactivation of endogenous peroxidases was performed before the coating with normal rabbit serum. Counterstaining with hematoxyline was performed.

The electron microscopic immunocytochemistry was performed according to the pre-embedding method of Nakane et al. [7]. As we have shown in the experimental results, a distinct positive reaction was attained when the tissues of the phenobarbital exposed animals were tested. This positive reaction was completely inhibited when phenobarbital was added to the antiserum before use.

## Experimental Results

### *General Remarks*

*The Effects of Fixatives on the Phenobarbital Fixation*—As we have described above in the Method section, the only effective fixative was that containing glutaraldehyde. We also examined, as in the case of immunocytochemistry of amphetamines, the effects of other fixatives including formalin, paraformaldehyde, and other organic substances such as methanol and acetone, but no indication of drug fixation was found. We are now investigating the histologic demonstration of morphine by the same method and have found that glutaraldehyde is the only effective reagent for this purpose.

*Macroscopic Findings of Stained Slides*—As shown in Fig. 1*a, b, c* (brain, liver, kidney), positive staining was found in the tissues from the animals that were exposed to phenobarbital. The positive reaction was so distinct that macroscopic observation of the stained slices is sufficient for a clearcut diagnosis of intoxication. As for the sensitivity of the reaction, we could discern a positive reaction in the animals that were administered about 0.3 mg. Hence, since the body weight of the experimental animals was about 30 g, the concentration of phenobarbital in the whole body was about 10 mg/kg. This sensitivity was raised to 0.1 mg, provided that the control tissues (animal without intoxication) were stained simultaneously on the same slide. This definitely indicates that tissues from autopsy materials from phenobarbital intoxication in the acute stage can be used immunohistochemically.

### *Staining Aspects of Phenobarbital in Various Tissues in the Immunocytochemistry*

*Brain*—Figure 2 shows the frontal cut surface of the brain from an animal given 1 mg of phenobarbital. The cut surface lies in the level running from the frontal lobe to the bulbus olfactorius. A very strong reaction was noticed in the nerve cells of the stratum pyramidale internum, while some cells in other regions showed strong staining, but others were devoid of the reaction. The glial cells in all regions gave positive reactions of varying strength. The myelin sheaths were also stained, but the glomeruli in the stratum glomerulosum gave no staining even in the animals that were administered 10 mg of phenobarbital. The cortex in Fig. 2 represents the same pattern of staining as in Fig. 3.

Figure 3 demonstrates the frontal cut surface at the level that runs from the parietooccipital lobe to the tuber olfactorium. In the cortex region, we always found a negative reaction in the lamina zonalis. With respect to the reactivity of nerve cells in the cortex layers, it was found, contrary to amphetamine intoxication, that the positive reaction was discerned in

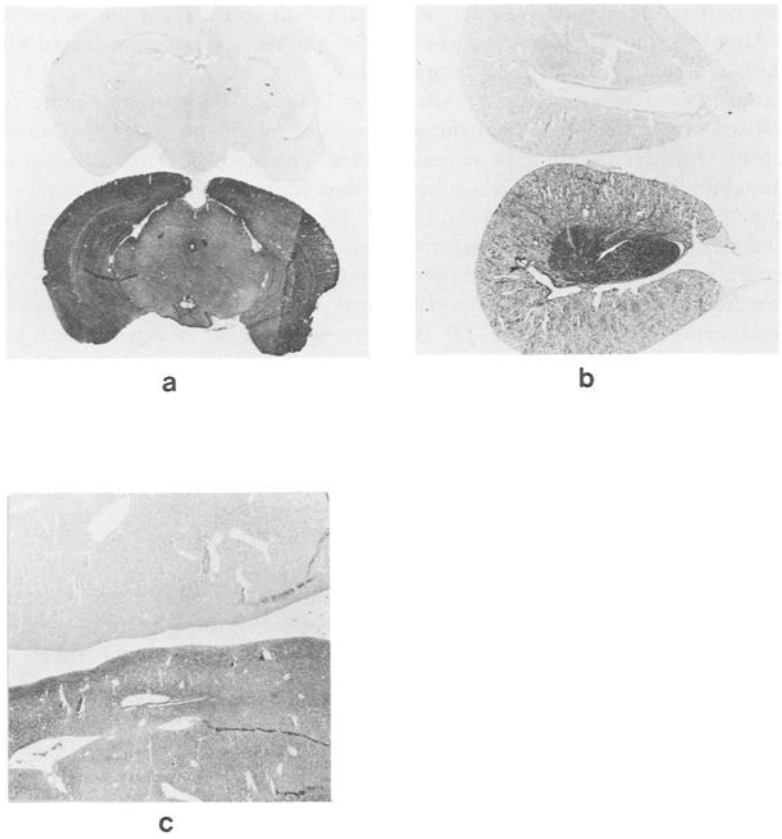



FIG. 1—*Macroscopic immunohistochemistry of the stained slides:* (a) *brain,* (b) *kidney, and* (c) *liver.* Upper image: *control animal without phenobarbital.* Lower image: *experimental animal with 1 mg of phenobarbital. Positive reaction* (dark brown color) *was noticed in the tissues from the experimental animals, while no positive finding was found in the control samples (Mag. 1 : 4).*

