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Histochemical Demonstration of Methamphetamine by Immunocytochemistry

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ABSTRACT: A method for the demonstration of methamphetamine (MA) by immunocytochemistry was established. The tissues of intoxicated mice, administered various amounts of MA in single doses of from 0.01 to 1 mg of MA-HCl, were fixed in glutaraldehyde-containing fixatives. Cryostat and paraffin slices gave a positive reaction of MA localization by staining the brain, liver, kidney, lung, stomach, spleen, and so forth, with the aid of the indirect immunperoxydase technique. Those of animals administered a single dose of 0.1 mg or more (over 3 to 4 mg/kg—the usual dose of MA in acute intoxication death in forensic medicine), in particular, gave a strong strong reaction, so that the diagnosis of MA intoxication can be performed by macroscopic observation of stained slices. The histochemical diagnosis of MA intoxication in clinical toxicology and pathology might be regarded as a useful tool, especially in forensic pathology. The following cells gave a strong positive reaction: nerve cells and myelin sheaths, hepatocytes, epithelial cells of the distal part of the renal tubule and of the collecting tubule, alveolar and bronchial epithelial cells of the lung, chief and parietal cells of the gastric gland, capillaries of the renal glomerulus, macrophages in the blood and tissues, and striated muscle cells including cardiocytes. The morphological evidence of the pharmacodynamics and pharmacokinetics of MA can be determined at the cellular level by immunocytochemistry.

KEYWORDS: pathology and biology, methamphetamine, cytochemistry, histology

Histochemical investigations of the biogenic endogeneous amines, such as serotonin [*1,2*]; histamine [*3*], epinephrine, norepinephrine [*4*], and 5-, 6-, and 5,6-hydroxytryptamine [*5*], have been performed by immunocytochemistry and become some of the fundamental techniques in neurochemical research. These experiments indicate clearly that the topographical distribution of drugs in the tissues might be demonstrated in the same way, using various antisera against drugs, which have been developed in clinical medicine for the application of immunoassay.

Based on this hypothesis, we have investigated the immunocytochemistry of methamphetamine (MA) in the tissues. Abuse of this stimulant has become a serious social problem in Japan. By testing various measures for this purpose, we have established a method of staining MA in the tissues.

The results seem to be very important not only for the histological diagnosis of MA intoxi-

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cation in forensic science autopsy, but also for morphological evidence of the pharmacodynamics and pharmacokinetics of this drug.

Materials and Methods

Preparation of Antiserum Against MA

Antigen—The antigen for the induction of anti-MA was prepared according to the method of Cheng et al. [6]. Methamphetamine hydrogenchloride (HCl) (2.00 g) was dissolved in 30 mL of 10% sodium hydroxide (NaOH) and extracted with 100 mL of ether. This extract containing free MA was dried with magnesium sulfate ($MgSO_4$) and evaporated. MA thus obtained (1.59 g) was dissolved in 40 mL of benzene. To this, *N*-(4-bromobutyl)phthalimide (3.80 g) and anhydrous sodium carbonate (Na_2CO_3) (1.80 g) were added and refluxed for 30 h at 80°C. After this, the mixture was filtered, and the filtrate was acidified with 48 mL of 0.1*N* HCl, and stirred continuously. Subsequently, the water layer was extracted by adding 8 mL of chloroform at each of four times.

This chloroform extract was washed with 10 mL of saturated NaCl and dried by adding anhydrous $MgSO_4$. This chloroform layer was evaporated. The yield thus obtained was 3.01 g. This was added to 40.9 mL of 95% ethanol and 0.2 mL of 90% hydrazine hydrate and refluxed at 90°C for 2 h. This solution was then acidified by adding 1*N* HCl and filtered. The filtrate was evaporated, and the residue was dissolved in 1*N* HCl and washed with chloroform several times until the phthalhydrazine disappeared in the chloroform layer. The water layer was brought to an alkaline pH by adding 5*N* NaOH and extracted by adding 5 mL of chloroform each time for five times. This chloroform layer (about 25 mL) was dried by anhydrous $MgSO_4$ and filtered. The filtrate was evaporated. The yield of *N*-(4-aminobutyl)-MA was 1.28 g. The chemical confirmation of this substance was performed by nuclear magnetic resonance (NMR) analysis.

Conjugation of the N-(4-aminobutyl)-MA and Bovine Serum Albumin—The conjugation of the bovine serum albumin (BSA) and MA derivative was performed according to the method of Aoki et al. [7]. A mixture of 20 mg of *n*-(4-aminobutyl)-MA in 40 μL of dimethylformamide, 6 mL of 0.1*M* phosphate-buffer solution at pH 7.0, and 45 mg of BSA (Miles Scientific, 30 W475 North Aurora Road, Naperville IL, 60566) was treated dropwise with 0.5 mL of 1% aqueous solution of glutaraldehyde with gentle stirring. The reaction mixture was allowed to stand for 2 h. After dialysis against phosphate buffer solution, pH 8.0, at 4°C for 8 h, sodium borohydride (2 mg) was added. The reduction was allowed to proceed at 4°C overnight. The aliquot mixture was centrifuged and the sediment was discarded. This BSA-conjugate solution was used for the antibody production.

