um status produced a marked change of appetite, as expected, the IVT infusions of blocking agents of the renin-angiotensin system at doses that eliminate effects of IVT renin and NGF had no significant quantitative effect on salt appetite in response to sodium deficiency. In addition, AII added to CSF of sodium-deficient sheep did not cause further increase in sodium intake, despite the fact that more severe sodium depletion did. By contrast, lowering the concentration of sodium in CSF with IVT mannitol consistently and rapidly doubles sodium intake (*15*).

While not being completely conclusive, these data suggest that the sodium appetite caused by IVT renin or AII may not be a physiological regulatory action. It could result from direct pharmacological actions of AII or be secondary to effects AII may have on systemic sodium balance and ionic concentrations.

Thus, the data with IVT renin and AII may reflect the effect of AII in vitro in altering a variety of ionic fluxes between cells and tissue fluid (*16*), including sodium fluxes between extracellular and intracellular fluid, as described for jejunum and skin (*17*). In supraphysiological concentration, the induction of such sodium movements in the neuronal systems subserving sodium appetite may mimic events occurring physiologically with sodium deficiency. Such ionic movements within the physiological range may also be contrived by IVT infusion of mannitol (*15*), a proven stimulus for sodium appetite in sheep.

We have measured the sodium concentration of CSF and plasma in sodium-replete sheep that were treated with IVT AII (3.8 μg/hour) and had access to water and 0.6*M* $NaHCO_3$. The CSF and plasma sodium concentration fell 11 ± 1 and 13.5 ± 2.6 m*M* ($N = 4$), respectively, over 24 hours. This occurred also in control experiments in which AII-treated animals were subjected to water restriction (0.6 liter in the 24 hours) and were not allowed access to 0.6*M* $NaHCO_3$. Therefore, the fall of the sodium concentration is not due solely to increased water intake (Fig. 2). Because IVT AII causes a rapid onset of natriuresis in which the sodium concentration of the urine is usually greater than that of plasma, this phenomenon will contribute to lowering the sodium concentration of CSF and plasma caused by IVT AII (Fig. 2). The increased water drinking and vasopressin release attributable to AII will augment this effect. In the light of our recent data in sheep showing the powerful sodium appetite–inducing effect of lowering the sodium concentration of CSF (*15*), this may be an important consideration in sodium appetite induced by IVT renin or AII. Caution is urged before assigning any primary role for cerebral AII in the physiological induction of sodium appetite in sodium deficiency. Evidence over and above results derived from introduction of pharmacological amounts of renin and angiotensin into the CSF or brain substance will be required to give a credible basis to such a hypothesis.

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12. The lowest rate of infusion of AII into the third ventricle that increased sodium intake was 16 fmole/min (16 pg/min). The volume of the third ventricle of the rat is 1 to 2 μl based on a stereotaxic atlas of rat brain [J. F. R. König and R. A. Klippel, *The Rat Brain* (Williams & Wilkins, Baltimore, 1963)]. After 2 minutes of infusion the concentration of AII may be about 16,000 pg/ml. Concentrations of AII up to 359 pg/ml have been measured in rat CSF [J. B. Simpson, W. A. Saad, A. N. Epstein, in *Regulation of Blood Pressure by the Central Nervous System*, G. Onesti *et al.*, Eds. (Grune & Stratton, New York, 1976), pp. 191–202].
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Vitamin D–Dependent Calcium Binding Protein: Immunocytochemical Localization in Chick Kidney

Abstract. *A vitamin D–dependent calcium binding protein in the chick kidney that was detected by immunocytochemical techniques was localized exclusively in the distal convoluted tubule, the initial collecting tubule, and the early part of the collecting tubule. The intercalated (mitochondria-rich) cells in these tubular segments were negative for the calcium binding protein. Subcellularly, the protein was found in the cytosol and the nucleus of the tubular cells. The results suggest a role for vitamin D–dependent calcium binding protein in intracellular calcium metabolism rather than a direct involvement in membrane-mediated calcium reabsorption in the avian kidney.*