almost all cells located in the deeper layers (lamina multiformis and intima), while those in the upper layers (lamina corpuscularis to ganglionaris) were sometimes lacking in reactivity.

The nerve cells in the stratum and thalamus showed a positive reaction, but some cells were free from the staining. Interesting findings were obtained in the Ammon's horn and fascia dentata. The nuclei of the nerve cells in these regions were stained positively, so that their frameworks were finely demonstrated by immunocytochemistry. The cells with no reaction were also scattered throughout. Glial cells were stained positively. The myelin sheaths were also stained and a somewhat strong reaction was found in the region of the striatum (Fig. 4).

The findings of Purkinje's and granular layer cells in the cerebellum were very interesting. In other brain regions, as described previously, the cells gave a variety of positive reactions, but no positive reaction was found in the cells located in the cerebellum. On the other hand, a very strong reaction was discerned in the surrounding Bergmann's glial cells. These results are shown in Fig. 5. Figure 6 indicates the reaction pattern of the medulla oblongata. This region is considered to be very important, as the affect on this region by phenobarbital induces paralysis of the respiratory and circulatory centers. Generally, all nerve cells located in

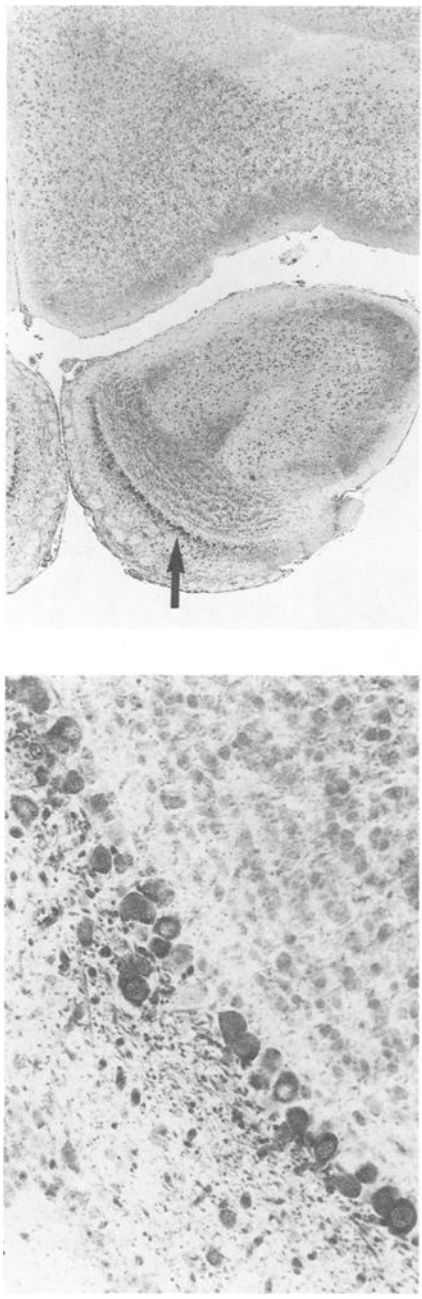



FIG. 2—*Light microscopic immunohistopathology of the brain.* (a) *The olfactory bulb together with the cerebral cortex was shown. As indicated by arrow, a strong positive reaction was shown in the nerve cells located at the region of stratum pyramidale internum. The dots in the figure indicate the nerve cells with a positive reaction (Mag. 1:20).* (b) *The cells located at the stratum pyramidale internum are indicated. The cytoplasm was positively stained. Some nuclei were also stained. A few cells, as indicated by arrow, are negative (Mag. 1:400).*

