

Improvement in the Histochemical Localization of Leucine Aminopeptidase with a New Substrate, *L*-Leucyl-4-Methoxy-2-Naphthylamide*, †

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PLATES 129 TO 134

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ABSTRACT

A new method for the histochemical demonstration of leucine aminopeptidase in fresh frozen sections was developed with the substrate *L*-leucyl-4-methoxy-2-naphthylamide. The superior enzyme localization is due to the more rapid rate of coupling of the hydrolysis product, 4-methoxy-2-naphthylamine as compared to 2-naphthylamine itself, and to the low lipid solubility and high substantivity for protein of the copper chelate of the dye formed on coupling with tetrazotized diorthoanisidine. A comparison of the old and the new method is illustrated, and a description is given of the localization of leucine aminopeptidase in the tissues of the rat and man.

INTRODUCTION

So far it has not been established that leucine aminopeptidase is a distinct entity, and there is evidence of some overlap of substrate specificity among the group of aminopeptidases (9, 15). However, until it has been demonstrated that leucinamides are hydrolyzed extensively by a wide variety of distinct aminopeptidases, we prefer to follow the precedent of earlier investigators (9, 15) and call the enzyme that hydrolyzes leucinamides, *leucine aminopeptidase* rather than just *aminopeptidase* (5, 6). Since the main specificity requirement of the enzyme was provided by the leucine moiety (9, 15), two laboratories independently synthesized *L*-leucyl-2-naphthylamide in order to provide a substrate with strong chromogenic potential (6, 8). This reagent has been used by other investigators as well for the colorimetric estimation of leucine aminopeptidase in tissue homoge-

nates, serum, and urine (1, 3, 7, 14). In the adaption of this chromogenic substrate for histochemical methodology (5, 11) it was noted that the enzymic sites could be visualized in the tissue sections, but evidence of significant diffusion of the primary reaction product was demonstrated (11). Diffusion resulted from the fact that the rate of coupling of 2-naphthylamine and the diazonium salt was slow as compared to the coupling rate with 1-naphthol (12), thus permitting the amine, which has considerable aqueous solubility, to diffuse from the site of its production before it had been precipitated as a dye by the coupling reaction. Improvement in the histochemical localization of leucine aminopeptidase would be expected if the diffusion of the primary reaction product could be minimized by a faster capture reaction. This consideration led to a study of the influence of the chemical structure of both the diazonium salt and of the amine upon the coupling rate (12). It was noted that variations in the structure of the diazotate had much less effect upon the speed of coupling than did the introduction of certain groups into the amine. In particular, 4-methoxy-2-naphthylamine was found to couple with tetrazotized diorthoanisidine approximately 40 times faster than the unsubstituted amine. Therefore, *L*-leucyl-4-methoxy-2-naphthylamide

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was synthesized (13) to serve as a more satisfactory substrate than those used previously for the histochemical localization of leucine aminopeptidase. That far better localization was obtained with the new substrate will be illustrated below.

Materials and Method

The tissues examined were those of freshly killed rats, and of humans obtained at operation or at post-mortem examination within several hours of death. After quick freezing of small blocks in isopentane, cooled in a mixture of dry ice and acetone at -70° to -80° C., sections 12 microns thick were cut in a cryostat using a rotary microtome. Fixation in cold formalin may be used with very active tissues (11). The sections were then incubated in the following solution:

<i>L</i> -leucyl-4-methoxy-2-naphthylamide	1.0 ml.
HCl (stock)	
Acetate buffer (0.1 M) pH 6.5	5.0 ml.
Sodium chloride (0.85 per cent)	3.5 ml.
Potassium cyanide (2×10^{-2} M)	0.5 ml.
Tetrazotized diorthoanisidine (fast blue B powder)	5.0 mg.

The stock solution of the substrate was made by dissolving the reagent in distilled water at a concentration of 4 mg./ml. This solution could be kept in the refrigerator for several months without spontaneous hydrolysis of the substrate. The final concentrations of the substrate and the diazonium salt in the incubation medium were 0.4 mg./ml. and 0.5 mg./ml. respectively. The incubations were carried out at 37° C. for 5 minutes in very active organs and for 0.5 to 2 hours with many tissues, depending upon the enzymatic activity. Incubations longer than 4 hours produced an unsatisfactory dark yellow background stain due to the decomposition of the diazonium salt. After the sections were stained adequately, they were rinsed in saline for 2 to 3 minutes, and then transferred to a solution of 0.1 M cupric sulfate for a few minutes. Cupric ions chelate with the dye formed on coupling 2-naphthylamine with tetrazotized diorthoanisidine producing a shift in color from red to purple (11). In addition, chelation with copper conferred positive charge on the molecule and made the pigment insoluble in lipid and more substantive to tissue protein so that it was not removed when the sections were passed through the organic dehydrating agents. Copper chelation produced no shift in color of the dye formed on coupling 4-methoxy-2-naphthylamine and tetrazotized diorthoanisidine. That chelation did occur was supported by the observation of decreased lipid solubility and increased substantivity of the pigment for protein. The sections were dehydrated in graded alcohol solutions, cleared in xylol, and mounted in permount. Counterstaining was not used in this study.

