ULTRACYTOCHEMICAL AND BIOCHEMICAL INVESTIGATIONS OF ALKALINE PHOSPHATASE AND 5'-NUCLEOTIDASE ACTIVITIES IN CARCINOGEN-INDUCED WELL- AND POORLY-DIFFERENTIATED ADENOCARCINOMA OF THE RAT STOMACH

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Ultracytochemical localizations and biochemical activities of alkaline phosphatase (ALPase) and 5'-nucleotidase (5'-Nase) in carcinogen-induced adenocarcinomas of the rat stomach were investigated. Immunoprecipitations and isozyme patterns of ALPase revealed a significant increase of liver/bone type ALPase in well-differentiated adenocarcinomas. Ultracytochemically, the reaction product of ALPase activity appeared on the basal plasma membrane of glandular carcinoma cells, whereas 5'-Nase was on the apical plasma membrane; poorly-differentiated carcinoma cells were devoid of these enzyme activities. Both activities of ALPase and 5'-Nase were conspicuous in the extracellular matrix (ECM), and especially on the fibroblasts adjacent to carcinoma cells. The pronounced activities of these enzymes may possibly be involved in the interaction between neoplastic cells and their ECM. For ultrastructural demonstration of 5'-Nase, the cerium-based method appeared to provide little nonspecific deposits of the reaction product, being superior to the conventional lead-based method.

As normal cells undergo malignant transformation, their surface properties are altered. This recognition is based upon accumulated evidence from a great number of experimental studies, delineating some significant pathophysiological changes in cell-to-cell communication, permeability or transport, lipid and protein composition, and dynamic behavior of membrane constituents of transformed cells (8, 21, 32, 34). Some membrane-associated enzyme activities show changes in malignantly transformed cells, often in relation to their differentiation (5, 20, 26). The present study concerned electron microscopic cytochemical changes or expressions of alkaline phosphatase (ALPase) and 5'-nucleotidase (5'-Nase) activities in well- and poorly-differentiated carcinomas of the rat stomachs. ALPase is normally absent in the glandular epithelium of the stomach, but the appearance of its enzyme characterizes intestinal epithelial metaplasia of the stomach; this has frequently been described as a significant functional change preceding the development of stomach carcinoma (23, 24). In view of this relationship, identification of ALPase isozymes was biochemically carried out. 5'-Nase, which is widely distributed in various tissues, has been used as a marker enzyme for plasma membrane in subcellular fractions (6, 39). Cytochemical localization of cytosolic 5'-Nase is not well understood (14); it is even difficult to differentiate the activity of 5'-Nase from that of ALPase by the conventional method using lead as a capture agent (9, 35). In the present study, a cerium-based method (36) was employed for comparison in rigorous investigation of the localization of 5'-Nase and ALPase activities in experimental gastric carcinomas.
MATERIALS AND METHODS

Induction of gastric carcinoma

The experiment for induction of gastric carcinomas in rats was done with a modification of the original design with a direct acting carcinogen, N-methyl-N-nitrosourea (MNU), which we have recently described as a useful experimental model for gastric carcinogenesis (16). F344 male rats of inbred strain (Charles River Japan Inc., Kanagawa) divided into 2 groups (Groups I and II) were used at the age of 8 weeks. Each group was composed of 10 rats. MNU (Nacalai Tesque Co., Kyoto) was dissolved in distilled water at a concentration of 200 ppm, twice a week, as daily drinking water for group I. They were given the MNU solution ad libitum for 15 weeks; the drinking water was then switched to tap water and maintained for a further 15 weeks. Group II was given weekly gavage for 8 times with 4 mg of MNU in 3 ml of distilled water and then maintained for 60 weeks. They were fed a commercial CE-2 animal diet (CLEA Japan Inc., Tokyo) during the experiment. At the end of experiments, tumorous lesions in the glandular (pyloric) portion of the stomach occurred in all rats in Groups I and II. Prior to histochemical and ultracytochemical investigations, frozen tissue sections from part of each tumor were quickly examined. Small portions of the tissue with gastric lesions were processed for histocytochemical and biochemical studies, and most portions of each stomach from all rats were processed for histopathologic evaluations by routine formalin fixation and paraffin embedding. The lesions of the stomach in Group I were well-differentiated adenocarcinoma which were invasive to the submucosal layer of the stomach. In Group II, poorly-differentiated (signet-ring cell type) adenocarcinoma occurred in 3 rats and well-differentiated adenocarcinoma in other 7 rats.