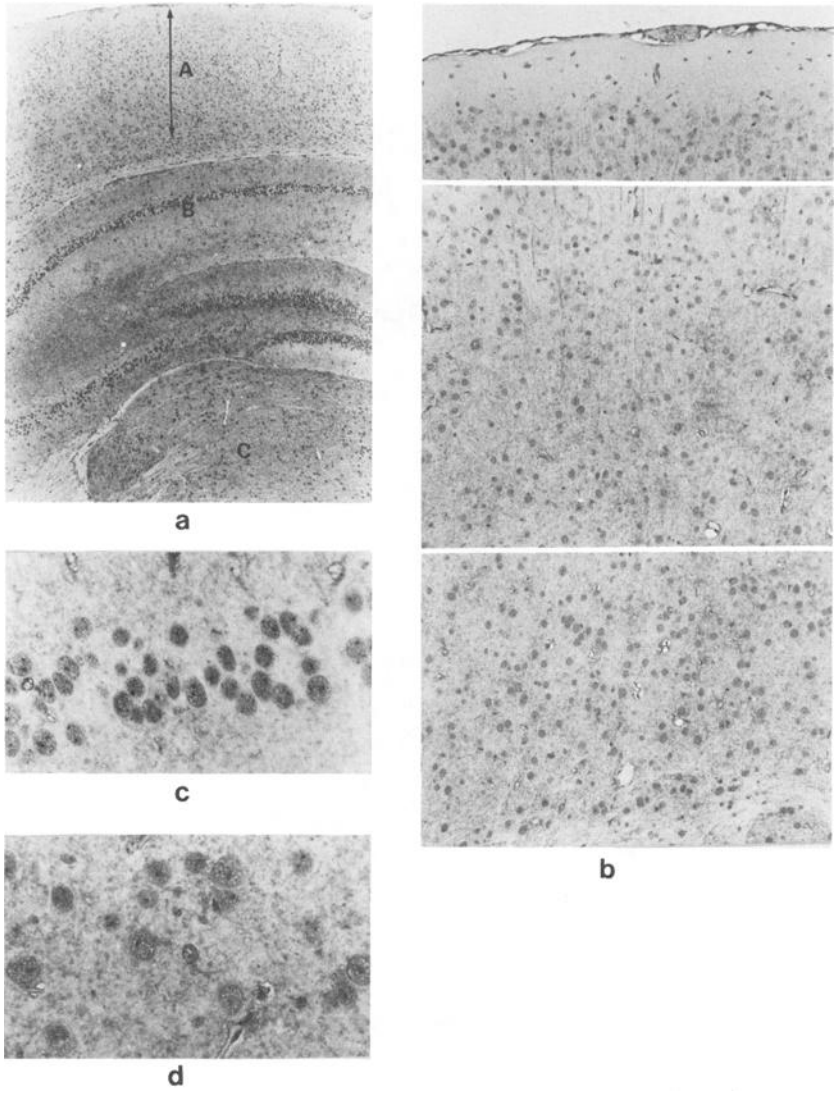



FIG. 3—*Light microscopic immunohistochemistry of the brain.* (a) *The cerebral cortex, Ammon's horn, fasciculus dentatus and brain stem are shown. The dots in the figure show the cells stained positively.* A, B, *and* C *indicate the sites in the following series of figures (Mag. 1 : 20).* (b) *The area* A *in* (a) *is shown. Although the cells are stained diffusely in the cerebral cortex, the ones located in the deeper layers react more strongly than in the superficial layers (Mag. 1 : 100).* (c) *The area* B *in* (a) *is shown. The nuclei of the nerve cells were positively stained (Mag. 1 : 400).* (d) *The area* C *in* (a) *is shown. The cells in the brain stem were also stained like those in Ammon's horn.*

the middle part of the medulla reacted very strongly, while those in the ventrolateral region were completely negative as a group. At present, no definite evaluations as to the function of the cells with negative findings are known. A precise investigation based on the anatomy of the mouse brain is in progress.

*Liver*—We had already noted in the experiments with amphetamines that paraffin and

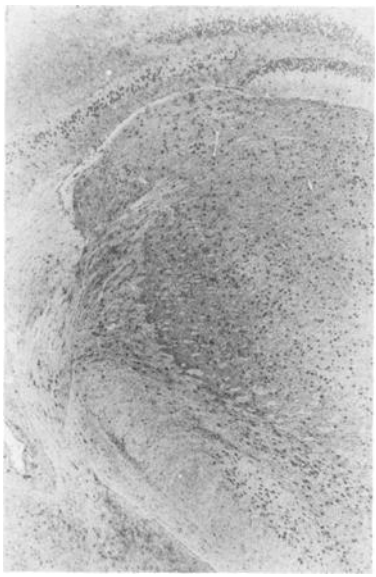


FIG. 4—*Light microscopic immunohistochemistry of the striatum (Mag. 1:20). Some nerve fibers were strongly positive; the nuclei in this region showed the same images, as indicated in Fig. 3*(d).

