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A Novel Calcium-Independent Enzyme Capable of Incorporating Putrescine into Proteins

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A Ca^{++}-independent enzyme capable of incorporating [^{3}H]-putrescine into proteins was detected in the rat intestine mucosa. The Ca^{++}-independent incorporation of [^{3}H]-putrescine into proteins was temperature-, pH-, time-, and dose-dependent. However, this enzyme was absent in the gastric mucosa. Similar to testicular Ca^{++}-dependent transglutaminase, the optimal pH of intestinal Ca^{++}-independent enzyme was 9.0. At 10^{-5} M or less putrescine concentrations, the Ca^{++}-independent enzyme in an intestinal cytosol preparation showed a greater activity than did the Ca^{++}-dependent transglutaminase. However, at higher putrescine concentrations, the latter showed a greater activity than did the former. Both the intestinal Ca^{++}-dependent and independent enzymes were inhibited by cystamine, thermal labile at 50°C and precipitated by 30 to 50% saturation of ammonium sulfate. The fact that these two enzymes shared many similar characteristics, with the exceptions of Ca^{++}-requirement, suggests that they may have similar active site and intrinsic molecular function(s).

Key Words: **transglutaminase; putrescine; polyamines; intestine; testis; colon; stomach; mucosa.**

A large volume of literature documents the importance of diamine putrescine and polyamines (spermidine and spermine) in normal cell growth, proliferation and differentiation [1–3]. These aliphatic organic cations are found in all living cells and are involved in the regulation of macromolecular biosynthesis and the stabilization of nucleic acids as well as cell membranes [2–4]. Recent data demonstrat that polyamines play crucial roles in plasma membrane ion trnsport (5) and NMDA receptor function (6). However, the precise mechanism of polyamine action in each cell type remains to be delineated.

Recent studies have demonstrated that polyamines are essential during gastrointestinal adaptation [7-10] and healing of induced gastric and duodenal damage [11-12]. Data from Wang and Johnson revealed that the inhibition of transglutaminase-catalyzed protein cross-linking with dansylcadaverine prevented the effect of polyamines on the healing of gastrointestinal mucosa [13]. This observation implies that protein cross-linking catalyzed by transglutaminase may be part of the mechanism through which polyamines are required for the gastrointestinal repair process.

Transglutaminase is a Ca^{++}-dependent enzyme and forms (γ-glutamyl) lysyl isopeptide bonds between glutamine and lysine residues leading to the cross-linking of two protein molecules [14, 15]. Putrescine and polyamines also serve as natural substrates for transglutaminase to form γ-glutamyl-amine derivatives. Through both terminal amino groups, these aliphatic amines may also link two polypeptides or proteins at the glutamyl residues. The modification of proteins with di- and polyamines occurs intracellularly [16-18] and extracellularly [19].

Several different species of transglutaminases have been demonstrated in different tissues and body fluids. The biological roles of circulating transglutaminase, factor XIII, in blood coagulation [14] and epidermal particulate-bound transglutaminase in keratinocyte differentiation [20-22] have been established, although the processes are not completely understood. However, the functional role(s) of the tissue-transglutaminase which exists in a variety of cells remains to be defined. Tissue-transglutaminase has been suggested to associated with the regulation of cell proliferation [23, 24], receptor-mediated endocytosis [14, 24-26], secretion coupling [24], cell differentiation [24, 27,28], cellular senescence [29] or programmed cell death [24, 30], as well as the formation of the intracellular matrix [18, 24] and the process of aging [15, 31].

In this paper, the existence of a novel calcium-independent enzyme of rat intestinal mucosa, capable of covalently incorporating [^{3}H]-putrescine into proteins, is presented in addition to the calcium-dependent transglutaminase.