RESULTS

A comparison of the histochemical localization with fresh frozen sections of rat kidney and intestine was made between the old substrate and the new substrate. The striking improvement in preciseness of localization is illustrated in Figs. 1 to 4. In Fig. 1 the kidney tubules are diffusely stained using the old substrate and there is dye in the glomerulus as well. In Fig. 2, by comparison, the pigment is heavily concentrated in the brush-border of the proximal convoluted tubules, and other segments of the nephron and glomeruli are free of pigment. In Fig. 3, using the old substrate, the epithelial cells of the small intestine are diffusely stained with a gradient of staining extending from the epithelium into the lamina propria. By contrast, Fig. 4 shows enzyme activity to be confined to the striated border in the luminal portions of the columnar cells and there is no gradient of pigment into the lamina propria. This demonstration is consistent with the previous observation of a coupling rate for 4-methoxy-2-naphthylamine about forty times faster than that of 2-naphthylamine (12).

Tissue Survey

In general, the tissues of man contained more leucine aminopeptidase activity than those of the rat. The enzyme was confined to the cytoplasm, apparently unassociated with recognizable intracellular organelles. The descriptions of the histochemical localization given below apply to the tissues of both rat and man. When significant species variations occur they are noted. The sections mounted in either Canada balsam or permount remained histologically adequate for 4 to 6 months.

Digestive System: Submaxillary Gland (Rat).—Intense activity was found in some segments of the excretory system. The intercalated and secretory ducts were intensely stained. Secretory alveoli were not active (Fig. 5). The *esophagus* in the rat was entirely negative. The *stomach* showed no activity except for that present in small vessels and capillaries. The *duodenum* (rat) reacted like the other segments of the small intestine. Brunner's glands were entirely negative. In the remainder of the small intestine (rat and man) intense activity was found in the striated border of the columnar cells. Unstained goblet cell openings seeded clear areas in the densely active surface. The lamina propria and the muscle layers of the entire intestinal tract were negative (Figs. 4 and 6). The *large intestine* of man stained irregularly in that the surface epithelium and crypts were quite active in certain areas, and weak or more frequently negative in other areas (Fig. 7). The large intestine of the rat was devoid of leucine aminopeptidase. The *liver* (man) contained moderate

leucine aminopeptidase activity throughout the parenchymal cells with highest concentration being noted in the portal areas. Dense concentrations of active droplets appeared occasionally at the site of the bile capillaries (Fig. 8 *a*) and in a few areas, bile canaliculi appeared discretely stained (Fig. 8 *b*). The lumina of bile canaliculi were delineated by pigment lining their borders (Fig. 8 *c*). Epithelium of bile ducts contained intense activity, mostly concentrated in the luminal aspect of duct cells. In the rat liver, parenchymal cell activity was less than in man. Leucine aminopeptidase activity was present in the *pancreas* of man. Most of the acini were only moderately stained, but in some, prominent red globules were seen in the luminal aspect of acinar cells. The interlobular ducts were active, but the islands of Langerhans, connective tissue septi, and blood vessels were negative. In the rat, pancreatic cells were entirely negative whereas the stromal connective tissue and small blood vessels showed some activity.

Urinary System.—Intense leucine aminopeptidase activity of proximal convoluted tubules was apparent in the *kidney* within 5 minutes of incubation (Fig. 9). In the rat, strongest reaction appeared in the lower segments of the proximal tubules. Thus, the inner cortex appeared clearly delineated. The glomeruli as well as other segments of the nephrons were unstained (Fig. 10). The epithelium of the *bladder* and *urethra* were not reactive although the adjacent connective tissue and small blood vessels contained some activity.

Genital System.—The *testis*, and epithelium of the *epididymis*, *seminal vesicles*, and *vas deferens* (rat) were non-reactive. The connective tissue elements of these organs had some activity, especially in the fibroblasts and mast cells (Fig. 11). The *prostate* (rat) showed intense activity in the luminal aspect of the epithelial cells (Fig. 12); the stroma contained active mast-cells. In the human *ovary* (Fig. 13), the theca interna of the corpus luteum in early stages of vascularization and hemorrhage showed very intense activity corresponding to its proliferating cells. Aside from activity in the follicular epithelium and stromal tissue and small vessels, the remaining histological elements of rat ovary were negative. Human *uterus* (proliferative phase) showed activity only in the endometrial stroma and blood vessels. In contrast to the marked activity in the guinea pig uterus (11), the endometrial glands and myometrium of both humans and rats were devoid of leucine aminopeptidase. The cervix was inactive.

Respiratory System.—The bronchial epithelium contained intense activity, but the cartilage was not reactive. The alveolar cells were positive but less active.

Endocrine Glands.—The *thyroid* follicles in the rat did not contain leucine aminopeptidase. Some fibroblasts and mast cells in the intervening connective tissue displayed activity. Intense reaction was shown by the glandular cells of the rat *parathyroid*. The *adrenals* of man were entirely devoid of this enzyme. In

the rat, the juxtamedullary area of the *zona reticularis* was particularly active (Fig. 14).