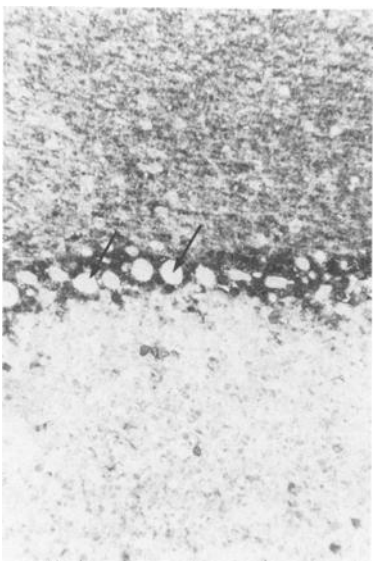


FIG. 5—*Light microscopic immunohistochemistry of the cerebellum (Mag. 1:200). The Purkinje's and granular layer cells were entirely negative, while the cerebellar cortex was stained positively.*

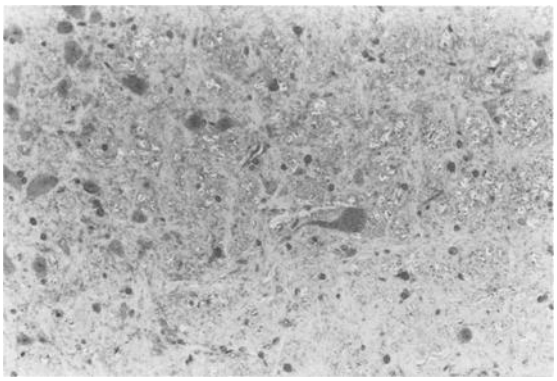


FIG. 6—*Light microscopic immunohistochemistry of the medulla oblongata (Mag. 1 : 400). Predominantly positive reaction was found in the large nerve cells located in the medulla oblongata.*

frozen sections gave some entirely different images. This result was found with phenobarbital also, as shown in Fig. 7. The frozen sections gave predominantly positive staining in the cytoplasm with some vesicular conformation, and the sinusoids were faintly positive. On the other hand, paraffin sections gave no remarkable staining in the cytoplasm, while the sinusoids were apparently positive. This clearly indicates that tissue fixed drugs in the liver are likely to be dissolved by treating the tissue with organic solvents during the preparation of paraffin sections. This artifact seemed, however, to be exceptional, and no tissue other than the liver showed this phenomenon. Electron microscopic immunocytochemistry of the liver tissue will be reported later in this paper.

*Kidney*—The same images as described with amphetamines were found. A positive reaction was noted in the glomeruli and distal part of the renal tubules, especially in the outer zone of the medulla (Fig. 8). No conspicuous reaction was seen in the proximal part of the renal tubules.

*Lung*—Alveolar and bronchial epithelial cells gave a positive reaction. In some alveoli, we found a remarkable excretion of the drug. Some of these findings are shown in Fig. 9.

*Spleen*—Macroscopically, all of the tissue seemed to be stained positively. But on microscopic examination, the positive reaction was found predominantly in the red pulp, while that in the white pulp was very faint.

*Others*—As indicated previously, the endothelial cells of the capillaries gave a distinct positive reaction. Red cells were also stained positively. This is presumably due to the nonspecific absorption of the drug onto their surface. A positive reaction was found rarely in a few cardiocytes (Fig. 10).

### *Electron Microscopic Immunocytochemistry of Liver Tissue*

Previous investigations in biochemical toxicology have shown that the xenobiotics are mainly biotransformed in the smooth endoplasmic reticulum in the hepatocytes. We have confirmed this by electron microscopic immunocytochemistry.

According to the method of Nakane et al. [7], the frozen section of the liver in a thickness of 6 $\mu$m was prepared and processed by the procedure of preembedding. The results are shown in Fig. 11.

As expected, only positive images were found in the smooth endoplasmic reticulum; no trace of phenobarbital was present in other intracellular compartments. The endothelial cells of the capillaries gave distinct positive staining.