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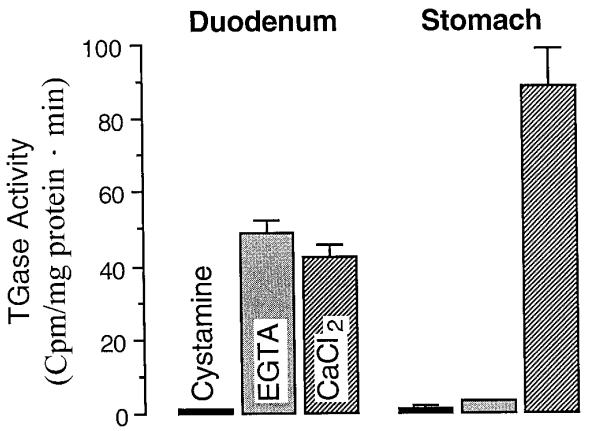

FIG. 1. Incorporation of [^{3}H]-putrescine into proteins by duodenal and gastric mucosal enzyme preparations. Mucosal tissues from 5 rats were homogenized separately in 20 mM Tris - HCl, pH 7.5 - 1 mM EDTA - 1% β-mercaptoethanol. Aliquots of 30,000 $\times$ g supernatants were used for the assay of transglutaminase (TGase) activities in the presence of 0.25 mM [^{3}H] - putrescine (1 μCi/100 μl reaction), 50 mM Tris - HCl, pH 9.0 at 37°C for 15 min. The incubation mixture contained either 5 mM EGTA or 5 mM $CaCl_2$ with or without 10 mM cystamine. Only one cystamine bar was presented in each group since 5 mM EGTA + cystamine and 5 mM $CaCl_2$ + cystamine resulted in similar activities.

MATERIALS AND METHODS

Young male Sprague Dawley rats (150g up) were housed at 22°C with a 12 h light-dark cycle and allowed free access to tap water and a standard laboratory rat diet. Putrescine dihydrochloride-[2, 3-^{3}H(N)-1] (40.3 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Putrescine-dihydrochloride, leupeptin, pepstatin and phenylmethylsulfanyl fluoride (PMSF), and β-mercapto-ethanol were purchased from Sigma (St. Louis, MO).

Preparation of enzymes. Rats were sacrificed in a CO_2 gas chamber. Gastric oxyntic glands, 4 cm segments of duodenum (2 cm distal from the pylorus), jejunum (4 cm distal from the ligament of Treitz) or distal ileum (4 cm proximal to the ileocecal valve) were collected, flushed thoroughly with ice-cold saline (0.9% NaCl) and kept in ice-cold saline separately until the collection of mucosa. The mucosa from each tissue was scraped with a glass slide over an ice-cold glass plate and placed into a Beckman 12 ml plastic centrifuge tube. The mucosal tissues were homogenized in 10 volumes (v/w) of 20 mM Tris-HCl, pH 7.5, 1 mM EDTA and 1% β-mercaptoethanol [32] containing protease inhibitors (leupeptin 10 μg/ml, pepstatin 10 μg/ml, PMSF 10 mM) or in other buffers as stated in the text. The homogenates were centrifuged at 30,000 $\times$ g at 4°C for 45 min. The supernatants were transferred to new tubes for subsequent assays.

Transglutaminase assays. A 100 μl reaction mixture containing 1 μCi [^{3}H]- putrescine, 2 mg/ml dimethylcasein, 5 mM $CaC1_2$ or 5 mM EGTA, 50 mM Tris-HCl, pH 9.0 (or as otherwise stated) and 60 μl of the 30,000 $\times$ g mucosal supernatant was incubated at 37°C for 15 min or as stated. At the end of incubation, an aliquot of 50 μl was spotted onto a piece of 3 MM Whatman filter paper and plunged immediately into ice-cold 10% trichloroacetic acid (TCA) for 30 min. The filter was washed 3 more times (5 min each) in ice-cold 5% TCA followed by 3 rinses with ice-cold 95% ethanol and air dried. The incorporated radioactivity was counted in 5 ml scintillation fluid [EcoLite (+), ICN Biomedicals, Inc., Irvine, CA] in a Beckman LS 5000TA (Fullerton, CA). Supernatants were assayed for protein concentrations according to the Bradford method [33]. Enzymatic activity was expressed as pmol incorporated per mg protein per hour or as cpm incorporated per assay tube (30 μl of enzyme).