Miscellaneous Tissues.—The epidermis did not contain activity. Connective tissue cells in the corium, and in the vicinity of hair follicles were active. In the rat these fibroblasts were delineated as a delicate network due to the demonstration of enzymatic activity in their delicate cell processes (Figs. 15 and 16). The sweat glands (Fig. 17) in human skin contained abundant enzyme in the luminal border of the epithelial cells of the coiled segments and at the site of the basement membrane. The basal layers of the covering epithelium showed focal activity, while sebaceous glands were unstained. All varieties of muscle in both species were essentially negative. The adventitia of most blood vessels and the perineurium of nerves were active. In the rat, neutrophilic leucocytes were usually active. Tissue eosinophils, lymphocytes, and red cells were negative. Rat mast cells frequently contained activity (Fig. 18). In man, only polymorphonuclear leucocytes displayed intense activity.

DISCUSSION

The first histochemical methods for aminopeptidase were dependent upon the coupling properties of 2-naphthylamine (5, 11). Although the claim has been made that the localization was improved by using frozen-dried sections rather than fresh frozen sections (4, 5), and by using a copper chelate rather than the simple azo dye (11), neither of these modifications alone produced enough improvement to provide us with a really satisfactory method for leucine aminopeptidase. The reason for inadequacy was the limitation in coupling rate between 2-naphthylamine and either tetrazotized diorthoanisidine (fast blue B) or diazotized 4-*o*-tolylazo-*o*-toluidine (fast garnet GBC). The claim that fast garnet GBC coupled faster than fast blue B (4), was shown not to be correct in a study of coupling rates (12). In this study it was also shown that for those enzymes hydrolyzing an ester link, increase of coupling rate might be accomplished by modifying the structure of either the coupling component used in the substrate or the diazonium salt. However, for enzymes splitting amide linkages, the only possibility of improving the localization is by modifying the structure of the coupling component, naphthylamine (12). The synthesis of naphthylamines with additional strong ortho or para directing groups, would provide an increased coupling rate. In order to use the amine as part of a substrate for histochemical purposes, however, the degree of reactivity afforded by the new substituents must be limited so that the intact amide

would not couple before its enzymatic splitting had occurred. This was done successfully by the synthesis of 4-methoxy-2-naphthylamine (13), which couples about 40 times as fast as 2-naphthylamine (12), and from it the synthesis of the substrate, *L*-leucyl-4-methoxy-2-naphthylamide (13). The present method has also been used in a study of tumors and pathologic tissue (10). Because lipid in fresh tissue has a solvent effect upon the azo dye formed, superior, permanent sections were prepared by taking advantage of the increase in substantivity for protein and decrease in lipid solubility of the dye after chelation with cupric ions. This had been demonstrated and used with the former method (11), and was found to be helpful with the present agent as well. The superiority of the new method was predicted (12) and is clearly illustrated in Figs. 1 to 4.

No significantly different localization in tissues was noted with the new substrate, except for the marked decrease in diffusion artifacts which may be seen in published photographs (5, 11). Although counterstaining nuclei with blue dye tends to obscure some of the diffuse red dye, diffusion may be seen in published colored illustrations (5). The precise localization of enzymatic activity in granules scattered throughout the cytoplasm and extending into the processes of fibroblasts in skin is shown in Figs. 15 and 16. The illustrations with this histochemical technique suggest the possible syncytial nature of these fibroblasts in the dermis (2), an anatomical phenomenon which is not seen with ordinary staining techniques.

BIBLIOGRAPHY

1. Arst, H. E., Manning, R. T., and Delp, M., Serum leucine aminopeptidase activity: Findings in carcinoma of the pancreas, pregnancy, and other diseases, *Am. J. Med. Sc.*, 1959, **238**, 598.
2. Bloom, W., Handbook of Hematology, New York, Hoeber, Inc., 1938, **2**, 1344.
3. Bressler, R., and Forsyth, B. R., Serum leucine aminopeptidase activity in normal pregnancy and in patients with hydatidiform mole, *New England J. Med.*, 1959, **261**, 746.
4. Burstone, M. S., The relationship between fixation and techniques for the histochemical localization of hydrolytic enzymes, *J. Histochem. and Cytochem.*, 1958, **6**, 322.
5. Burstone, M. S., and Folk, J. E., Histochemical demonstration of aminopeptidase, *J. Histochem. and Cytochem.*, 1956, **4**, 217.
6. Folk, J. E., and Burstone, M. S., Chromogenic leucyl substrates for aminopeptidase and papain, *Proc. Soc. Exp. Biol. and Med.*, 1955, **89**, 473.
7. Goldbarg, J. A., and Rutenburg, A. M., The colorimetric determination of leucine aminopeptidase in urine and serum of normal subjects and patients with cancer and other diseases, *Cancer*, 1958, **11**, 283.
8. Green, M. N., Tsou, K. C., Bressler, R., and Seligman, A. M., The colorimetric determination of leucine aminopeptidase activity with *L*-leucyl- β -naphthylamide hydrochloride, *Arch. Biochem. and Biophysics*, 1955, **57**, 458.
9. Johnson, M. J., and Berger, J., The enzymatic properties of peptidase, *Adv. Enzymol.*, 1942, **2**, 69.
10. Monis, B., Nachlas, M. M., and Seligman, A. M., Study of leucine aminopeptidase in neoplastic and inflammatory tissues with a new histochemical method, *Cancer*, 1959, **12**, 601.
11. Nachlas, M. M., Crawford, D. T., and Seligman, A. M., The histochemical demonstration of leucine aminopeptidase, *J. Histochem. and Cytochem.*, 1957, **5**, 264.
12. Nachlas, M. M., Goldstein, T. P., Rosenblatt, D. H., Kirsch, M., and Seligman, A. M., Influence of chemical structure on the rate of azo coupling and its significance in histochemical methodology, *J. Histochem. and Cytochem.*, 1959, **7**, 50.
13. Rosenblatt, D. H., Nachlas, M. M., and Seligman, A. M., Synthesis of *m*-methoxynaphthylamines as precursors for chromogenic substrates, *J. Am. Chem. Soc.*, 1958, **80**, 2463.
14. Siegel, I. A., Leucine aminopeptidase in obstetrics, *Obstet. and Gynecol.*, 1959, **14**, 488.
15. Smith, E. L., The specificity of certain peptidases, *Adv. Enzymol.*, 1951, **12**, 191.