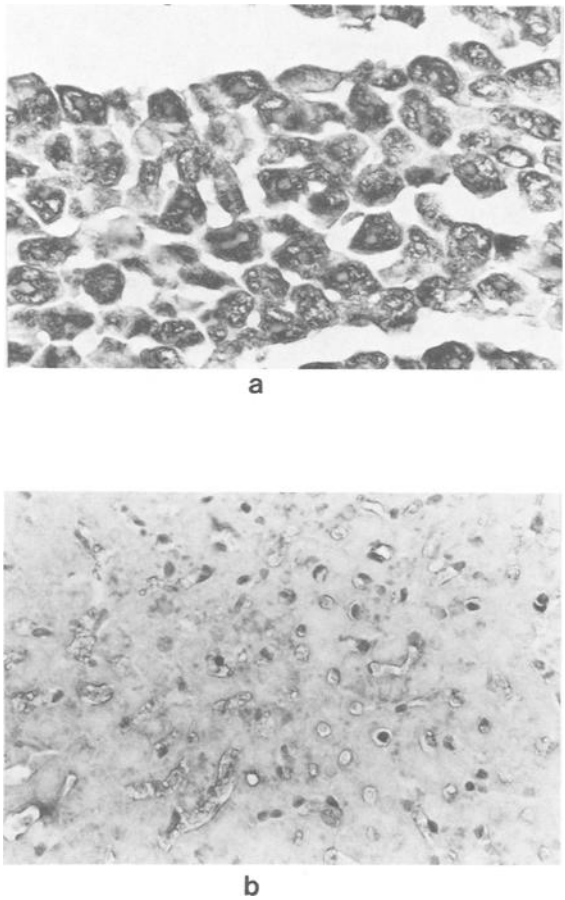
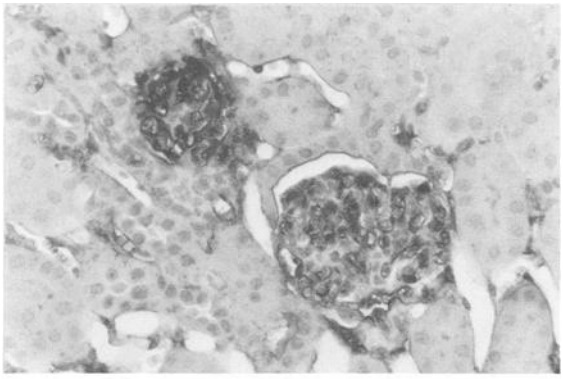



FIG. 7—*Light microscopic immunohistochemistry of the liver (Mag. 1 : 400).* (a) *Frozen section: the hepatocytes were stained positively. Positive images were located in the granular compartment in the hepatocyte.* (b) *Paraffin section: positive reaction was not found in staining the paraffin section. The endothelial lining of sinusoid was demarcated by a distinct positive line.*

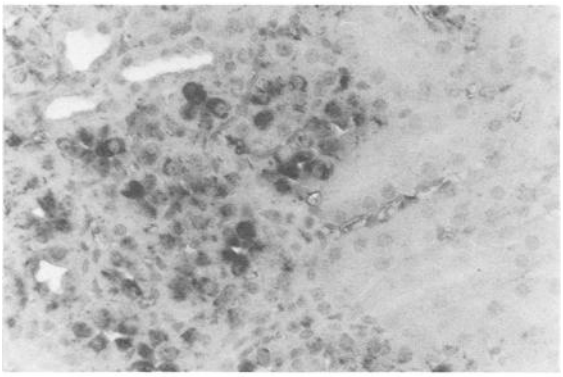
## Discussion

It is evident from our experiments that phenobarbital administered in lethal doses, as encountered in forensic autopsy, can be detected by staining the tissues with the usual indirect immunoperoxidase method. The results are so distinct and specific that macroscopic observation of the stained slides gives a clearcut diagnosis of drug intoxication. In this manner, forensic pathomorphology can be performed more precisely. The most important point in staining the drugs in the tissues is fixation. If death as a result of drug intoxication is suspected, small pieces of various tissues, especially the brain, liver, and kidney, should be preserved in fixatives containing glutaraldehyde, so that the immunohistochemical examinations can be conducted in parallel with the chemical procedures. We have succeeded in demonstrating tissue fixed amphetamines, phenobarbital, and morphine by the same technique.

As for the fixation mechanism of drugs in tissues by glutaraldehyde, no reasonable elucidation has been attained, although this process is one of the most important steps in applying immunocytochemistry in the research fields of toxicology and pharmacology. To eluci-



a



b

FIG. 8—*Light microscopic immunohistochemistry of the kidney (Mag. 1:400).* (a) *The glomeruli were stained positively; no reaction was observed in the proximal tubules.* (b) *Positive finding was observed in the distal tubules in the outer zone of medulla.*

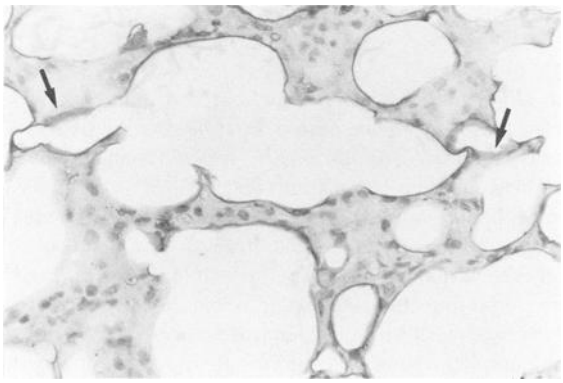


FIG. 9—*Light microscopic immunohistochemistry of the lung (Mag. 1:400). The alveolar lining was stained distinctly; the cells indicated by arrows are Clara cells, which were stained remarkably.*