Histochemical and ultracytochemical procedures

Six cases of well-differentiated adenocarcinoma from Group I, 2 of poorly-differentiated carcinoma from Group II and the normal glandular stomach from 2 untreated controls at the age of about 30 weeks were used for histochemical and ultracytochemical studies. Some frozen tissue samples were cut on a cryostat for histochemical ALPase reaction with conventional azo-dye method (3) and for 5'-Nase reaction with lead-based method (43). For ultracytochemistry, fresh tissues which were cut into longitudinal strips of approximately 0.2 cm width with razor blades were fixed in cold (4°C) 2% paraformaldehyde plus 1% glutararadehyde in 0.1 M cacodylate buffer (pH 7.4) for 1.0 hr. Then the tissues were washed overnight at 4°C in cacodylate buffer containing 8% sucrose followed by sectioning at 40 μm thickness with a microslicer (Dosaka EM Co., Ltd., Kyoto). The tissue sections were then immediately incubated at 37°C for 30 min in the medium for ALPase at pH 9.2-9.4 (lead-based method) or pH 7.4 (cerium-based method) and for 5'-Nase at pH 7.2 (lead-based method) or pH 7.4 (cerium-based method). In the lead-based method, the medium for ALPase (27) contained 28 mM Tris-HCl buffer (pH 8.5), 20 mM β-glycerophosphate (Sigma Chem. Co., St. Louis), 3.9 mM MgSO₄, 2.0 mM lead citrate and 8% sucrose; the medium for 5'-Nase (43) consisted of 78.2 mM Tris-malate buffer (pH 7.2), 1.4 mM adenosine-5'-monophosphate (AMP, Sigma Chem. Co., St. Louis), 10 mM MgSO₄, 1.8 mM Pb(NO₃)₂ and 6% sucrose. In the cerium-based method, the medium for ALPase (36) contained 100 mM Tris-malate buffer (pH 8.0), 1 mM β-glycerophosphate, 2 mM CeCl₃, and 6% sucrose; the medium for 5'-Nase (35) consisted of 100 mM Tris-malate buffer (pH 7.4), 1 mM AMP, 2 mM MgCl₂, 2 mM CeCl₃ and 6% sucrose. After incubation, the sections were postfixed with 1% osmium tetroxide solution for 30 min, followed by graded ethanol and propylene oxide and embedded in epoxy resin. Ultrathin sections were double-stained with both uranyl acetate and lead acetate.

To observe the possible intracellular localization of ALPase and 5'-Nase activity, saponin was added to the incubation medium at the final concentration of 0.005% for some of the tissue sections (41, 42). Incubation was in substrate-free media and/or in media in which levamisole (Sigma Chem. Co., St. Louis), 5 mM and 2 mM for ALPase and 5'-Nase, respectively, were used as control reactions.

Biochemical assays

Part of the same stomach tumors form 6 rats in Group I as those used for cytochemical investigations was biochemically assayed for ALPase and blood samples from 4 out of those 6 rats were also assayed. ALPase activity in the samples was defined as released p-nitrophenolate molar absorption at 405 nm. The reaction mixture contained 2 mM disodium p-nitrophenoyle phosphate and 50 mM carbonate/bicarbonate buffer (pH 10.0), in the presence of 1 mM MgCl₂, at 37°C (25). Conventional electrophoresis for ALPase isozymes was carried out on the agarose gel. ALPase-active bands were detected with 5 mM 5-bromo-3-indolyl phosphate p-toluidine salt, 1 mM MgCl₂, and 10 μM Zn (CH₃COO)₂ in 1 M 2-amino-2-methyl-1,3-propanediol buffer (pH 10.2), and fixed with 5% acetic acid. ALPase isozyme in the immunoprecipitation tests was identified by the use of

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monoclonal ALPase antibodies as described earlier (30) compared with the control tube without the antibodies.