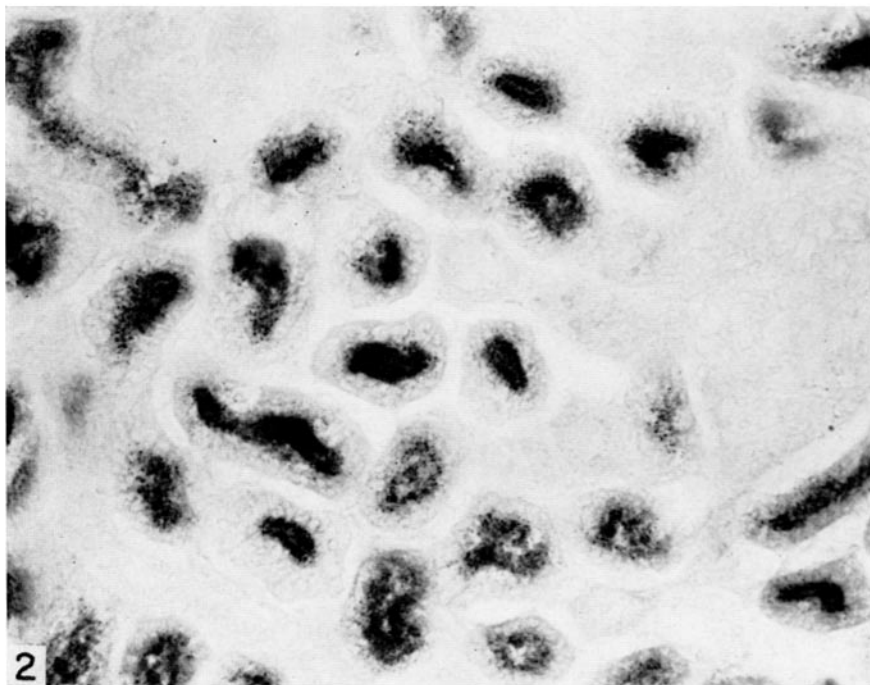
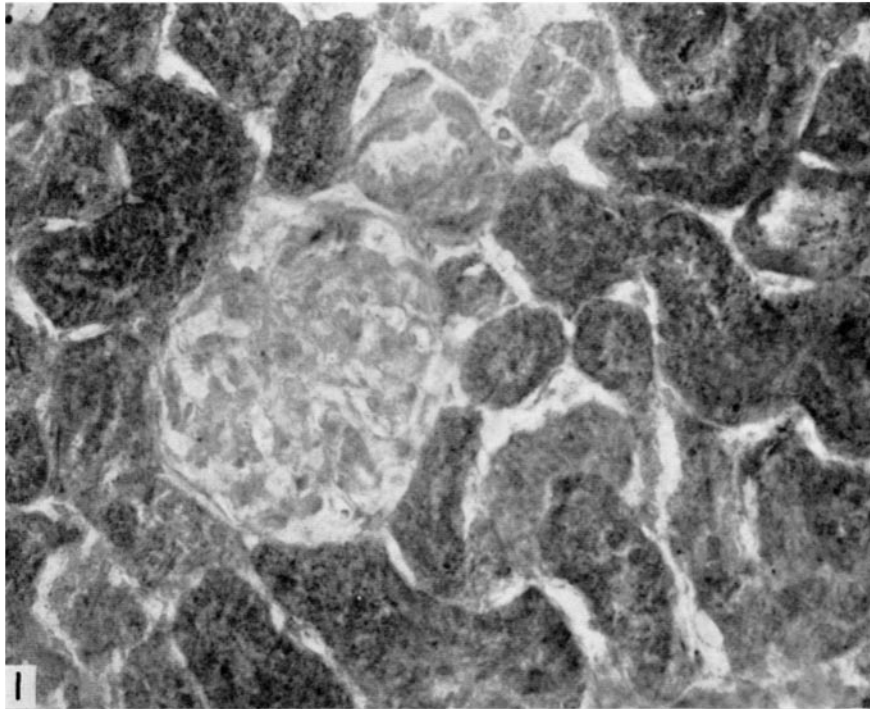
EXPLANATION OF PLATES

Leucine Aminopeptidase in Normal Tissues

PLATE 129

FIG. 1. Rat kidney. Old substrate, *L*-leucyl-2-naphthylamide. Renal tubules are diffusely stained as well as the glomerulus. $\times 400$.

FIG. 2. Rat kidney. New substrate, *L*-leucyl-4-methoxy-2-naphthylamide. Compare serious diffusion artifacts in the section incubated with the former substrate (Fig. 1) with the new substrate which here reveals enzymatic activity confined to the luminal portion of the cytoplasm of cells of the proximal convoluted tubules. The glomerulus and other segments of the nephron are negative. Incubation with both methods was for 5 minutes. $\times 400$.