The maintenance of the calcium and phosphorus homeostasis is essential for the normal functioning of cells and tissues (*1*) and principally involves the integrated actions of the intestine, bone, and kidney. This is accomplished by an endocrine system in which the peptide hormones calcitonin and parathyroid hormone (PTH) interact with vitamin D and its two chief biologically active metabolites 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] and 24,25-dihydroxyvitamin D_3 [$24,25(OH)_2D_3$] (*2, 3*). Both of these dihydroxylated metabolites are produced in the proximal tubule of the avian (*4*) and mammalian kidney (*5*). $1,25(OH)_2D_3$

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is a steroid hormone that interacts with a specific receptor protein in a large number of vitamin D–related target tissues (*2*) including the intestine and kidney (*6*) to stimulate the synthesis of several macromolecules responsible for the characteristic vitamin D–related biological response of that tissue. One such 1,25-$(OH)_2D_3$–induced macromolecule is a calcium binding protein (CaBP) (*7, 8*) that is present in a variety of tissues including the kidney (*2, 9*). In view of the prominent role of the kidney tubule in calcium reabsorption as well as vitamin D metabolism, an intriguing problem has been to identify the precise location of the CaBP in the kidney. In this report we describe both the cellular as well as subcellular distribution of the CaBP in the chick kidney.

Table 1. Intensity of the staining for CaBP in the chick kidney. The kidneys of three animals were used for the evaluation. A total of 60 micrographs (20 for each kidney and each tubular region) were taken on 70-mm film at the primary magnification of 11,000 calibrated with a carbon grating replica (2160 lines per millimeter). The surface of the cytosol and the euchromatin, as well as the number of gold particles present over these compartments, were recorded on a graphic tablet (Tektronix, type 4973) connected to a microprocessor system (IMSAI, type 8080) programmed to calculate the number of particles per unit area (square micrometers) of the compartment (*22*). The values, which are expressed as gold particles per square micrometer (± standard error), were compared statistically by Student's *t*-test.

	Distal convoluted tubule		Collecting tubule	
	Cytosol	Nucleus	Cytosol	Nucleus
Antiserum to CaBP*	50.19 ± 1.80†	43.69 ± 3.86	30.55 ± 2.86	36.87 ± 2.39
Antigen-adsorbed antiserum to CaBP‡	4.71 ± 0.89	5.06 ± 0.39	5.13 ± 0.75	5.43 ± 0.15
Normal serum‡	3.36 ± 1.01	3.09 ± 1.30	4.41 ± 1.07	4.35 ± 1.13
Antiserum to CaBP, protein A, pAg‡	1.99 ± 0.26	1.88 ± 0.30	2.89 ± 1.17	3.25 ± 1.75

*Comparison with all control values, $P < .001$. †Comparison with cytosol of collecting tubule, $P < .005$. ‡Control incubations.

Normally fed, 1-week-old male White Leghorn chicks and 2- to 4-week-old chicks which had free access to a standard rachitogenic diet (*8*) were used as a source of kidney tissue. The rachitic animals were housed in electrically heated brooders in an air-conditioned room from which sunlight was excluded. The CaPB was detected immunocytochemically as follows: (i) sections (5 μm thick) of paraffin-embedded kidneys or semithin sections of Epon-embedded tissue were stained by an indirect immunocytochemical technique with antiserums to chick intestinal CaBP and peroxidase-labeled protein A (*10*); (ii) thin sections from Epon-embedded kidney were stained for CaBP by the protein A–gold (pAg) technique (*11*) which allows the precise intracellular identification of a variety of antigens at the ultrastructural level (*11, 12*).

Under the light microscope, specific immunoreactive sites were localized in the cytoplasm and the nuclei of the epithelial cells of the convoluted part of the distal tubule, the early part of the collecting tubule, as well as the initial collecting