RESULTS

Histochemical and ultracytochemical localization of ALPase

Lead-based method

Histochemical reaction for ALPase was unequivocally positive in the fibrous stroma of all carcinomas examined both for well-differentiated and poorly-differentiated types, being more markedly positive in the former type (Figs. 1a, b). Non-neoplastic areas outside of the carcinomas were almost completely negative for ALPase except for blood vessels, as were the pyloric gland mucosa of the normal controls. Electron microscopic cytochemistry revealed distinct localization of ALPase activity on the plasma membrane of fibroblasts adjacent to carcinoma cells, and diffuse reaction product of ALPase appeared in the collagenous extracellular matrix (ECM). The basal plasma membrane of well-differentiated carcinoma cells in close proximity to the collagenous ECM and fibroblasts showed a positive reaction, but no reaction product of ALPase was detected on the apical or microvillous membrane or on the lateral plasma membrane adjacent to which ECM components were almost absent. A very weak positive reaction, if any, was found on the plasma membrane of fibroblasts in the control ECM with no reaction product on any aspect of plasma membrane of normal glandular epithelial cells (Figs. 2a, b; Table 1). In contrast to well-differentiated type, no reaction product was demonstrated on the surface membrane of carcinoma cells of poorly-differentiated type (Fig. 3). Tissue sections from adenocarcinomas which were incubated with saponin-containing media demonstrated much increased ALPase activity in the fibroblasts surrounding carcinoma cells; that is, lead precipitates as reaction products on the plasma membrane with partial pitting into the cytoplasm were intensified (Fig. 4). The reaction products in the subcellular organelle of fibroblasts, however, were not evident. There were no lead precipitates in any areas of control sections in substrate-free media and/or in media with levamisole addition.

Cerium-based method

No reaction product of ALPase was demonstrable

Table 1. Ultracytochemical localization of ALPase and 5'-Nase in normal pyloric gland mucosa [A] and alteration of both enzymes in MNU-induced well-differentiated adenocarcinoma [B] of the rat stomach

<table>
<thead>
<tr>
<th></th>
<th>Epithelial cells (PM)</th>
<th>Fibroblasts (PM)</th>
<th>Extracellular matrix</th>
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<tbody>
<tr>
<td></td>
<td>Apical</td>
<td>Lateral</td>
<td>Basal</td>
</tr>
<tr>
<td>ALPase</td>
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<td></td>
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<tr>
<td>(Lead)*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(Cerium)*</td>
<td>-</td>
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<tr>
<td>5'-Nase</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(Lead)*</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>(Cerium)*</td>
<td>(-)</td>
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<thead>
<tr>
<th></th>
<th>Carcinoma cells (PM)</th>
<th>Fibroblasts (PM)</th>
<th>Extracellular matrix</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Apical</td>
<td>Lateral</td>
<td>Basal</td>
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<tr>
<td>ALPase</td>
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<tr>
<td>(Lead)*</td>
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<tr>
<td>(Cerium)*</td>
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<tr>
<td>5'-Nase</td>
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<td>(Lead)*</td>
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<tr>
<td>(Cerium)*</td>
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</table>

PM, plasma membrane; *, capture agents (see MATERIALS AND METHODS); (−), almost negative.
on the plasma membranes of either fibroblasts or carcinoma cells.

Histochemical and ultracytochemical localization 5'-Nase
Lead-based method

No histochemical positive reaction for 5'-Nase was present in the glandular structure of adenocarcinoma.