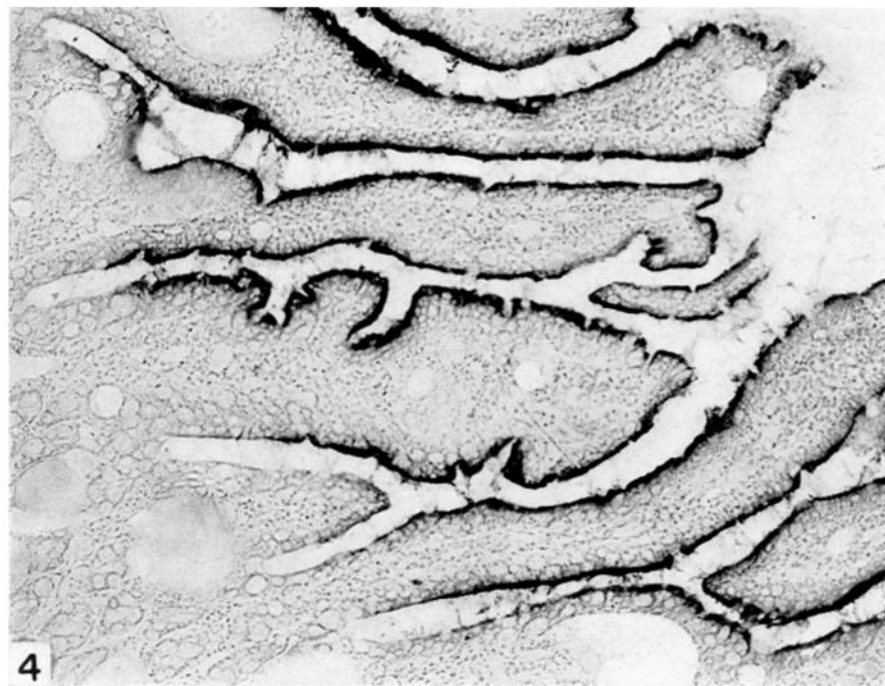


(Nachlas *et al.*: Localization of leucine aminopeptidase)

PLATE 130

FIG. 3. Rat small intestine. Old substrate, *L*-leucyl-2-naphthylamide. Note strong staining of epithelial cells with a diffusion gradient into the lamina propria. $\times 150$.

FIG. 4. Rat small intestine. New substrate, *L*-leucyl-4-methoxy-2-naphthylamide. Compare improved localization with the new substrate. Aminopeptidase activity is confined to the brush border of the epithelial cells. The lamina propria is essentially negative. Incubation with both methods was for 5 minutes. $\times 150$.



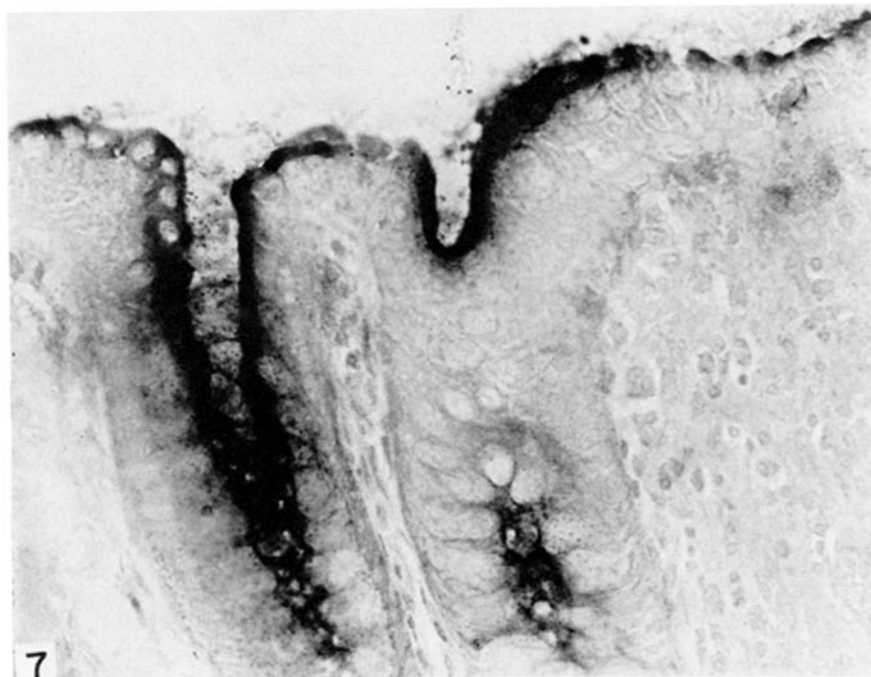
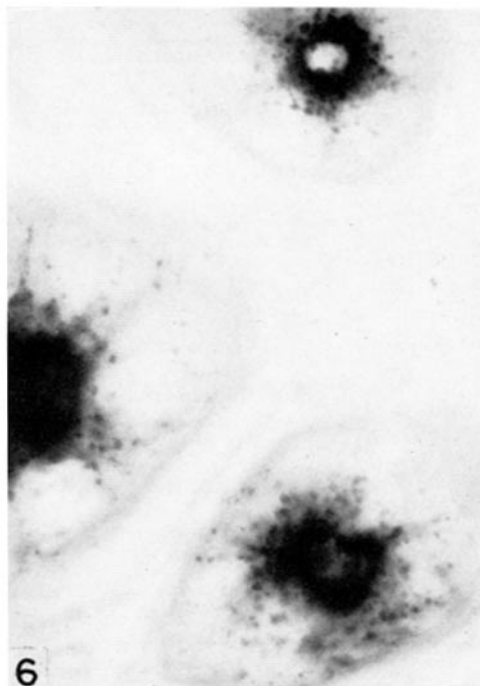
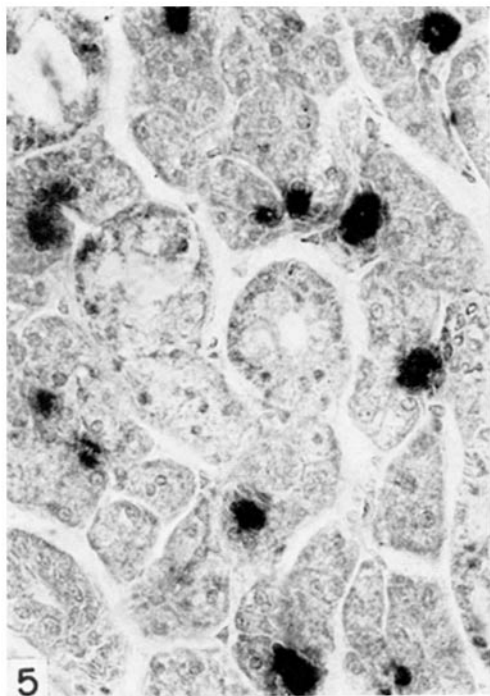
(Nachlas *et al.*: Localization of leucine aminopeptidase)