Immunization—Of the BSA-conjugate solution, 0.6 mL was diluted by adding 1.4 mL of phosphate buffered saline (PBS). To this, the same volume of complete Freund's adjuvant was added and mixed thoroughly. The mixture was injected subcutaneously into three rabbits (each animal received about 1.3 mL). The immunization was repeated eight times at intervals of one month (first to second injections) and two weeks (second to eighth).

The titer of the antiserum thus obtained was estimated by the method of the mixed agglutination reaction of Shibata et al. [8]. All sera from various animals gave the titer of 1:32 000. No remarkable difference was found in these antisera.

The specificity of this anti-MA was assayed by the enzyme immunoassay for methamphetamine according to the method of Aoki and Kuroiwa [9]. The levels of amphetamine derivatives, which induced the 50% inhibition of the antigen-antibody reaction, were as follows:

N-(4-aminobutyl)methamphetamine	0.000 24 m*M*,
methamphetamine	0.11 m*M*,
amphetamine	1.2 m*M*, and
ephedrine	2.5 m*M*.

Animals

Black mice B10.A(3R) were kindly supplied by the Department of Immunology, University of Tokyo. They were administered 1, 0.1, or 0.01 mg of MA intraperitoneally or subcutaneously. After 30 min, the animals were killed and the tissues were removed, cut to a thickness of 2 mm, and immersed in the fixatives at 4°C.

Fixation of Tissues

The tissues were fixed in the following four kinds of fixatives:

(1) 10% formalin,
(2) 4% paraformaldehyde-containing phosphate buffer solution in 0.1*M* at pH 7.4,
(3) 4% glutaraldehyde-containing phosphate buffer solution in 0.1*M* at pH 7.4, and
(4) 2% glutaraldehyde and 4% paraformaldehyde-containing phosphate buffer solution in 0.1*M* at pH 7.4.

Solutions 2, 3, and 4 were prepared before use and precooled at 4°C. The tissues were fixed in these fixatives for 3 h. Fixation by Solution 1 was continued at 4°C overnight. After fixation, they were washed with 15% sucrose-containing PBS at 4°C overnight.

Preparation of Histological Slices

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

Immunoperoxydase Staining

The indirect immunoperoxydase technique of Nakane and Pierce was applied [*10*]. The slices ready for the staining were washed thoroughly with 0.1% Triton X-100 containing phosphate buffered saline several times and coated with normal goat serum at the dilution of 1:10 for 30 min. After this, the goat serum was discarded and coated with anti-MA at the dilution of 1:200 for an hour at room temperature. After this, the samples were thoroughly washed with Triton X-phosphate buffered saline and sensitized with 1:100 diluted peroxidase conjugated goat antirabbit immunoglobulin G (IgG) (Cappel Laboratories Inc., U.S.A.) for 15 min at room temperature.

The samples were washed with Triton X-containing phosphate buffered saline and then stained with 3-3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co. Ltd., U.S.A.)-hydrogen peroxide (H_2O_2) mixture. Inactivation of endogeneous peroxidases was performed before the coating with normal goat serum. Counter staining with hematoxylin was performed.

As we have shown in the experimental results, a distinct positive reaction was attained when the tissues of the MA exposed animals were tested. This positive reaction was completely inhibited when MA was added to the anti-MA before use.

Experimental Results

Effects of Various Fixatives on MA Fixation

Comparison of the fixation of MA by testing four kinds of fixatives revealed that the fixation of MA in the tissue could be detected only when the fixative contained glutaraldehyde. On the other hand, as had been previously expected, some difficulties were found in prepar-

ing the histological slices from the samples fixed with 4% glutaraldehyde-containing phosphate buffer solution. On the basis of these results, we have used the glutaraldehyde-paraformaldehyde-containing phosphate buffered fixative in this experiment.

Staining Aspects of MA in Various Tissues in the Immunocytochemistry

As shown in Figs. 1 (brain), 4 (liver), 6 (kidney), 10 (lung), and 12 (stomach), strong positive reactions were observed in the cases of MA intoxication at doses of 1 and 0.1 mg (about 30 to 40 and 3 to 4 mg/kg, respectively), and samples from the intoxicated animals could be easily discerned by macroscopic observation of the stained slices. A distinct positive reaction could be attained even in the cases of 0.01-mg administration (about 0.3 to 0.4 mg/kg) by comparing the staining results of the control sample on the same slide glass.

The following immunohistochemical findings were noted.

Brain—A diffuse positive reaction was observed macroscopically in the acute intoxication induced by the doses of 1 and 0.1 mg (Fig. 1). The findings of the nerve tissue included positive reactions in the cerebral cortex white matter. There was a negative finding in the region of the cerebral cortex, which corresponds to the zona marginalis of the cortex layers of the human brain (Fig. 2). On further magnification (Fig. 3), somewhat distinct aspects of the MA localization in the brain were noticed. The nerve cells of the outer layers of the