Figs. 1a, b. Histochemical reactions for ALPase activity of the stomach.
1a. Strong positive reaction in the fibrous stroma of an intramucosal neoplastic lesion in well-differentiated adenocarcinoma.
1b. Marked positive reaction in the fibrous tissue stroma of invasive poorly-differentiated adenocarcinoma.
Azo-dye method, counterstained with hematoxylin. 1a. ×92, 1b. ×135
cinomas nor in the pyloric gland of the normal stomach, whereas the fibrous connective tissue stroma around the neoplastic lesions stained positive. Electron microscopic cytochemistry for 5'-Nase revealed the reaction product in the ECM and fibroblastic plasma membrane adjacent to the neoplastic epithelium (Fig. 5a; Table 1). Fibroblasts in the normal stomach also occasionally showed weak positive

Figs. 2a, b. Electron micrographs of ALPase activity of the stomach.
2a. No reaction product on the plasma membrane of pyloric glandular epithelial cells from the normal control rat. × 6,940
2b. Note the clear reaction product on the basal plasma membranes (arrows) of carcinoma cells and fibroblasts (double arrows) in well-differentiated adenocarcinoma with glandular structure. No reaction product on the lateral and apical or microvillous membrane of carcinoma cells. Fib: fibroblast. × 5,470
reaction. These reaction products were considerably diminished when levamisole was added to the reaction medium, yet such products were still observed on the fibroblastic plasma membrane and the ECM (Fig. 5b); there was no reaction product on any aspect of the plasma membranes of either carcinoma cells or glandular epithelial cells of the normal stomach. Control sections in substrate-free media showed no lead

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**Fig. 3.** An electron micrograph of ALPase activity in poorly-differentiated adenocarcinoma (signet-ring cell type) of the stomach. Note the marked reaction product both on the plasma membrane of the fibroblast and collagenous matrix adjacent to carcinoma cells; no reaction product on the plasma membrane of carcinoma cells. Sig: signet-ring cell carcinoma, Fib: fibroblast. ×5,400

**Fig. 4.** An electron micrograph of ALPase activity with saponin-containing medium. Note much increased reaction products on the plasma membrane with partial pitting into the cytoplasm of fibroblasts. ×10,880
Figs. 5a, b. Electron micrographs of 5'-Nase activity in well-differentiated adenocarcinoma of the stomach using the lead-based method.

5a. Note the marked reaction product on the fibroblastic plasma membrane and extracellular matrix adjacent to neoplastic epithelium without levamisole-containing medium. Fib: fibroblast. × 4,520

5b. Note the drastic diminution of the reaction product with levamisole-containing medium. Fib: fibroblast. × 5,570
Figs. 6a–d. Electron micrographs of 5'-Nase activity of the stomach using the cerium-based method.

6a. Note little reaction product on the plasma membrane of the glandular epithelial cells and fibroblasts from the normal rat. × 6,430

6b, c. Note fine and linear electron-dense precipitates on the fibroblastic plasma membrane, and basal and apical or microvillous membrane of well-differentiated adenocarcinoma cells without levamisole-containing medium. 6b. × 6,280, 6c. × 8,250

6d. Note the reaction product only on the apical or microvillous membrane of well-differentiated adenocarcinoma cells and no reaction product on the basal plasma membranes with levamisole-containing medium. × 7,050
Fig. 7. An electron micrograph of 5'-Nase activity in signet-ring cell carcinoma of the stomach. Reaction products on the plasma membrane of carcinoma cells are not demonstrated although the minimum products are occasionally seen on the microvillous membrane. Cerium-based method with levamisole-containing medium. ×5,730