PLATE 131

FIG. 5. Rat submaxillary gland. Intense staining of glandular ducts, presumably intercalated and secretory segments. The remainder is stained yellow by diazonium salt and does not represent enzymatic activity. Incubation 15 minutes. $\times 400$.

FIG. 6. Human small intestine. The luminal aspect of the cytoplasm of the epithelial cells covering villi and crypts of Lieberkühn are intensely stained. In some cells (upper right) the decrease in pigment concentration from luminal to basal zones of the cell suggests an intracellular diffusion gradient; either diffusion of enzyme, or of reaction product. Against this interpretation is the perinuclear distribution of fine granules in a pattern unlike simple diffusion. The lamina propria is not active. Incubation 15 minutes. $\times 750$.

FIG. 7. Human colon. Intense activity in the apical aspects of the cytoplasm of the columnar cells of the surface epithelium and of the cells of some crypts of Lieberkühn. However, this activity is erratic. Some activity can also be seen in the lamina propria in the vicinity of the surface epithelium. Otherwise, the lamina propria is largely devoid of activity. Incubation 30 minutes. $\times 400$.



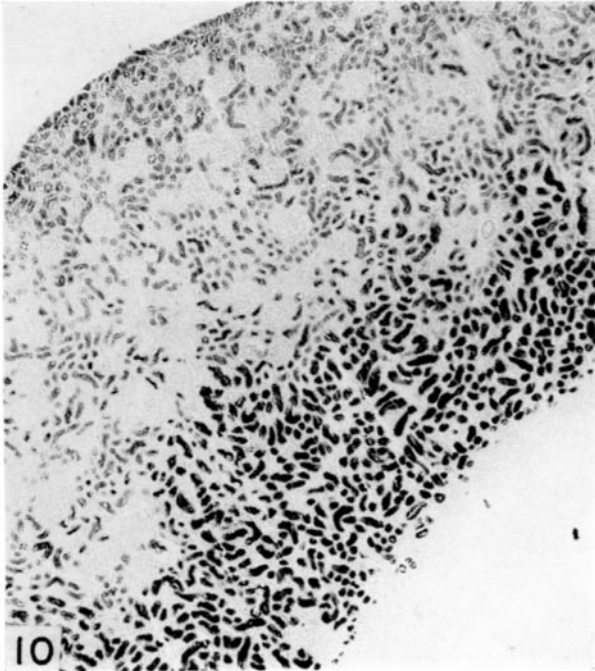
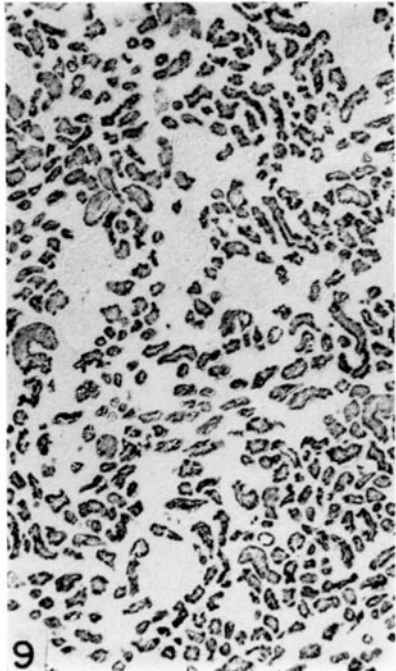
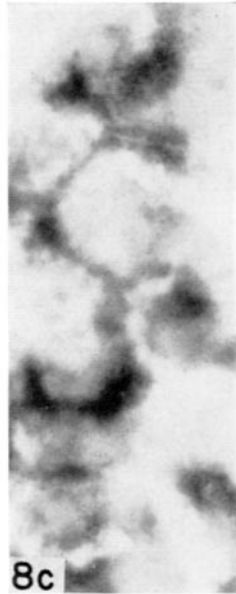
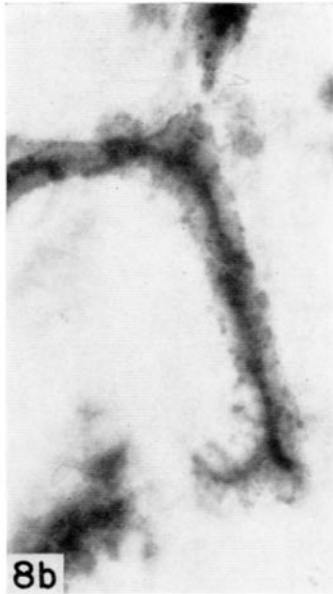
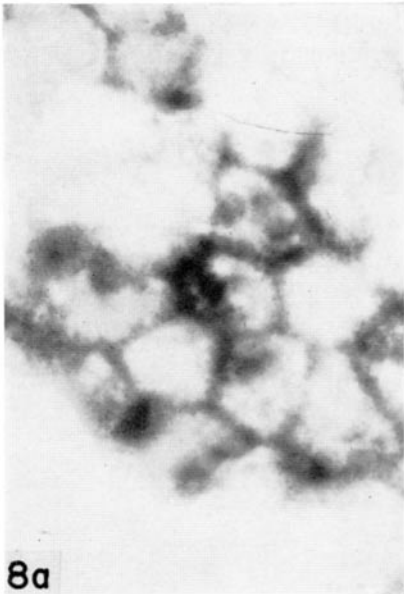
(Nachlas *et al.*: Localization of leucine aminopeptidase)