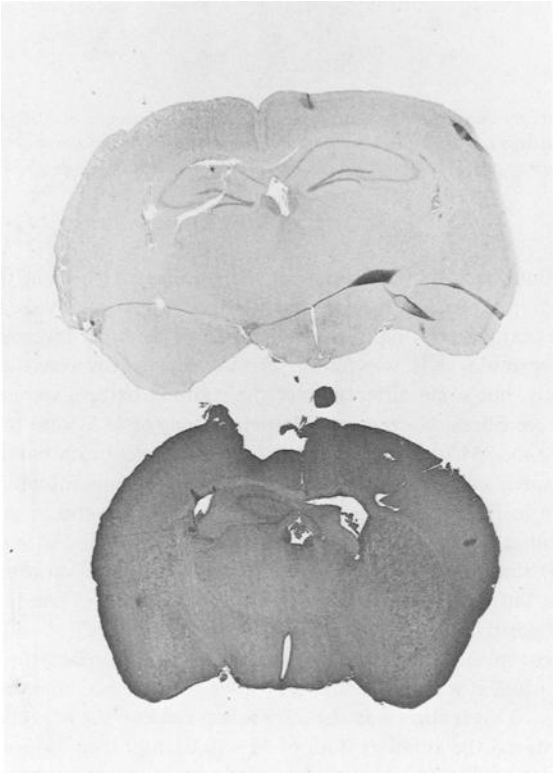

FIG. 1—*Macroscopic finding of MA-exposed brain tissues.* Upper tissue: *the brain from the control animal.* Lower tissue: *the brain from the experimental animal. Tissues are stained by indirect immunoperoxidase technique. Counterstain is hematoxylin staining (×10).*

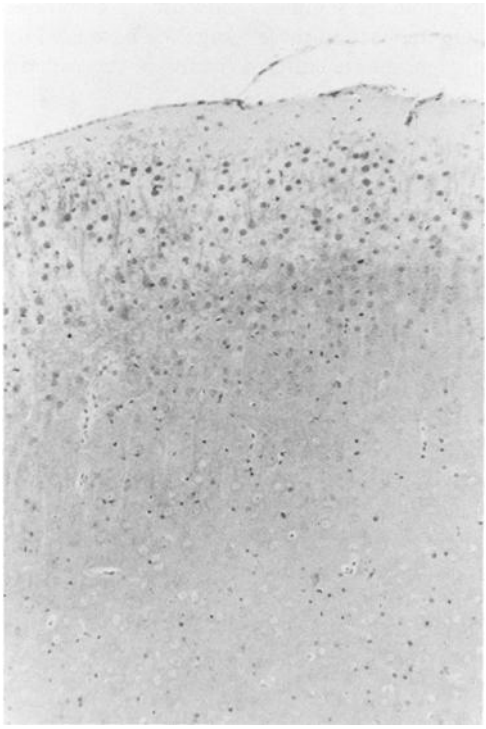

FIG. 2—*Positive reaction of the nervous tissue indicates the presence of MA. Zona marginalis of the cerebral cortex is entirely negative. The small dark clumps indicate the nerve cells possessing MA. The white matter is diffusely positive. Staining was performed with the same method as described in Fig. 1 (×100).*

cerebral cortex contained the particles with the positive reaction in the intracytoplasmic spaces. Interestingly, nuclei of the nerve cells gave a strongly positive reaction. On the other hand, no definite positive staining of the nerve cells in the inner layers of the cortex, corresponding to the pyramidal cells, was found. The myelin sheaths were stained predominantly in the same regions, but some differences of the staining pattern were found in comparing the bundles of nerve fibers. No remarkable depositions of MA were found in the cerebral vessels, suggesting that MA passes easily through the blood-brain barrier.

Liver—The positive aspect of the liver tissue was found macroscopically in the cryostat sections, as shown in Fig. 4, while no distinct staining was found in the paraffin sections. Microscopic examination revealed that the hepatocytes were filled with rigid particles in the cytoplasm (Fig. 5) throughout in the liver. This pattern was remarkable predominantly in the cryostat slices, but not in the paraffin sections. On the other hand, the sinusoids of the liver were stained positively in the paraffin slices.

Kidney—Macroscopically, all of the kidney tissues seemed to be strongly positive (Fig. 6). By careful observation, it was found that a positive reaction was localized mainly in the region of the medulla. This feature was also very remarkable in the renal tissues of the animals that were administered the smallest dose of MA (0.01 mg) (Fig 7). Some glomeruli, especially the glomerular capillaries, were stained distinctly, suggesting that the endothelial cells have an abundant uptake of MA (Fig. 8). On the other hand, only a slight positive reaction was observed in the proximal part of the renal tubuli. A strikingly positive reaction was found in the epithelial cells of collecting tubules in the cortex and medulla (Fig. 9).

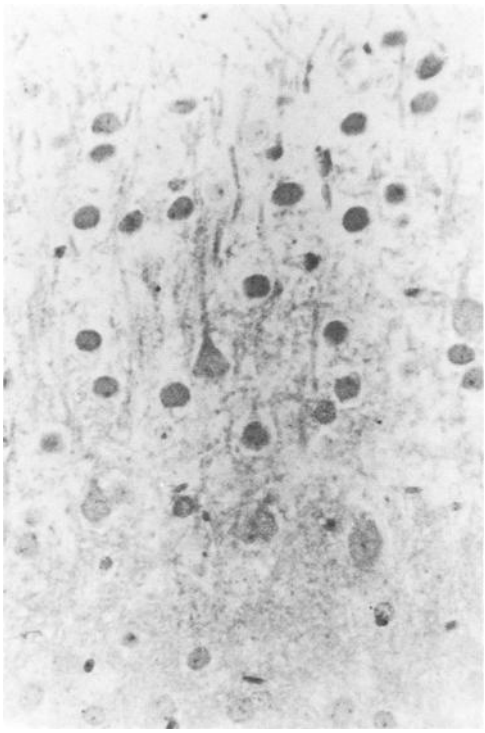

FIG. 3—*Almost all nerve cells are swollen and stained intensely, suggesting the localization of MA. Nerve fibers are also remarkably positive. Staining was performed with the method as described in Fig. 1 (×400).*