<table>
<thead>
<tr>
<th>Samples (rat no.)</th>
<th>Total ALPase activity (μmol/hr/mg)</th>
<th>% of ALPase isozymes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>Carcinomas</td>
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<td></td>
</tr>
<tr>
<td>(1)</td>
<td>0.058</td>
<td>92</td>
</tr>
<tr>
<td>(2)</td>
<td>0.039</td>
<td>89</td>
</tr>
<tr>
<td>(3)</td>
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<td>30</td>
</tr>
<tr>
<td>(4)</td>
<td>0.113</td>
<td>22</td>
</tr>
<tr>
<td>(5)</td>
<td>0.134</td>
<td>33</td>
</tr>
<tr>
<td>(6)</td>
<td>0.099</td>
<td>31</td>
</tr>
<tr>
<td>Normal stomachs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(7)</td>
<td>0.018</td>
<td>45</td>
</tr>
<tr>
<td>(8)</td>
<td>0.016</td>
<td>47</td>
</tr>
<tr>
<td>Sera from carcinoma-bearing rats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1)</td>
<td>0.201</td>
<td>43</td>
</tr>
<tr>
<td>(2)</td>
<td>0.190</td>
<td>29</td>
</tr>
<tr>
<td>(3)</td>
<td>0.212</td>
<td>68</td>
</tr>
<tr>
<td>(4)</td>
<td>0.195</td>
<td>59</td>
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<tr>
<td>Sera from normal rats</td>
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<td></td>
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<tr>
<td>(7)</td>
<td>0.189</td>
<td>22</td>
</tr>
<tr>
<td>(8)</td>
<td>0.171</td>
<td>9</td>
</tr>
</tbody>
</table>

The data were the mean of 2 determinations.
precipitates.

Cerium-based method

In the normal controls, there was little, if any, reaction product of 5'-Nase in the glandular epithelial cells and ECM including fibroblasts (Fig. 6a). In well-differentiated adenocarcinoma, however, a positive reaction was sometimes demonstrated without levamisole-containing medium on the basal and apical or microvillous plasma membranes of carcinoma cells and the plasma membrane of fibroblasts adjoining these cells. The reaction product on the fibroblastic plasma membrane was invariably prominent where intercellular spaces were almost absent (Figs. 6b, c). Unlike the lead-based method, a fine and linear electron-dense precipitate appeared only on the plasma membrane; the ECM was almost devoid of the reaction product. This enzymatic positive reaction was found to be on the apical microvillous membrane of adenocarcinoma cells with a negative reaction on the basal plasma membrane, in levamisole-containing medium (Fig. 6d; Table 1). The intracellular localization of the enzymatic activity was not detected with saponin-containing media. 5'-Nase activity in poorly-differentiated adenocarcinoma, on the other hand, was mostly absent, although minimum reaction products were occasionally seen on the microvillous membrane (Fig. 7).

Biochemical assays

An approximately 7-fold increase of total ALPase activity yielded in carcinomas, on the average, compared to the normal controls. Immunological and electrophoretical data showed that ALPase in both carcinomas and normal stomachs mainly comprised the liver/bone type (tissue-unspecific) isozyme, while the intestinal isozyme was minimal or imperceptible (Table 2; Fig. 8a). In the sera of those rats, on the

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Fig. 8a, b. Isozyme patterns of ALPase from rat stomachs (8a) and their sera (8b). 1–8, same rat numbers as those in Table 2. L: liver ALPase, B: bone ALPase, I: intestinal ALPase. Anodal migration is toward the left.
other hand, regardless of the presence or absence of stomach carcinomas, the intestinal isozyme was clearly identified in addition to the tissue-unspecific isozyme (Fig. 8b).