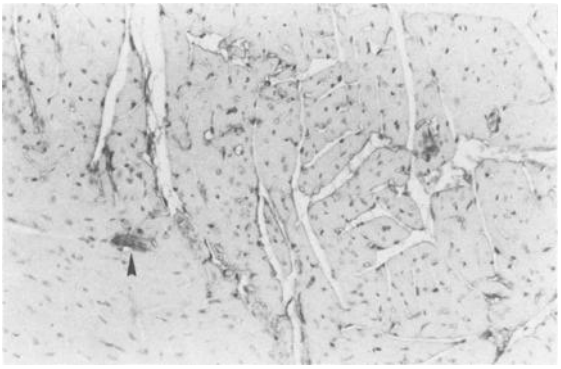


FIG. 10—*Light microscopic immunohistochemistry of the myocardium (Mag. 1:200). The positive reaction was found in the endothelial cell linings of capillaries. The arrow indicates the cardiocyte with a positive reaction.*

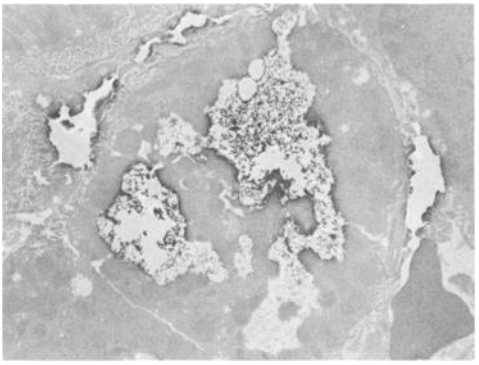


FIG. 11—*Electron microscopic immunohistochemistry of the liver (Mag. 1:5600). The positive images were strictly located in the glycogen area or the smooth endoplasmic reticulum. The endothelial cells were stained positively.*

date the true mechanism of this reaction, we are now conducting investigations using various kinds of amphetamine derivatives (amphetamine, methamphetamine, *N*-dimethyl amphetamine, aminoethyl amphetamine, aminopropyl amphetamine, and so forth). In considering these experimental results, which will be reported elsewhere, the formation of Schiff's complex does not seem to be an essential one. The specific combination of drugs and their cell receptors is likely to be the first step for the fixation. Thus, the effect of glutaraldehyde might be the conversion of this combination to the irreversible state. In this respect, the fixation with glutaraldehyde may be considered to be a theoretically optimal procedure in analyzing the drug fixation to the target cells. The application of immunocytochemistry to drug intoxication has a distinct advantage in demonstrating the topographic distribution in vivo. For example, previous histochemical investigations in this field have been performed with the aid of autoradiography, but the results obtained by this method permit solely the analysis of drug distribution at the level of submicroscopic examination, and practical application in forensic pathology is impossible. Hence, immunocytochemical investigation to de-

termine the topographic distribution of drugs might be expected to be a fundamental tool in analyzing the pharmacodynamics at a cellular level based on morphological evidence.

This preliminary experiment revealed several interesting findings as to the pattern of the topography of drugs in the tissues, which may provide information concerning the action of drugs. They are as follows:

1. Some remarkable difference between amphetamines and phenobarbital with respect to the topographic distribution in the cerebral cortex should be noted. The amphetamines are distributed more densely in the cells located in the superficial layers of the mouse cerebral cortex, while phenobarbital is found more densely in those in the deeper layers. The localization of drugs in the superficial layers of the cerebral cortex might be explained by the anatomical specificity of the cerebral cortex, that is, by the abundant supply of blood as a result of the rich anastomotic vasculature in the superficial layers. But this assumption is contradictory, since some drugs are distributed in a reverse manner. The selective uptake into some nerve cells might be a more reliable explanation for such phenomena.
2. Some nerve cell systems, such as Purkinje's and granular layer cells in the cerebellum, were entirely negative in our experiment. As the surrounding Bergmann's glial cells contained an abundant amount of phenobarbital, the findings in these cells can be explained solely by the blockage of drug uptake.
3. With respect to the topographic distribution of phenobarbital in the nerve cells, the most characteristic finding is that the nuclei of the cells are predominantly stained. The same results were obtained in the intoxication caused by methamphetamine. It might be possible that this finding is some sort of artifact that arises during the preparation of the histologic slides. The nuclei of other cells, such as liver, renal, and endothelial cells, were, however, devoid of positive staining. Furthermore, the staining pattern of nerve cell nuclei demonstrating the fine intranuclear frameworks reveals that the preference of the nuclei of nerve cells to phenobarbital is a more reliable one. The pharmacological findings as a result of the acute phenobarbital intoxication are very scanty. The change of synaptic curvature [*8,9*], mitochondrial degeneration [*10*], and myeline degeneration [*10*] are reported, but the practical application in the human pathology is uncertain. Such findings, when they are investigated together with the topographic distribution of phenobarbital in the nerve tissue, may support their pathological significances.

