Effect of Thiocyanate and Isothiocyanate Esters on H^+ Secretion and Microsomal Adenosine triphosphatase of Bullfrog Gastric Mucosa* (33919)

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Thiocyanate has long been known as a potent inhibitor of gastric acid secretion. From studies with SCN^- and other ionic compounds such as OCN^- and NO_2^-, LeFevre *et al.* (1) proposed that a common structural feature of these compounds, *i.e.*, a pair of unshared electrons on the nitrogen atom, represents the basis for their inhibitory action. However, Durbin (2) demonstrated a competition between Cl^- and SCN^-, *vis à vis* H^+ secretion in the isolated frog gastric mucosa, implicating anionic competition as the basis for inhibition of H^+ secretion. These studies were further extended by Forte *et al.* (3), who showed a competition between Cl^- and SCN^- binding by isolated gastric microsomes. Kasbekar and Durbin (4) demonstrated that SCN^- inhibits the activity of frog gastric microscomal ATPase, and suggested that this inhibition may represent the site of action of SCN^- in the sequence of reactions leading to H^+ secretion.

This communication reports the results of studies carried out with "nonpolar" analogues and isomers of SCN^- to test whether the anionic charge *per se* and/or the pair of unshared electrons on the nitrogen atom is a prerequisite for the inhibitory action. Some interesting observations relative to the effects of these nonpolar derivatives on the transport properties of intact mucosae and on the gastric microsomal ATPase are discussed.

Methods. Gastric mucosae from bullfrogs (*R. catesbeiana*) were isolated and mounted between Lucite chambers as described previously (5). The tissue was bathed on the serosal side with Ringer's solution containing (m*M* final concentration) NaCl, 85.3; KCl, 3.4; $CaCl_2$, 1.8; KH_2PO_4, 0.9; $NaHCO_3$, 17.6; histamine, 0.1; and dextrose, 11. The mucosal side was bathed with an unbuffered secretory solution comprised of 95.2 m*M* NaCl, 20.9 m*M* sodium isethionate ($HO-CH_2CH_2SO_3^-$) and 2.5 *M* K_2SO_4. Both solutions were aerated and stirred continuously with 95%O_2–5%CO_2 The various compounds whose effects were being studied were added to the nutrient bathing medium.

The rate of H^+ secretion was determined by using the pH stat method and was computed from the amount of 20 m*M* NaOH required to maintain the secretory solution at pH 5.0, as a function of time. The transmucosal electrical potential difference (PD) was monitored through saturated agar–KCl bridges placed in close juxtaposition to the gastric mucosa; the bridges were in electrical contact via saturated KCl with calomel electrodes, which were in turn connected to a Sargent MR recorder to obtain a continuous trace of PD. Separate Ag–AgCl electrodes imbedded in agar–saline were threaded into the distal end of each chamber. These were used to pass current through the mucosa employing the voltage clamp device reported previously (6). The current required to reduce the mucosal PD to zero was referred to as the short-circuit current and was recorded by a Honeywell-Electronic 19 recorder.

For studies of microsomal ATPase, the mucosa was chilled at 0° in a solution containing 175 m*M* mannitol, 60 m*M* sucrose and 0.15 m*M* EDTA, adjusted to pH 7.4 with 150 m*M* Tris. After briefly blotting the tissue on filter paper, the cells were scraped from the connective tissue layer, placed in 2 ml of the above solution and homogenized with a Teflon homogenizer. The homogenate

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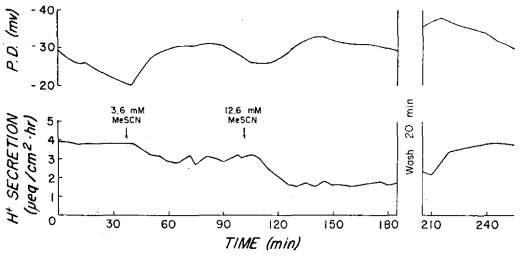

FIG. 1. Effect of methyl thiocyanate (MeSCN) on transmucosal PD. and H+ secretion by isolated bullfrog gastric mucosa. During the 20-min wash period the bathing solutions were replaced three times. All values of PD are given for the secretory side with respect to the nutrient side.