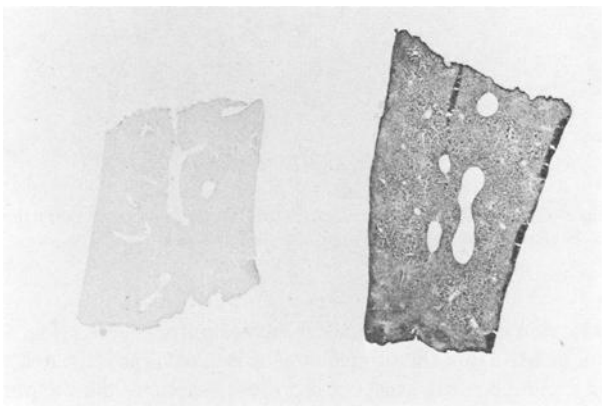

FIG. 4—*Macroscopic finding of liver.* Left tissue: *the liver from the control animal.* Right tissue: *the liver from the intoxicated animal. Staining was performed with the method as described in Fig. 1 (×10).*

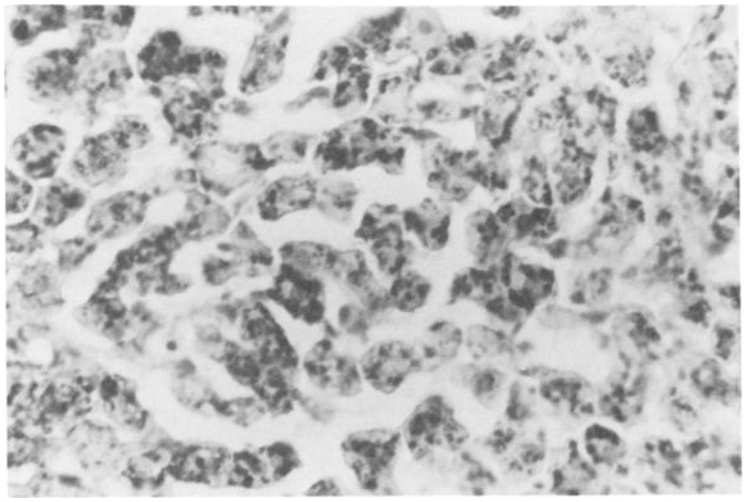

FIG. 5—*Dense positive particles are found in the hepatocytes. Staining was performed with the method as described in Fig. 1 (×400).*

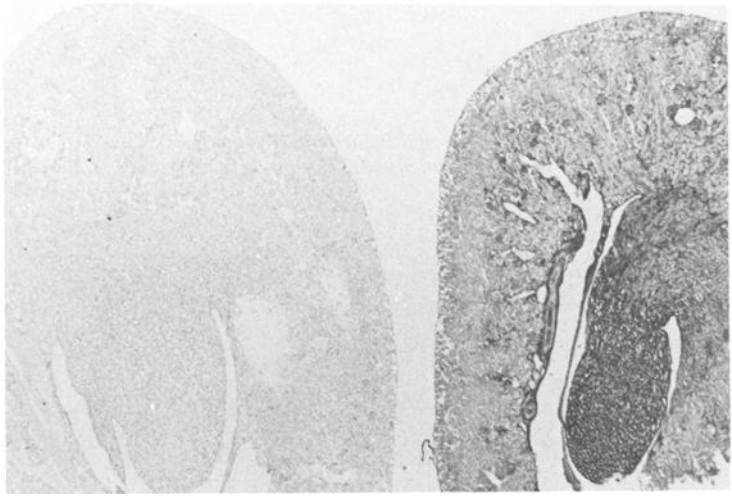

FIG. 6—*Macroscopic finding of the kidney.* Left tissue: *the kidney tissue from the control animal.* Right tissue: *the kidney tissue from the experimental animal (×20).*

Lung—MA was located in the alveolar and bronchial epithelial cells (Fig. 10). The typical pattern of excretion of MA from the bronchial cells is shown, as indicated in Fig. 11. The cryostat slices gave a more distinct aspect of a positive reaction than the paraffin sections.