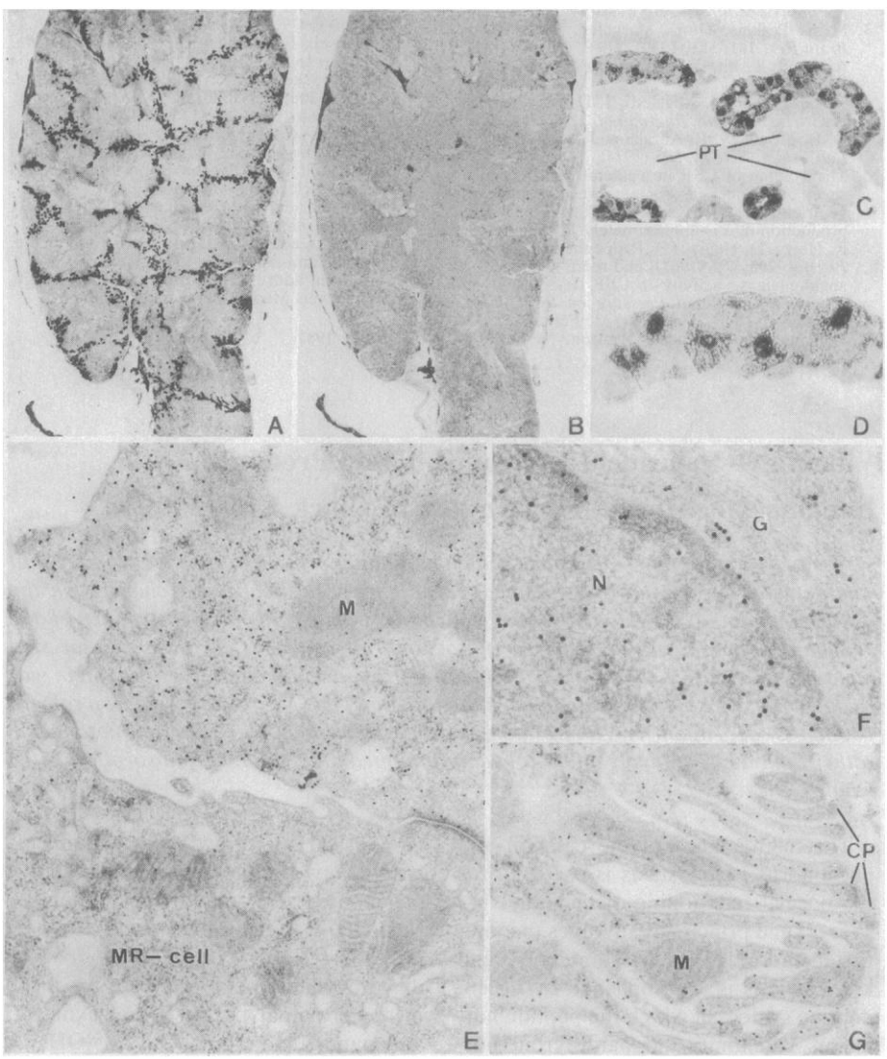

Fig. 1. (A) Immunoperoxidase staining for CaBP on a paraffin section showing positive reaction (black) at the periphery of renal lobules (×13). See (C) and (D) for higher magnification. (B) Paraffin section cut consecutively after (A) but incubated with antigen-adsorbed antiserum. No reaction is detectable. The remaining black areas represent, unspecifically, peroxidase-stained connective tissue [these are also visible in (A)] (×13). (C and D) Semithin sections from Epon-embedded material. (C) Positive tubular profiles are visible among negative tubules. Negative structures in this field are proximal tubules (*PT*) (×400). (D) In positive tubules, stained cells (cytoplasm and nucleus) alternate with unstained cells (×1000). (E to G) Thin sections stained for CaBP with the pAg technique. (E) Apical regions of two adjacent cells of a distal tubule. One of the cells shows a cytosol heavily labeled by gold particles revealing CaBP antigenic sites while the other is almost free of labeling. The latter corresponds to an intercalated (mitochondria-rich) cell (*MR-cell*); *M*, mitochondrion (×30,000). (F) Small field of a distal tubular cell. CaBP immunoreactive sites are present over the cytosol and the nucleoplasm but are absent from the Golgi cisternae (*G*); *N*, nucleus (×57,000). (G) Basal region of a distal tubular cell. The thin cytoplasmic processes (*CP*) delimiting the deep basal invaginations are distinctly labeled by gold particles (×40,000).

tubule joining these two tubular regions (Fig. 1, A, C, and D). Cells similar to the intercalated (mitochondria-rich) cells of these segments of the mammalian urinary tubule did not show immunoreactivity nor did any other part of the nephron, collecting tubule, or interstitial elements. In the CaBP-positive segments, the distal tubules stained more intensely than collecting tubules. These results confirm and extend previous observations (*13*). Specific staining was absent under all control conditions (see Fig. 1B). In the kidneys of rachitic animals, specific but less intense staining for CaBP was observed in the same segments of the nephron as in nonrachitic chicks (data not shown).