Ammonium sulfate fractionation of mucosal cytosol. The cytosol preparations were fractionated with saturated ammonium sulfate prepared in the homogenizing buffer. The fractions precipitated by 0 to 30%, 30 to 50% and 50 to 70% saturated ammonium sulfate and were collected by centrifugation at 10,000 $\times$ g for 10 min. The pellets

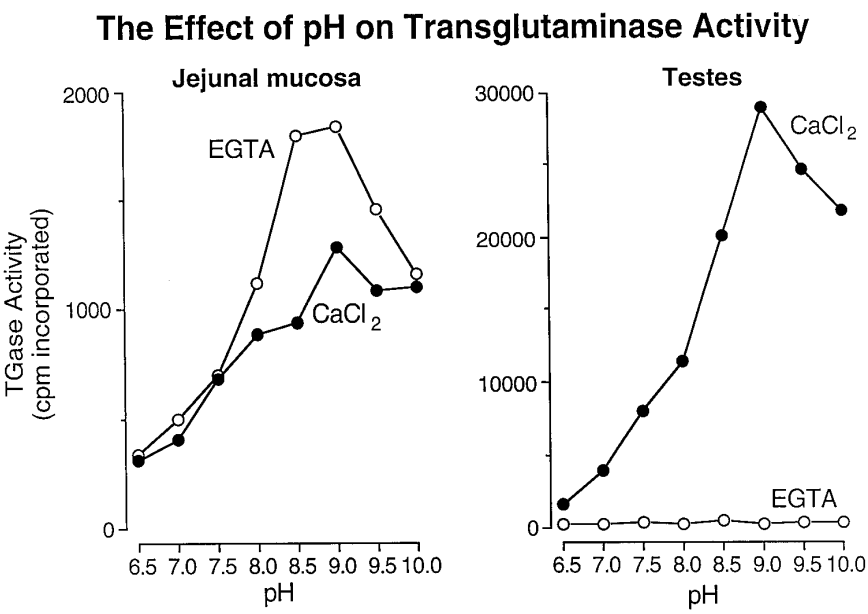

FIG. 2. The effect of pH on the enzyme activities. Ten μl of 500 mM Tris - HCl - 1 % β-mercaptoethanol of various pHs was included in every 100 μl reaction mixture containing 50 μl of jejunal mucosal or testicular enzyme prepared in the presence of protease inhibitors. The assay was performed in the presence of 5 mM $CaCl_2$ or 5 mM EGTA at 37°C for 30 min. Data represent means of duplicate determinations in one of many experiments.

were redissolved in 1 ml of homogenizing buffer, and dialyzed at 4°C against 20 volumes of homogenizing buffer, with 3 changes, for 1 h each. After dialysis, the cytosol fractions were clarified by centrifugation prior to enzyme assay.

RESULTS AND DISCUSSION

Transglutaminase Activity in the Gastric and Duodenal Mucosa

During the studies of transglutaminase activities in gastrointestinal mucosa, it was found that the incorporation of [^{3}H]-putrescine into proteins by cytosol preparations of duodenal mucosa occurred either in the presence of 5 mM $CaCl_2$ or 5 mM EGTA (Fig 1). This implied that the enzymatic incorporation of [^{3}H]-putrescine into proteins did not require calcium ion. On the contrary, only the Ca^{++}-dependent incorporation of [^{3}H]-putrescine was detected in gastric mucosal preparations. All putrescine-incorporation by either duodenal or gastric cytosol preparations was abolished in the presence of 10 mM cystamine as was that by the Ca^{++}-dependent transglutaminase reported in the literature. This suggests the requirement of a reduced sulfhydryl structure for active enzyme function. The inclusion of a sulfhydryl reducing agent in the assay and isolation buffer is essential.

The Effects of pH and Temperature

To further characterize the enzyme, its activity was measured in reaction mixtures of different pHs. The intestinal mucosal enzyme(s) either in the presence of $CaCl_2$ or EGTA showed a maximal activity around pH 9.0, identical to that exhibited by the Ca^{++}-dependent testicular soluble tissue-transglutaminase (Fig 2). The overall intestinal enzyme activity on a per mg protein basis was only 1/40 to 1/10 of that of testicular preparations. Furthermore, all preparations of duodenal, jejunal and ileal mucosal cytosol showed Ca^{++}-independent activities of [^{3}H]-putrescine incorporation. The Ca^{++}-independent incorporation of [^{3}H]-putrescine, measured in the presence of 5 mM EGTA, was a temperature dependent reaction. At 4°C, the activity was negligible as compared to that measured at 37°C (Fig 3).