Stomach—The histological aspects of the gastrointestinal tract from the esophagus to the stomach are illustrated in Fig. 12. The stratified epithelia of the esophagus are, for the most part, free of MA, while cells at the region near the cardia are remarkably stained. On the other hand, all of the gastric glands were filled with positive staining. Upon precise observation of the gastric glands, we found a remarkable positive reaction in the chief and parietal

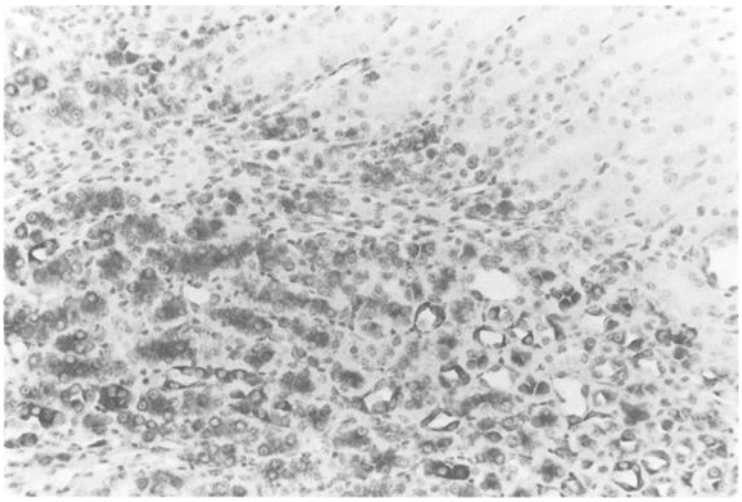

FIG. 7—*The distinct localization of MA is found in the renal cortex, while the strong positive reaction is noticed in the medulla (×200).*

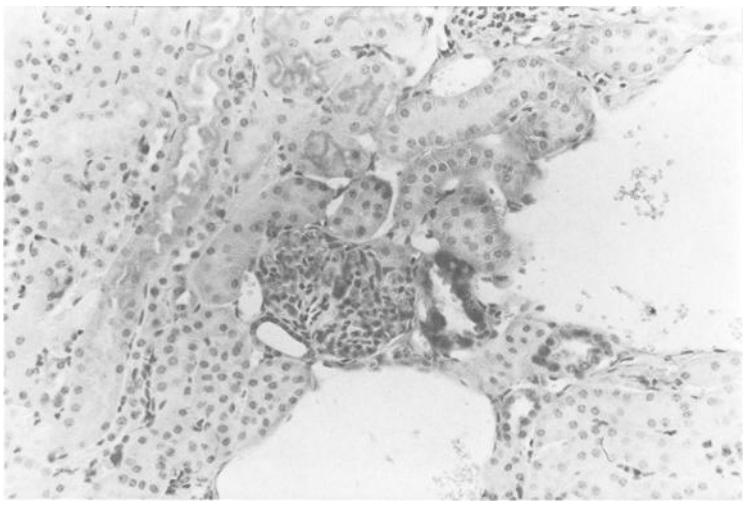

FIG. 8—*Glomerulus is stained remarkably, while the proximal tubules are almost negative (×200).*

cells. Negative staining was noted in the surface mucous epithelia. In addition, the cells at the base of the gland were shown to be negative. Dense conglomerate was found in the lumina of the gastric glands, definitely indicating the finding of excretion (Fig. 13).

Spleen—All of the tissue was stained diffusely, but on careful examination, the staining of the red pulp was more prominent as compared with the white one. Some white pulp, however, revealed a strong positive reaction. Such a discrepancy may have been induced as a kind of artifact by the unequal distribution of the blood at the moment of sacrifice of the animals. Perfusion of the spleen in vivo may be the inevitable procedure for the investigation of the true aspects of the uptake of MA in this organ.

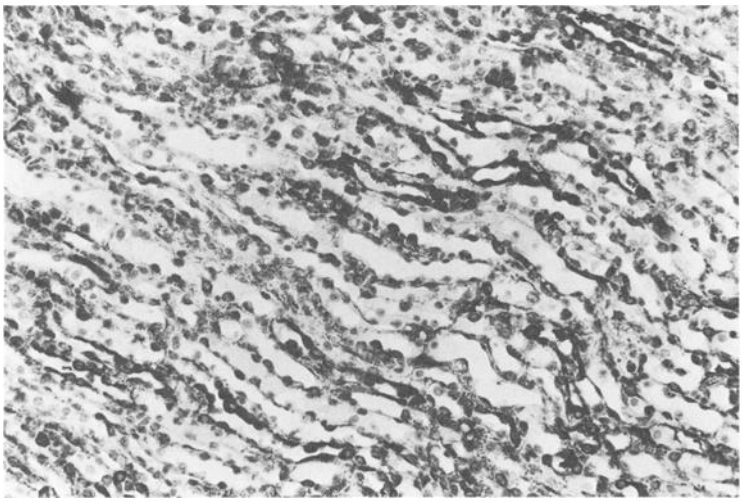

FIG. 9—*The epithelial cells in the region of pyramid are stained, suggesting the localization of MA (×200).*