Ultrastructurally, the different parts of the urinary tubule in the chick kidney were similar to corresponding regions of the mammalian kidney (*14*); only Henle's thin loop was absent (*15*). In cells showing a positive pAg reaction, the gold particles indicative of CaBP were present in the cytosol and nucleus (Fig. 1, E, F, and G). In the nucleus, euchromatin was intensely labeled. Labeling was absent from the cisternae of the rough endoplasmic reticulum and the Golgi apparatus, from mitochondria, lysosomes, and membrane-bound vesicles in the apical cytoplasm. Membrane labeling was similarly not observed.

Extracellular spaces were not labeled above background nor were the intercalated cells of the positive tubular segments. There was no significant difference in the labeling of the cytosolic and nuclear compartments from positive cells in the distal and collecting tubules (Table 1). However, the amount of labeling in the cytosol of the distal tubular cells was significantly higher than in the collecting tubule. A low degree of cellular background labeling was observed. These data clearly indicate that in the avian kidney the vitamin D-dependent CaBP is present only in the distal convoluted tubule, in the initial collecting tubule, and early part of the collecting tubule (Fig. 2).

The differentiation of the urinary tubule with respect to its involvement in the vitamin D endocrine system is impressive. Although both of the enzymes responsible for the production of $1,25(OH)_2D_3$ (*4*, *5*) and $24,25(OH)_2D_3$ (*5*) are found exclusively in the proximal tubule, the distal and collecting tubules are the only sites of localization of tritiated $1,25(OH)_2D_3$ as determined by autoradiography (*16*, *17*) and of CaBP as described here. These data suggest that although $1,25(OH)_2D_3$ is produced in the proximal tubule, the effects of $1,25(OH)_2D_3$ that require steroid receptor–mediated transcriptional processes occur in the distal and collecting tubule. The precise role of CaBP in the renal calcium uptake mechanism is not clear. The bulk of renal calcium reabsorption (approximately 80 percent) occurs in the proximal tubule (*18*), the region that did not show immunoreactive sites for CaBP. However, a selective calcium reabsorption for regulation of calcium excretion takes place in the distal and collecting tubules (*18*), segments in which CaBP was detected immunocytochemically. In addition, immunoreactivity was found at the subcellular level only in the cytosol and the nucleus suggesting an involvement of the vitamin D–dependent kidney CaBP in processes related to regulation of the intracellular translocation of calcium ions rather than to regulation of calcium reabsorption. Although few attempts have been made to locate CaBP in the kidney, the question of whether intestinal CaBP is extracellular, intracellular and soluble, or intracellular and membrane bound has been controversial (*13*, *19*). Since the biochemical properties of chick intestinal CaBP and renal CaBP are identical (*2*), it is not surprising that application of the new pAg technique (*11*) to the intestinal CaBP has shown that it, too, is located in the cytosol of intestinal epithelial cells (*20*, *21*). This is consistent with the proposal that the widely distributed vitamin D–dependent CaBP's (*8*) participate in processes related either to regulation of the intracellular calcium concentration or to the intracellular translocation of this ion.

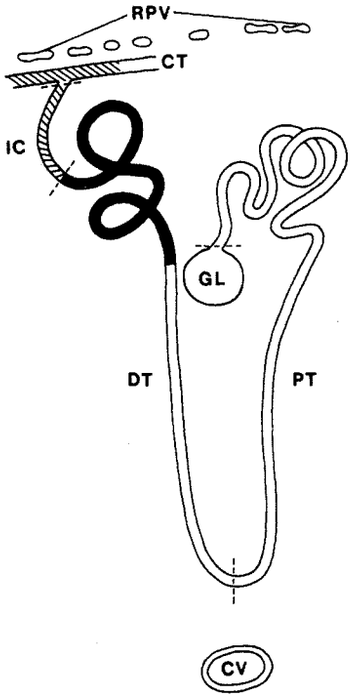

Fig. 2. Schematic representation of the chick urinary tubule [modified from (*15*)]. Abbreviations: *Gl*, glomerulus; *PT*, proximal tubule; *DT*, distal tubule; *IC*, initial collecting tubule (the initial collecting tubule of the avian kidney corresponds to the connecting segment of the mammalian kidney); *CT*, collecting tubule; *CV*, central vein; and *RPV*, branches of the portal vein between two renal lobules. Dashed segments of the tubule indicate an intermediate intensity of immunostaining; black segments, a high intensity. White segments are unreactive.