**DISCUSSION**

The high content of biochemical activity of ALPase in the homogenates of MNU-induced stomach carcinoma is considered to represent the marked increase of histochemical and ultracytochemical activity in the fibroblasts and ECM surrounding carcinoma cells. Since a higher content of ALPase activity in the stomach carcinoma induced by a higher dose of MNU was reported in our previous studies (18, 19), the pronouncement of this ALPase activity may possibly be dependent upon the dose of the carcinogen. In view that ALPase activity was absent on the apical or lateral plasma membrane of carcinoma cells as well as their surrounding non-neoplastic glandular epithelium and that a high content of ALPase was composed mainly of liver/bone (tissue-unspecific) type isozyme, it was less likely that intestinal metaplasia was involved during carcinogenesis in the present experiment, although such metaplasia has been suggested as an important condition and/or precursor for stomach carcinoma by some investigators. Demonstration of the intestinal isozyme of ALPase in the sera in the present experiment was consistent with the previous reports (28) that the appearance of intestinal isozyme in the sera is a phenomenon common to the rats, but not to the human.

We recently described pronounced ALPase activity in the stroma of neoplastic lesions of the stomach (18, 19); the present investigation with saponin as a membrane pit permeator did not clearly demonstrate that the main producer of ALPase was fibroblasts, since the enzymatic activity was not detected in their organelle, such as endoplasmic reticulum, Golgi apparatus and others. It is conceivable that ultracytochemically positive reaction for ALPase in the collagenous ECM between fibroblasts and carcinoma cells represents a soluble form of tissue-unspecific ALPase derived from fibroblastic plasma membrane. The mechanism of this transition from membrane-bound to soluble enzymes such as ALPase and 5'-Nase has recently been elucidated; that is, the released and/or soluble form is produced by enzymatic cleavage of the glycan-phosphatidylinositol-anchoring moiety in the membranous form (10, 17, 31, 33). The prominent activity of 5'-Nase observed on the plasma membrane of fibroblasts devoid of intercellular spaces might represent well preservation of membrane-bound form because of far less cleavage of the anchoring moiety under the paucity of ECM. Much increased ALPase activity in the ECM and the basal plasma membrane of well-differentiated carcinoma cells may be a phenotypic expression with involvement in some epithelial-stromal interaction during stomach carcinogenesis. The enzymatic activity restricted to the basal plasma membrane is possibly related to the abundance of ECM and fibroblasts adjacent to these membranes. This is probably supported by the recent studies by Zackson and Steinberg providing evidence that the candidate cell guidance associated molecule, identified as ALPase, plays an important role in directing embryonic cell migration (45, 46); furthermore, two ALPase (embryonal and tissue-unspecific) isozyme genes are expressed and the switch in predominance from embryonal to tissue-unspecific ALPase occurs during early development in mouse embryo (13).

5'-Nase, an intrinsic membrane glycoprotein, is highly concentrated in the plasma membranes of many eukaryotic cells (4, 6). Yet, the localization of the enzyme in the pyloric gland epithelium or gastric carcinoma, has not, thus far, been reported. In the present ultracytochemical study with the cerium-based method and the conventional lead nitrate method, clear activity of 5'-Nase was specifically demonstrated on the fibroblastic plasma membrane and the apical or microvillous membrane of gastric adenocarcinoma cells (well-differentiated type) only by the former method after levamisole treatment. The plasma membrane of the carcinoma cells were invariably negative for 5'-Nase with the latter method, but nonspecific precipitates were in the ECM and on fibroblastic plasma membrane in the stroma of the carcinomas. The disappearance of these precipitates after levamisole treatment indicates that they were some activities of tissue-unspecific ALPase (2). These findings are in agreement with the proposal by Robinson and Karnovsky (35) of the usefulness of a new method with cerium-based cytochemical reaction for 5'-Nase that does not rely upon lead. Actually, lead has several drawbacks in some instances, such as an inhibitory effect to the enzyme, nonenzymatic breakdown of substrate, and nonspecific deposits of lead or lead phosphate (9, 35, 36). The only problem with the cerium-based techniques, however, is that the reaction product is not readily visible at the light microscope level (36, 40). That the cerium-based technique failed to demonstrate ALPase activity in the present study was ascribed possibly not only to increased acidity (pH 7.4) to retain stability of cerium
ACKNOWLEDGMENTS

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