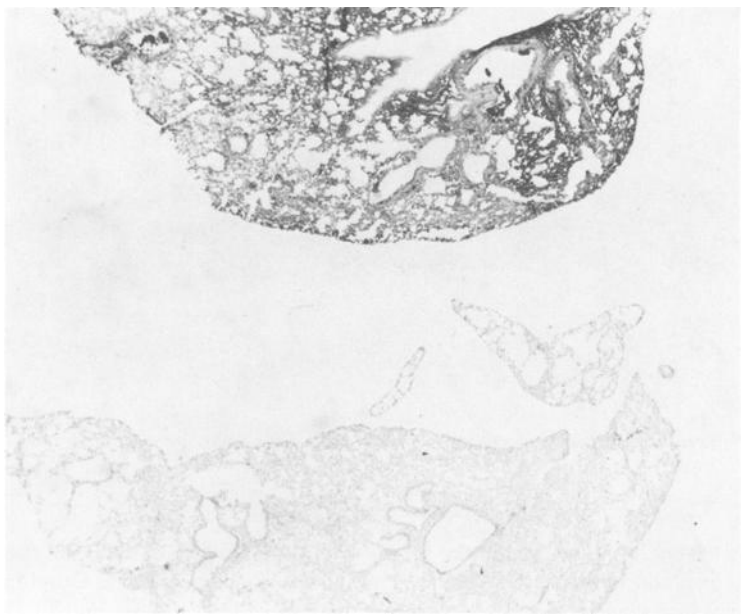

FIG. 10—*Macroscopic finding of the lung.* Upper tissue: *lung from the experimental animal, which exhibits a strong positive reaction.* Lower tissue: *lung from the control animal, which exhibits the negative reaction (×40).*

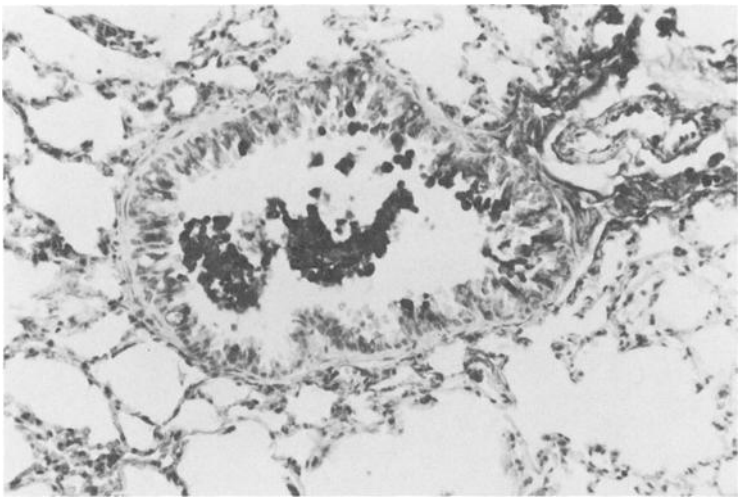

FIG. 11—*Some epithelial cells of the bronchi are excreting the densely positive staining material. In the bronchial lumen, a positive secrete is found (×100).*

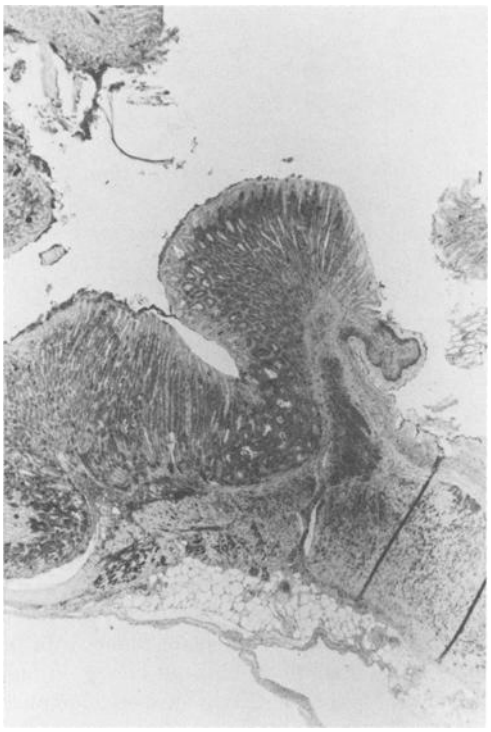

FIG. 12—*Almost all of the gastric glands stain positively, which can be discerned by macroscopic observation. The surface mucous epithelium is negative (×40).*

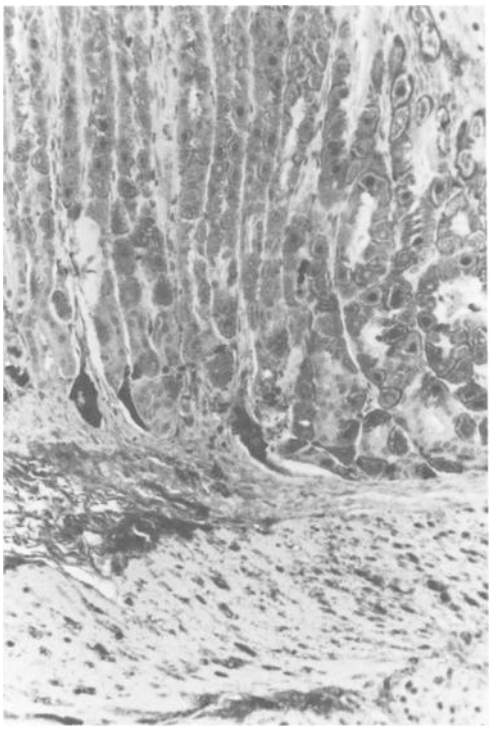

FIG. 13—*The cells of the gastric glands are stained positively (×200).*

Others—The blood in the vessels showed a positive reaction. This can be elucidated by the finding that the blood level of 1 mg of MA administered was about 1.8 to 2.3 μg/mL. In the case of 0.1-mg administration, only a slight positive reaction was observed (the blood contained a trace amount of MA), while no distinct reaction was found in the animals administered 0.01 mg.