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10. The kidneys of 1-week-old chicks were fixed by perfusion of the whole animal through the left ventricle with a solution of phosphate-buffered isotonic saline (PBS) (*p*H 7.4) for 3 minutes, followed by Bouin's fixative for 15 minutes. The kidneys were then removed from the animals, fixed for a further 12 hours at room temperature, and embedded in paraffin. Deparaffinized and rehydrated sections (5 μm thick) or semithin sections from Epon-embedded tissue were incubated with a solution of 1 percent ovalbumin in PBS for 5 minutes and then reacted with the antiserum to chicken intestinal CaBP (diluted 1/100 in 1 percent ovalbumin in PBS) for 2 hours at room temperature. The sections were rinsed twice for 5 minutes with PBS and incubated with peroxidase-labeled protein A (a gift from E. Berger, University of Bern) for 1 hour at room temperature. The sections were then rinsed twice with PBS for 5 minutes and the peroxidase activity was revealed by 3-amino-9-ethylcarbazole (0.02 percent) in 0.05*M* acetate buffer (*p*H 5) first for 5 minutes without, then 5 minutes

with 0.01 percent H_2O_2. The sections were then rinsed and stained with 0.01 percent Evan's blue for 2 minutes, mounted in a mixture of glycerol and PBS (1:1 by volume) and examined. For cytochemical controls see (*11*).

11. Perfusion fixation was done as described in (*10*) but with 1 percent glutaraldehyde in PBS being used as the fixative for 2 hours at room temperature. Free aldehyde groups were blocked by subsequent incubation of small tissue fragments in 0.5*M* NH_4Cl in PBS for 2 hours. The fragments were rinsed in PBS, dehydrated in ethanol, and embedded in Epon 812. Thin sections (400 to 600 μm) were placed on parlodion-carbon coated nickel grids (200 mesh) and processed by the pAg technique as follows. The grids were placed, sections downward, on a droplet of 1 percent ovalbumin in PBS for 5 minutes and then transferred to a droplet of the antiserum against chicken CaBP (diluted 1/100) and incubated for 16 hours at 4°C. The grids were rinsed twice with PBS and reacted with the pAg solution for 1 hour at room temperature. After two further rinses in PBS and a final rinse in distilled water, the thin sections were counterstained with uranyl acetate and lead citrate. Controls for cytochemical specificity included (i) the replacement of the diluted antiserum by normal rabbit serum or by antiserum previously adsorbed with purified chicken duodenal CaBP (400 μg of CaBP per milliliter of undiluted antiserum), (ii) incubation of the sections with nonlabeled protein A (0.1 mg/ml) between the antiserum and pAg incubation step, and (iii) staining for endogenous peroxidase activity. The thin sections were examined with a Philips EM 300. For further details see J. Roth, M. Bendayan, L. Orci, *J. Histochem. Cytochem.* **26**, 1074 (1978); *ibid.* **28**, 55 (1980).
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21. Since submission of this manuscript, A. N. Taylor [*J. Histochem. Cytochem.* **29**, 65 (1981)], detecting immunofluorescence under the light microscope, reported that "the true *in situ* localization of CaBP in chick duodenum was that which was present in absorptive cell cytoplasm."
22. D. Bertrand and M. Amherdt, in preparation.
23. We thank M. Amherdt for help with the quantitative analysis, D. Brown for helpful discussions, and F. Fichard, M. Sidler-Ansermet, and I. Bernard for technical assistance. This work was supported in Geneva by Swiss National Science Foundation grant 3.668.80, and in Riverside by PHS grants AM-09012 and AM-14,750. Reprint requests should be addressed to J.R.