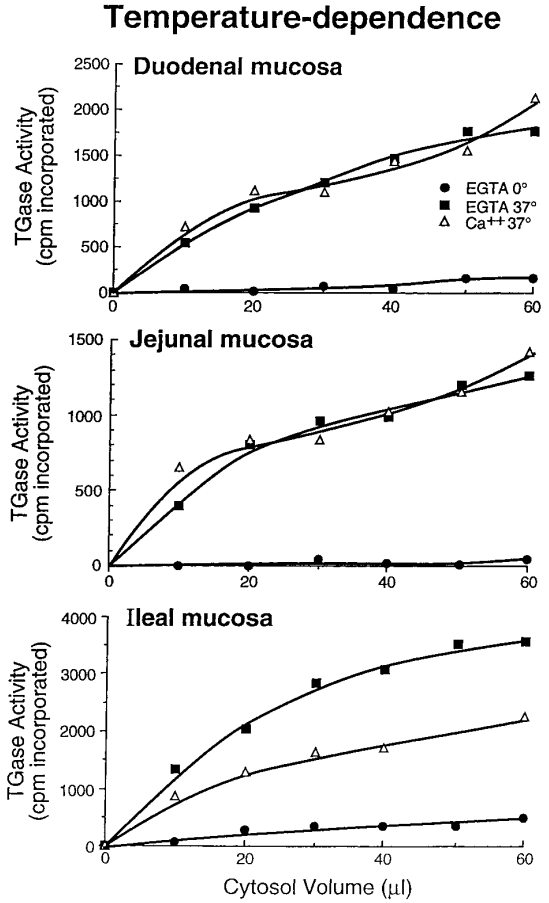

FIG. 3. Temperature-dependence of intestinal mucosal enzymes. Duodenal, jejunal and ileal mucosa were homogenized separately in 20 mM Tris - 1 mM EDTA - 1% β-mercaptoethanol, pH 7.5 containing protease inhibitors (leupeptin, 10 μg/ml; pepstatin, 10 μg/ml and PMSF, 10 mM). Aliquots of 30,000 × g supernatant were assayed as described in Methods. Each data point represents means of duplicate assays.

Time Course and Dose Dependency

When the reaction mixtures containing aliquots of intestinal cytosol and 5 mM $CaCl_2$ were incubated at 37°C for various time intervals, the activity of intestinal transglutaminase increased linearly during the first 10 min and leveled off afterwards (Fig 4A). The activity of the intestinal Ca^{++}-independent enzyme increased gradually and reached a plateau after 45 min of incubation (Fig 4A). When measured in the presence of either $CaC1_2$ or EGTA during a 15 min assay interval, both enzyme activities increased as a function of the enzyme concentration in the reaction mixture (Fig 4B). In addition, at a constant amount of enzyme and sufficient amount of dimethylcasein, the incorporation of [^{3}H]-putrescine into protein by both enzymes were also dose-dependent (Table 1). The Ca^{++}-independent enzyme exhibited a greater activity at lower substrate concentration than did the Ca^{++}-dependent enzyme. However, the former enzyme activity leveled off at lower putrescine concentrations as compared to that of the latter enzyme. The data imply that the Ca^{++}-independent enzyme may play a more crucial role, at a lower polyamine level, in maintaining the normal intestinal mucosal function than does the other enzyme.

Fractionation of Enzymes with Ammonium Sulfate

The intestinal cytosol fractions precipitated by 0 to 30%, 30 to 50%, and 50 to 70% saturated ammonium