The muscle fibers represent some peculiar aspects with regard to the permeability of MA into the cell body. In some striated muscles, as indicated in Fig. 14, the positive reaction was recognized in the fibers. The same finding was noted in the cardiocytes (Fig. 15). On the other hand, no striation was found in the nonstriated muscle fibers. This finding was shown in the figures of the gastrointestinal and respiratory tract and the vascular wall in various tissues. Pancreas tissues were negative.

Histochemical Comparison of MA and Amphetamine—Although this problem is considered to be an important point in the investigation of the pharmacodynamic aspects of amphetamines, we have investigated the staining pattern of amphetamines for the elucidation of the staining mechanism of MA. The animals were administered 0.01 to 1 mg of MA and amphetamine. The same or a somewhat weak positive reaction or both was observed with amphetamine as compared with MA. For example the kidney with 1 mg of MA exhibited a tissue level of MA of 82 μg/g and of amphetamine of 12 μg/g. In the case of 1-mg amphetamine, the same positive reaction was obtained (tissue level of amphetamine was 110 μg/g). A decrease of the doses of both agents gave the same pattern of reaction, with no discrepancy found between MA and amphetamine.

Effects of the Treatment with Sodium Borohydride—The treatment of the microscopic

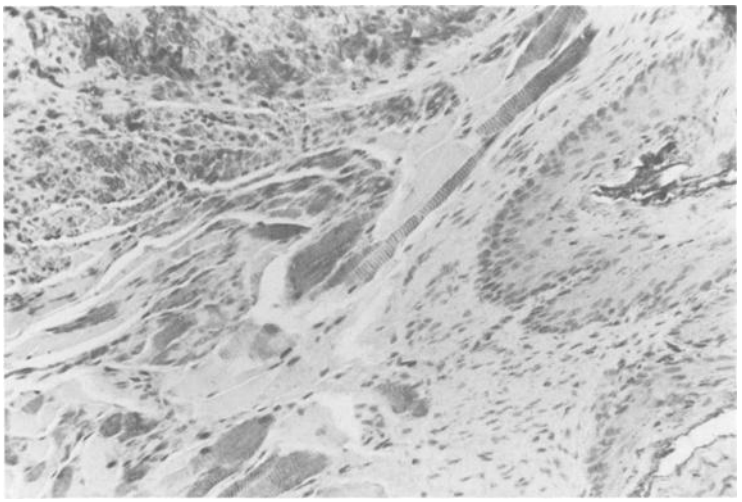

FIG. 14—*Epithelial cells of esophagus are negative. Some striated muscle fibers are stained positively (×200).*

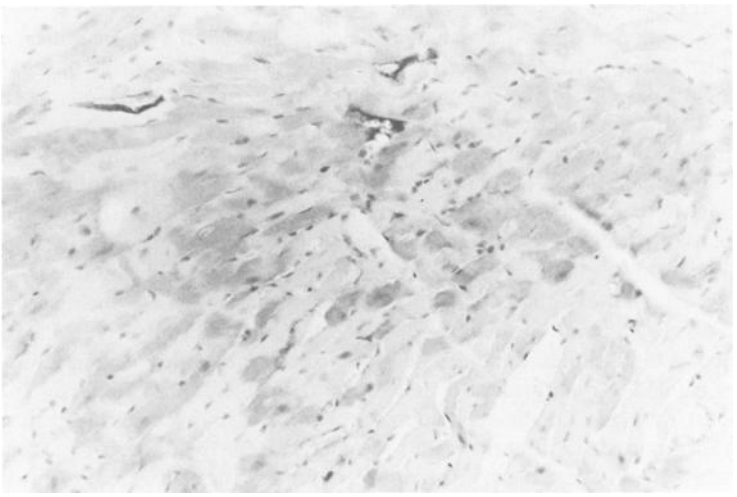

FIG. 15—*Patchy positive staining is found in many cardiocytes (×200).*

slides with sodium borohydride at the concentration of 0.5 mg/mL gave an increase of the image of a positive reaction in all tissues, especially the frozen slices, although the samples without borohydride treatment also gave a distinct positive response. From this finding, it was concluded that the borohydride treatment was not an essential procedure for the demonstration of MA, and that this procedure was instead interaction with the chemical structures surrounding the MA antigen. Thus, the unmasking of the MA antigen might be the factor of borohydride action.

Discussion

As shown in the figures illustrating the experimental results, a distinct positive reaction indicating the presence of MA in the tissues and tissue components could be observed by macroscopic examination of the stained tissues from the animals at the intoxication level, at which fatal cases of MA intoxication in forensic pathology are often reported (consumption of 30 to 200 mg). Furthermore, it was found that the presence of MA in animals given the substance at doses in the range of therapeutic application of MA for essential hypotension and narcolepsy (10 mg) can also be distinctly discerned by staining the tissues and comparing the staining pattern of the control on the same microscopic slides. Thus, it seems very probable that the tissues in a forensic science autopsy of acute MA intoxication can be analyzed histochemically with respect to the topographic distribution of MA, provided that they are fixed with fixatives containing glutaraldehyde.