PLATE 132

FIGS. 8 *a* to *c*. Human liver. The cytoplasm of the hepatic cells are most active in the peripheral portions, thus outlining some cell boundaries (8 *a*). Elsewhere the bile canaliculi are stained intensely (8 *b*), and in certain areas the lumina of the bile canaliculi can be easily identified by the pigment lining their borders (8 *c*, top). Incubation 1 hour. \times 750.

FIG. 9. Human kidney cortex. The proximal convoluted tubules contain intense activity. Glomeruli are unstained. Incubation 5 minutes. \times 35.

FIG. 10. Rat kidney. Proximal tubules are stained in all parts of the cortex. In the outer cortex, unstained glomeruli are interspersed between moderately active upper segments of the proximal tubules. Note higher activity in lower (distal) segments of proximal tubules in the inner cortex. Other segments of the nephron and vessels are inactive. The medulla is unstained (lower right). Incubation 5 minutes. \times 35.



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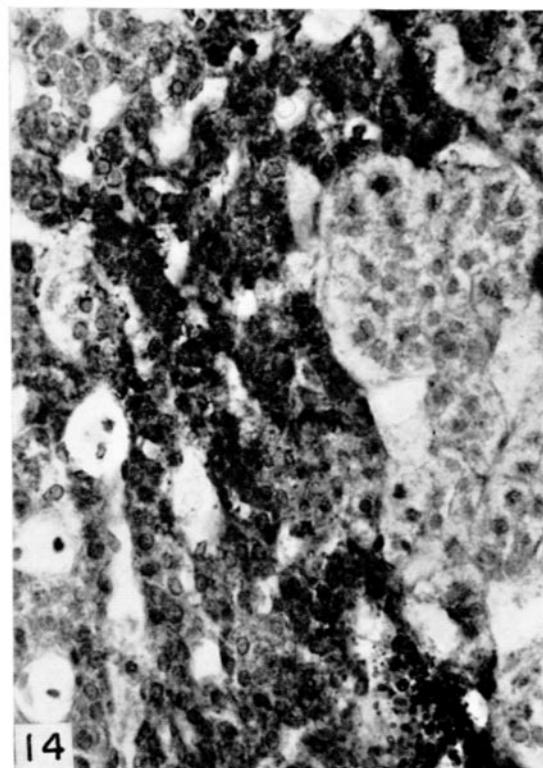
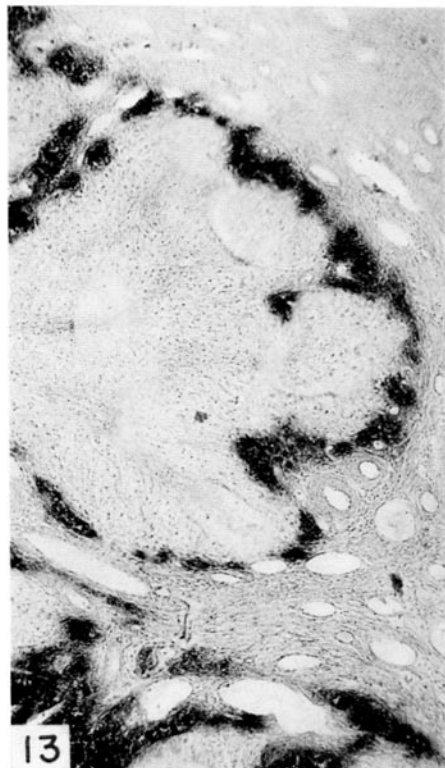
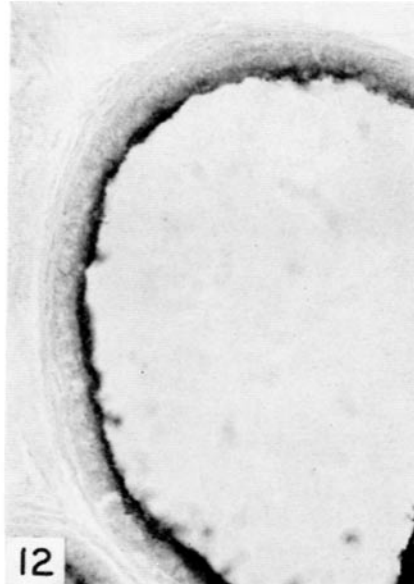
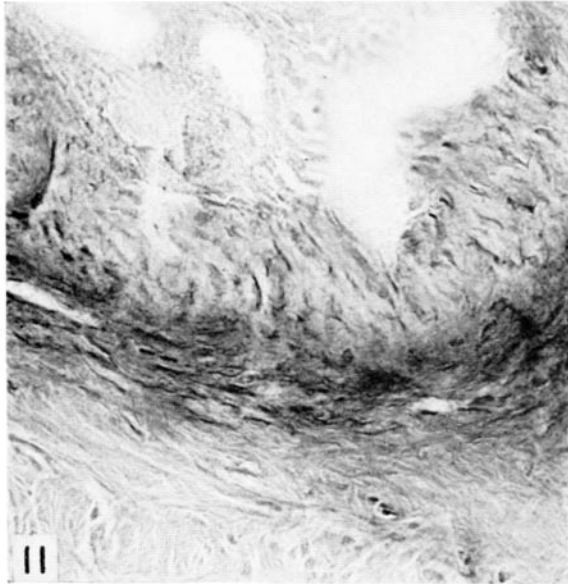
PLATE 133