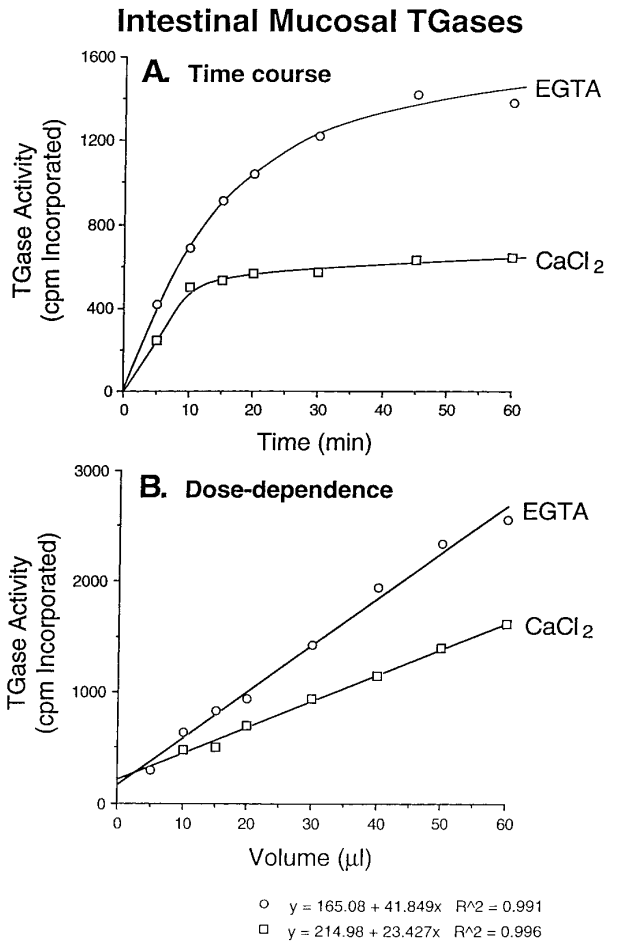

FIG. 4. Time course and dose-dependency of intestinal mucosal enzymes. Mixture of mucosa from equal lengths of jejunum and ileum was homogenized in Tris - EDTA - gb-mercaptoethanol containing protease inhibitors. A) Aliquots (30 μl) of samples were assayed at pH 9.0 in the presence of 5 mM $CaCl_2$ or 5 mM EGTA for intervals of time as indicated. B) Various amounts of the enzymes were assayed at 37°C for 15 min.

sulfate were analyzed for the activity of [^{3}H]-putrescine incorporation. The majority of both the Ca^{++}-dependent and independent enzyme activities were found in the fraction precipitated by 30 to 50% saturation of ammonium sulfate (Fig 5).

Thermal Stability of the Enzymes

The enzyme preparations were first isolated in the presence of protease inhibitors leupeptin, pepstatin and PMSF followed by fractionation with ammonium sulfate precipitation and dialysis. The partial purified Ca^{++}-dependent and Ca^{++}-independent enzymes also differed from each other in the thermal stability at 37 °C (Figure 6). The former was labile at 37°C while the latter was relatively more stable at this temperature. However, the activities of both enzymes were abolished at 50°C. The instability of Ca^{++}-dependent transglutaminase at 37°C partially explains why the activity of the intestinal Ca^{++}-dependent enzyme leveled off soon after 10 min of incubation, while the incorporation of putrescine catalyzed by the novel Ca^{++}-independent enzyme continued to increase during the 60 min interval. However, the time course of Ca^{++}-independent enzyme activity was not linear although the enzyme was relatively stable at 37°C.

TABLE 1

Substrate Concentrations and Enzyme Activities

Putrescine	Rate of [^{3}H]putrescine incorporation (pmol/mg protein/h) Assayed in 5 mM EGTA	Assayed in 5 mM $CaCl_2$
2.5×10^{-7} M	1.37 ± 0.04	0.65 ± 0.03
2.5×10^{-6} M	19.37 ± 0.24	10.47 ± 0.88
2.5×10^{-5} M	139.15 ± 3.01	84.16 ± 0.67
2.5×10^{-4} M	634.20 ± 10.08	679.82 ± 12.82
5×10^{-4} M	617.49 ± 18.71	783.05 ± 29.47
1.4×10^{-3} M	627.47 ± 13.91	1,004.60 ± 27.89

Note. Constant amounts of enzyme preparation were incubated in the presence of various concentrations of putrescine at 37°C for 15 min. Data represent means ± SE, $n = 3$ determinations.

The Ca^{++}-independent incorporation of putrescine into dimethylcasein was a typical enzymatic process since it exhibited temperature-, pH-, dose- and time-dependence. It can be enriched together with the Ca^{++}-dependent transglutaminase by ammonium sulfate fractionation at 30 to 50% saturation.. In comparison,