The most important point in the immunocytochemistry of MA is the selection of fixatives at the first stage of the staining. We have investigated this problem by examining the effects of fixation with four kinds of fixatives, that is, 10% formalin, 4% paraformaldehyde-containing phosphate buffer solution (PA), 4% glutaraldehyde-containing phosphate buffer solution (G), and 2% glutaraldehyde and 4% paraformaldehyde-containing phosphate buffer solution (PA+G), which are commonly used for routine histology, immunocytochemistry, and electron microscopy.

Of them, the (PA+G) and (G) fixatives gave a positive reaction, while others were inactive in fixing the MA. In comparing the two effective fixatives, the (PA+G) is much more useful, as the (G) fixative made the tissue very hard, making preparation of satisfactory microscopic slices very difficult.

As for the fixation mechanism, we had assumed at first that the amino radical of amphetamine, which is biotransformed in vivo from MA, might react with the glutaraldehyde to form the Schiff base. Actually, the positive reaction was stained intensively when the slices were treated with the sodium borohydride, suggesting the possibility that the Schiff base would be reduced to the *N*-alkylated amphetamine, which has the same chemical structure as the antigen for the induction of antibody against MA. This explanation was, however, contradictory to the finding that the animal tissues exposed to MA (0.1 and 1 mg) gave the same response to anti-MA as compared with the animals administered the same doses of amphetamine. This finding indicates clearly that the positive reaction in the MA experiment was induced essentially by the MA fixed in the tissues, but not its metabolite amphetamine. In histochemistry, fixation of the secondary and tertiary amines is very difficult, so that a new method is required for this purpose. It is expected that the primary amine of norepinephrine is fixed by paraformaldehyde in forming the Schiff base, as Wood and Barrnett have demonstrated [*11*]. On the other hand, the fixation of the epinephrine by nitrogen mustard has been reported [*12*], but the results are not so conclusive as the original report stated.

In this sense, the fixation mechanism of MA might be regarded as an exceptional one, as in the case of the Verhofstad report of fixation of epinephrine by paraformaldehyde [*4*]. One possibility in elucidating the fixation of the secondary amines is that the protons in the α-position of the aldehydes and ketons react easily with the *N* atom of the secondary amines; a reaction is described in organic chemistry books. We have examined this possibility using a mixture of glutaraldehyde, amphetamines, and bovine serum albumin, which was reduced after incubation for 5 h at room temperature by adding sodium borohydride. The chloroform extract of the reduced mixture at alkaline pH indicated that amphetamine, but not MA, could be conjugated with BSA. Although definitive elucidation of MA fixation cannot be given at present, this problem is a key point in the investigation of the immunohistochemistry of drugs in the chemical conformation of alkaloids. The investigation confirming the fixation of tertiary amines of amphetamine derivatives, such as *N*-dimethyl-amphetamine, may give some conclusive information on this subject.

Another significance of the immunohistochemical determination of the drugs in the tissues and tissue components was indicated. Topographic demonstration of amphetamines has been accomplished with the aid of autoradiography [*13–16*], but no conclusive histochemical information could be attained by this procedure. In this respect, the analysis of the detailed histological findings on the immunochemistry of MA in our experiment may reveal more interesting aspects of the pharmacodynamics and pharmacokinetics at the cellular level, which have been indicated by biochemical and neurochemical research. The selective uptake of MA by the nerve cells located at the outer layers of the mouse cerebral cortex, and the accumulation in the distal part of the renal tubule and collecting tubule, and also in the alveolar, bronchial, and gastrointestinal cells, might be the reflections of the morphological aspects of the pharmacodynamics and pharmacokinetics in these research fields. For example, the lung has been regarded as one of the chief organs of the accumulation and metabolism of xenobiotics [*17, 18*]. The elimination of amphetamine, as a kind of basic amine, in particular, has been carefully studied. Such a pharmacological framework can be demonstrated directly by our method. The selective staining of the outer cortical neurons could be due to perfusion by rich anastomotic surface cerebrovasculature rather than to the intrinsic preference for MA. The comparison of the distribution pattern of the phenobarbitone and MA examined by the same immunocytochemical method, however, distinctly reveals an indication that the latter one is more reliable, as the former drug is located in the entire layer of the cerebral cortex, including the lower cortical layers. These results will be reported precisely elsewhere.

With respect to the uptake of MA in the brain components, nerve cells, not only in the nuclei of the brain stem, but also of the cerebral cortex, gave a positive reaction indicating the presence of this drug. Distributions of the endogeneous biogenic amines, such as epinephrine, norepinephrine, serotonine, histamine, and so forth in the nerve cells are restricted solely in the nuclei and their projecting tracts, which are located in the region of brain stem [*19–22*]. It is reasonable to expect that the exogeneously administered amines are distributed more diffusely in the brain throughout.

In this study, we have examined the tissues immersed directly in fixative as we intended to apply the experiment to forensic science and pathology practice. As the fixative for electron microscopy is excellent for the immunocytochemistry of MA, investigations using perfused animal organs and electron microscopic immunocytochemistry are now in progress. For example, the MA in the hepatocyte is solely located in the spaces of the smooth endoplasmic reticulum, indicating the morphological evidence of the detoxification process in the biochemical toxicology. The granules in the cytoplasma of the nerve cell are also included in the endoplasmic reticulums. The precise observation using electron microscopy will be reported elsewhere.

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