FIG. 11. Rat vas deferens. The dark ring corresponds to the intensely active inner half of the lamina propria, though the epithelium (seen above this ring) is entirely inactive. The epithelium is stained yellow by the diazonium salt and is unrelated to enzymatic activity. Incubation 15 minutes. $\times 400$.

FIG. 12. Rat prostate. Intense activity in the apical border of glandular epithelium. Incubation 15 minutes. $\times 400$.

FIG. 13. Human ovary. A corpus luteum in an early stage of vascularization. Intense activity in the theca interna. Incubation 30 minutes. $\times 75$.

FIG. 14. Rat adrenal. The juxtamedullary area of the zona reticularis is most active. Cords of medullary cells can be seen. The yellow staining of the nuclei and cell membranes in these medullary cells (right) is due to the long incubation period which resulted in staining by decomposition products of the diazonium salts and not to enzymatic activity. Incubation 4 hours. $\times 400$.



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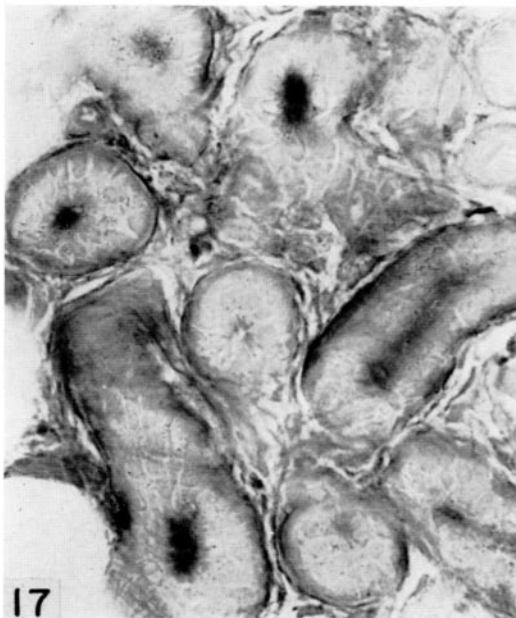
PLATE 134

FIG. 15. Rat dermis. Fibroblasts are discretely stained. Enzymatic localization reveals the delicate tapering processes of these cells. Incubation 2 hours. In this case, sections were mounted in glycerol-gelatin. $\times 250$.

FIG. 16. Same as Fig. 15. Aminopeptidase activity appeared as granules and droplet-like inclusions producing a beaded character to the fine cell processes. The processes appear to make contact with each other suggesting a syncytium. $\times 1000$.

FIG. 17. Human skin (leg). Section of coiled segments of sweat gland located deep in the corium. Intense staining of the apical border of secretory cells in most of the ducts. The remainder of their cytoplasm and nuclei are faintly pinkish. Another peak of activity is reached at the site of the basement membrane. Some staining is also present in fibroblasts in the intervening connective tissue. Incubation 45 minutes. $\times 400$.

FIG. 18. Rat mast cell. Four mast cells are seen in the interstitial connective tissue of the prostate. Prominent granules of different size are demonstrably stained and nuclei were unstained. The metachromatic granules which were demonstrated in adjacent sections were inactive for leucine aminopeptidase and were stained yellow by diazonium salt. Incubation 1 hour. $\times 2,000$.



(Nachlas *et al.*: Localization of leucine aminopeptidase)