The effects of phenobarbital on the prenatal nerve cells have been investigated more precisely [*11-14*]. Yanai et al. [*14*] have shown that the administration of phenobarbital to pregnant mice induces the tremendous reaction of the ($^3$H)-thymidine labeled nerve cells in various nuclei of the central nervous system, and that this autoradiography is very sensitive and accurate in estimating the toxic effects of various drugs in the prenatal stage. It is expected, therefore, that the combination of the direct immunohistochemistry of drugs and concomitant autoradiography using ($^3$H)-thymidine affords the precise information as for the actual aspects of drug action at embryonal stage. Furthermore, some similarity of the preferential uptake of phenobarbital by nerve cells at the prenatal and adult stages of mice was found. Yanai et al. [*14*] have confirmed that the nerve cells located in the deeper layer of mouse cerebral cortex are much more sensitive to phenobarbital action. The findings in our experiment with respect to the phenobarbital distribution in the cerebral cortex may be elucidated by the pharmacological specificity of the nerve cells, which are demonstrated in the prenatal stages of mice. In this respect, the histochemistry of drug distribution in the brain of adult animals might be indicative of the occurrence of drug induced malformation in fetal animals.

On the other hand, a distinct contradiction was found in the investigation of Purkinje's cells, which are the most vulnerable cells in the central nervous system of prenatal stages against phenobarbital action. The difference of the pattern of uptake of drugs in the adult and prenatal cells is the more reliable explanation, but the gender difference must be considered, as the Purkinje's cells from female prenatal mice are the target ones against this drug.

Another interesting finding in our experiment is the positive staining of the nuclei of nerve cells. As this drug is a potent inducer of gross malformations in mice [*15*], the fixation of phenobarbital with the nuclear matrix can be interpreted as direct evidence of the reaction inducing the malformation in the brain—although the uptake of the drug in the nuclei is not stable, but considerably transient, as the deposition of the drugs disappears within a short duration of several hours after the innoculation. The metabolic effect of the drug, based on morphology will be reported elsewhere.

Electron microscopic immunocytochemistry should be regarded as an essential tool for the investigation of the pharmacodynamics of the drug in vivo. It was found that the topographic distribution of phenobarbital in the hepatocytes is restricted to the intraluminal spaces of smooth endoplasmic reticulum. This supports the famous findings in the biochemical toxicology that the detoxication of xenobiotics occurs in this cell compartment, in which the major cytochrome P-450 dependent enzyme activity is located [*16*]. The positive findings in the lung may implicate the drug uptake by Clara and alveolar type-II cells, which contain the same enzymes abundantly [*17–21*]. Thus, the biochemical data are easily substantiated by morphological evidence.

## Conclusion

Finally, our experiment indicates some important aspects of the development of the new field of toxicological investigation. Because of the wide application of ELISA to the immunochemical assay of drugs in clinical medicine, we have many opportunities to obtain excellent sera against various drugs from commercial sources, which may be very useful in investigating the morphological evidence of topographic distribution of drugs in the tissue cells. This indicates clearly that the new research field of morphological toxicology, which is so far relatively untapped, can be advanced much more easily and exactly by introducing these commercial antisera.

## References

[*1*] Cassano, G. B., Gherti, B., Gliozzi, E., and Hansson, E., "Autoradiographic Study of 'Short Acting' and 'Long Acting' Barbiturates: $^{35}$S-Thiopentone and $^{14}$C-Phenobarbitone," *British Journal of Anaesthesia,* Vol. 39, 1967, pp. 11–20.

[*2*] Saubermann, A. J., Gallagher, M. L., and Hedley-Whyte, J., "Uptake, Distribution and Anaesthetic Effect of Phenobarbital-2-$^{14}$C after Intravenous Injection into Mice," *Anaesthesiology,* Vol. 40, 1974, pp. 41–51.

[*3*] Saubermann, A. J., Gallagher, M. L., Hedley-Whyte, E. T., and Hedley-Whyte, J., "Cellular and Regional Localization of Pentobarbital-2-$^{14}$C by Radioautography of Selected Area of Mouse Brain at Loss and Return of Withdrawal Response," *Anaesthesiology,* Vol. 42, 1975, pp. 254–266.

[*4*] Schechter, P. J. and Roth, L. J., "The Distribution of Thiopental-2-$^{14}$C in Maternal and Fetal Tissues of the Rat," *Journal of Pharmacology and Experimental Therapeutics,* Vol. 158, 1967, pp. 164–173.