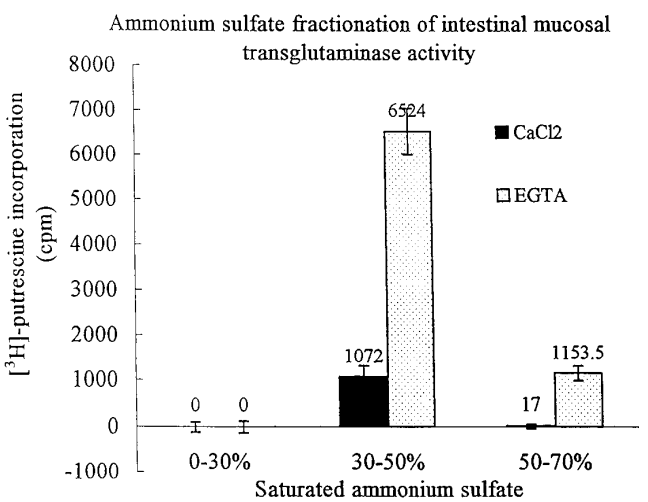

FIG. 5. Fractionation of the putrescine-incorporating enzymes by ammonium sulfate precipitation. The intestinal cytosol fractions were prepared and fractionated as described in Methods. The Ca^{++}-dependent and independent enzymes were assayed as stated previously. The greatest activities of both types of enzymes are shown in the fraction precipitated at 30 to 50% saturation of ammonium sulfate.

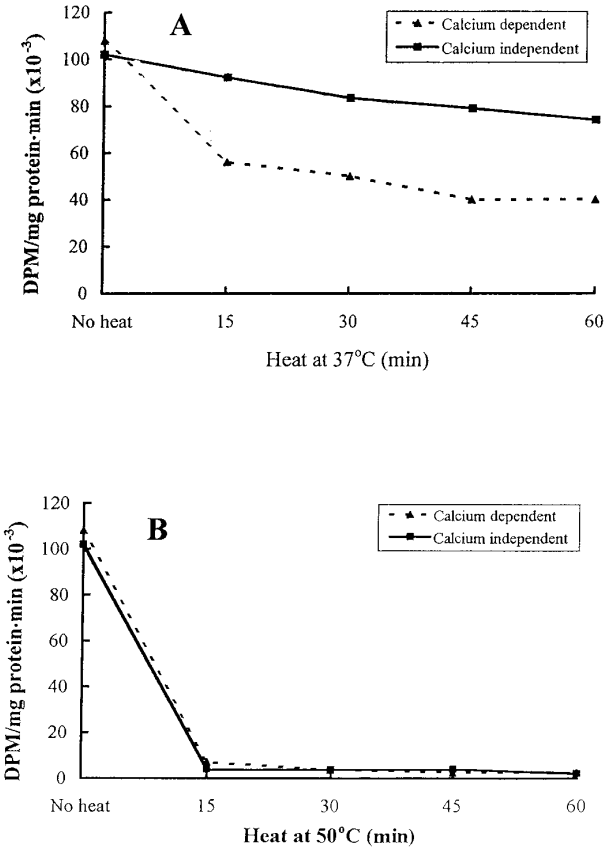

FIG. 6. Thermal stability of enzymes. Aliquots of partially purified enzyme preparation were heated at either 37 °C or 50 °C in assay tubes for designated time intervals. After cooling in ice water, the reaction mixture was added to each tube and enzyme activity was assayed as usual.

several characteristics were common for the novel intestinal Ca^{++}-independent, and both the testicular and intestinal Ca^{++}-dependent enzymes. These shared characteristics suggest that these enzymes may have similar molecular natures.

The association of polyamines with the maturation [8-10], adaptation [7] and healing [11-12] of gastrointestinal mucosal cells has been documented. Their mechanism of action has not yet been defined. Polyamines are able to modify proteins [16-19] by using their two amino terminals to crosslink two peptides or proteins via the action of Ca^{++}-dependent trasglutaminase. Wang and Johnson showed that the inhibition of Ca^{++}-dependent transglutaminase with monodansylcadaverine retarded the polyamine-dependent recovery of damaged gastric and duodenal mucosa [11]. This observation suggests that transglutaminase-catalyzed protein cross-linking may be involved in the healing of gastrointestinal erosion although no products of isopeptide bond formation between polyamines and gastrointestinal mucosal proteins have been demonstrated.

In conclusion, this study demonstrates the existence of a Ca^{++}-independent enzyme capable of incorporating [^{3}H]-putrescine into TCA precipitable proteins in the rat intestinal mucosa. The Ca^{++}-dependent and Ca^{++}-independent enzymes differed from each other in their molecular weights and their requirement for Ca^{++} as cofactor. However, the precise physiological role and the importance of this Ca^{++}-independent enzyme in the intestinal mucosa remain to be explored.

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