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Sulfhydryl Compounds May Mediate Gastric Cytoprotection

Abstract. *Ethanol induces hemorrhagic gastric erosions and causes a dose-dependent decrease in the concentration of nonprotein sulfhydryl compounds in rat gastric mucosa. Sulfhydryl-containing drugs protect rats from ethanol-induced gastric erosions, whereas sulfhydryl blocking agents counteract the mucosal cytoprotective effect of prostaglandin $F_{2\beta}$. These observations suggest that endogenous nonprotein sulfhydryls may mediate prostaglandin-induced gastric cytoprotection and that sulfhydryl drugs may have potential for preventing or treating hemorrhagic gastric erosions.*

Severe stress or ingestion of ethanol, aspirin, and other nonsteroidal anti-inflammatory agents are predisposing causes of acute hemorrhagic gastric erosions in humans (*1*). Whereas chronic ulcers with inflammation usually occur in the antral or duodenal mucosa and penetrate the mucosal muscle to involve deeper layers, hemorrhagic gastric erosions primarily involve the acid-producing portion of the stomach, are limited to the mucosa, and cause little or no surrounding inflammation (*2*). Morphologically identical superficial erosions can be consistently induced in the acid-secreting glandular stomach of laboratory rodents by intragastric administration of ethanol and aspirin (*3*).

Erosions of the rat gastric mucosa induced by noxious substances (such as ethanol and aspirin) are fewer and less severe if prostaglandins are first administered. This effect, termed cytoprotection (*4*), is shared by virtually all prostaglandins (types A, B, C, D, E, F, and I); inhibition of acid secretion is not involved. The dose of prostaglandins required for cytoprotection is much smaller than that required for inhibition of acid secretion. Furthermore, some prostaglandins that do not inhibit acid secretion (such as prostaglandin $F_{2\beta}$) induce cytoprotection (*4*).

The mechanism of cytoprotection is unknown (*4, 5*). Gastric mucus glycoproteins may play a role (*4*). Tissue injury produced by noxious agents may result in the accumulation of toxic free radicals in mucosal cells. The gastric mucosa contains unusually high concentrations of reduced glutathione (*6*), the major component of the endogenous nonprotein sulfhydryl pool. Thiols such as reduced glutathione are able to bind reactive free radicals (*7*) and may influence the physical properties of mucus, since its subunits are joined by disulfide bridges (*8*). Boyd *et al.* (*6*) reported that diethylmaleate, an agent that markedly depletes gastric glutathione, causes severe gastric ulceration, suggesting a possible modulatory role for glutathione in ulcerogenesis. We performed a series of experiments to determine the effects of ethanol, thiols, and sulfhydryl blocking agents on mucosal nonprotein sulfhydryls and on gastric cytoprotection. We found that ethanol lowers the concentration of nonprotein sulfhydryls in gastric mucosa, that thiols induce gastric cytoprotection while increasing nonprotein sulfhydryl levels, and that sulfhydryl blocking agents prevent the cytoprotective effect of prostaglandin $F_{2\beta}$ and decrease nonprotein sulfhydryl levels.

In the first experiment, female Sprague-Dawley rats (150 to 200 g) on a diet of Purina Lab Chow and tap water were fasted overnight, given 1 ml of water or various concentrations of ethanol by gavage (Fig. 1), and killed 5 minutes later (*9*). (This interval was selected since major mucosal lesions do not appear during the first 5 minutes after exposure to ethanol.) Mucosa from glandular stomach and part of the left lobe of the liver were rapidly removed, weighed, and frozen for subsequent homogenization and measurement of protein and nonprotein sulfhydryl concentrations (*10*).

The concentration of nonprotein sulfhydryls in the gastric mucosa decreased significantly after only 5 minutes of exposure to 50 or 70 percent ethanol (Fig. 1). Exposure to 100 percent ethanol caused even more severe depletion. In contrast, liver nonprotein sulfhydryls (Fig. 1) and protein sulfhydryls in liver and gastric mucosa were unchanged 5 minutes after intragastric administration of ethanol at each of the test concentrations.

Gastric washings collected before the administration of ethanol contained only trace amounts of nonprotein sulfhydryls; 5 minutes after 100 percent ethanol was administered, the amount of nonprotein sulfhydryls removed from the gastric lumen more than doubled (235 percent). This may represent leakage of nonprotein sulfhydryls from remaining mucosal cells or nonprotein sulfhydryls present in mucosal cells that were exfoliated into the lumen as a result of ethanol administration.

In the second study, rats received prostaglandin $F_{2\beta}$ (0.05 mg per 100 g, by gavage) or one of several sulfhydryl-containing drugs including dimercaprol

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