[*5*] Shin, T., Nakamura, S., and Shigematsu, A., "Macroautoradiographic Study of Distribution and Excretion of $^{14}$C-Pentobarbitone in Rats Pretreated with Phenobarbitone," *British Journal of Anaesthesia,* Vol. 49, 1977, pp. 847–853.

[*6*] Ishiyama, I., Mukaida, M., and Yoshii, T., "Histochemical Demonstration of Methamphetamine by Immunocytochemistry," *Journal of Forensic Sciences,* Vol. 32, No. 3, May 1987, pp. 658–672.

[*7*] Nakane, P. K. and Pierce, G. A., "Enzyme-Labeled Antibodies for the Light and Electron Microscopic Localization of Tissue Antigens," *Journal of Cell Biology,* Vol. 33, 1966, pp. 307–318.

[*8*] Cooke, C. T., Nolan, T. M., Dyson, S. E., and Jones, D. G., "Pentobarbital-Induced Configurational Changes at the Synapse," *Brain Research,* 1974, Vol. 76, pp. 330–335.

[*9*] Jones, D. G. and Devon, R. M., "An Ultrastructural Study into the Effects of Pentobarbitone on Synaptic Organization," *Brain Research,* Vol. 147, 1978, pp. 47–63.

[*10*] Fishman, R. H. B., Ornoy, A., and Yanai, J.: "Ultrastructural Evidence of Long-Lasting Cerebellar Degeneration after Early Exposure to Phenobarbital in Mice," *Experimental Neurology,* Vol. 79, 1983, pp. 212–222.

[*11*] Bergman, A., Rosselli-Austin, L., Yedwar, G., and Yanai, J., "Neuronal Deficits in Mice Follow-

ing Phenobarbital Exposure During Various Periods of Fetal Development," *Acta Anatomica*, Vol. 108, 1980, pp. 370-373.

[*12*] Yanai, J., Rosselli-Austin, L., and Tabakoff, B., "Neuronal Deficits in Mice Following Prenatal Exposure to Phenobarbital," *Experimental Neurology*, Vol. 64, 1979, pp. 237-244.

[*13*] Yanai, J. and Tabakoff, B., "Altered Sensitivity to Ethanol Following Prenatal Exposure to Barbiturates," *Psychopharmacology*, Vol. 68, 1980, pp. 301-303.

[*14*] Yanai, J., Woolf, M., and Feigenbaum, J. J., "Autoradiographic Study of Phenobarbital's Effect on Development of the Central Nervous System," *Experimental Neurology*, Vol. 78, 1982, pp. 437-449.

[*15*] Persuad, T. V. N. and Menderson, W. M., "The Teratogenicity of Barbital Sodium in Mice," *Arzneimittel-forschung*, Vol. 19, 1969, pp. 1309-1310.

[*16*] Peterson, F. J. and Holzman, J. L., "Drug Metabolism in the Liver—A Perspective," in *Extrahepatic Metabolism of Drugs and Other Foreign Compounds*, T. E. Gram, Ed., MTP Press Ltd., International Medicine Publishers, 1980, pp. 1-121.

[*17*] Bend, J. R., Serabjit-Singh, and Philpot, R. M., "The Pulmonary Uptake, Accumulation, and Metabolism of Xenobiotics," *Annual Review of Pharmacology and Toxicology*, Vol. 25, 1985, pp. 97-1985.

[*18*] Boyd, M. R., "Evidence for the Clara Cell As a Site of Cytochrome P-450 Dependent Mixed-Function Oxidase Activity in Lung," *Nature*, Vol. 269, 1977, pp. 713-714.

[*19*] Devereux, T. R. and Fouts, J. R., "Isolation of Pulmonary Cells and Use in Studies of Xenobiotic Metabolism," *Methods in Enzymology*, Vol. 77, 1981, pp. 147-154.

[*20*] Jones, K. G., Holland, J. F., Foureman, G. L., Bend, J. R., and Fouts, J. R., "Induction of Xenobiotic Metabolism in Clara Cell and Alveolar Type-II Cells Isolated from Rat Lungs," *Journal of Pharmacology and Experimental Therapeutics*, Vol. 225, 1983, pp. 316-319.

[*21*] Serabjit, C. J., Wolf, C. R., Philpot, R. M., and Plopper, C. G., "Cytochrome P-450: Localization in Rabbit Lung," *Science*, Vol. 207, 1980, pp. 1469-1970.

Address requests for reprints or additional information to
Ikuo Ishiyama, M.D.
Department of Forensic Medicine
University of Tokyo
113-Tokyo, Bunkyo-ku, Hongo 7-3-1
Japan