was centrifuged at 5000*g* for 5 min to remove cellular debris and nuclei. The pellet was washed once, by resuspending, rehomogenizing, and centrifuging as above. The supernatant fraction from both procedures were combined and centrifuged at 15,000*g* to remove mitochondria. The 15,000*g* supernatant fraction was recentrifuged at 40,000*g* for 1 hr. The resulting sediment, operationally referred to as the microsomal pellet, was washed once by resuspension in homogenizing medium and centrifugation as above and used for ATPase assay. The activity of the enzyme was assayed at 37° by incubating 0.05–0.1 ml of microsomal suspension with 5 m*M* ATP, 5 m*M* Mg^{2+} and 15 m*M* Tris (pH 8.4), all in a final volume of 2.0 ml. For studying the effect of inhibitors, the enzyme was preincubated with all ingredients except ATP; the time of preincubation with SCN^- was 15 min, and with other inhibitors, 1 hr. Appropriate controls were included in each case. The incubation period, following addition of ATP, was 10 min in all cases. The reaction was stopped with 1.0 ml of 15% TCA, the mixture was chilled in ice, and the denatured protein was removed by centrifuging at 11,000*g* for 15 min. The inorganic phosphate released was determined by the method of Dreisbach (7). Protein was determined by the method of Lowry *et al.* (8) and bovine serum albumin was used for a standard.

Assays of thiocyanate anion were carried out according to the spectrophotometric procedure described by Senise and Perrier (9) using sodium thiocyanate as standard.

Results. The effects of methyl thiocyanate (MeSCN) on the H^+ secretory rate and the PD for a typical experiment are shown in Fig. 1. The inhibition of H^+ secretion appears to be concentration dependent and is accompanied by a concomitant increase in PD. These effects, including the reversibility following removal of the inhibitor from the bathing medium, are qualitatively similar to those produced by SCN^- and described by others (10, 11). As one possibility, biological demethylation of MeCSN to SCN^- could be responsible for this similarity. However, following prolonged incubation of the mucosa with MeSCN, no SCN^- could be detected either in the bathing media or in the tissue. The observed effects of MeSCN, therefore, appear to be due to the intact molecule and not due to its degradation of SCN^-.

Methyl isothiocyanate (MeisoSCN), an isometer of MeSCN, showed certain differences in its effects on both H^+ secretion and PD. The results of a typical study are shown in Fig. 2. As with SCN^- and MeSCN, MeisoSCN inhibited H^+ secretion; however such inhibition could not be reversed following several washes of the tissue with fresh bathing medium. This effect of MeisoSCN stands in sharp contrast to that observed with either MeSCN or SCN^-.

The mucosal PD showed a consistent transient decline immediately following the addi-

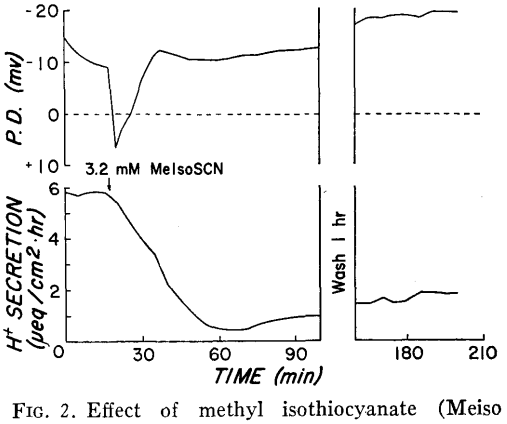

FIG. 2. Effect of methyl isothiocyanate (Meiso SCN) on PD and H^+ secretion by isolated bullfrog gastric mucosa. The bathing solutions were replaced three times during the 1-hr wash period.

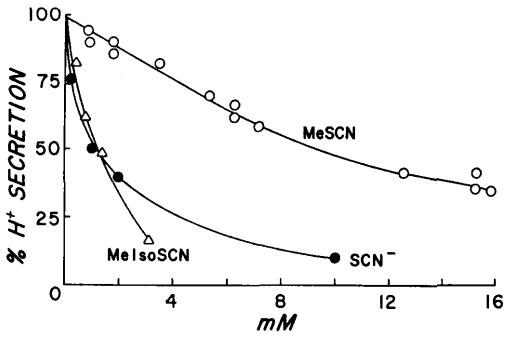

FIG. 3. Comparative effect of SCN^-, MeSCN, and MeisoSCN on H^+ secretion by bullfrog gastric mucosa. The indicated points were calculated as the % H^+ secretion occurring when compared to the preinhibitor rates.

tion of MeisoSCN, an effect not observed with SCN^- or MeSCN. The magnitude of the drop in PD appeared to be concentration dependent and at concentrations of Meiso SCN greater than about 2 m*M*, a transient reversal in sign was observed. In all instances, however, there was a gradual recovery of the polarity to preinhibitory values.

A comparative effect of SCN^-, MeSCN, and MeisoSCN on the inhibition of H^+ secretion is shown in Fig. 3. The MeisoSCN appears to be about as effective as SCN^+, while MeSCN was considerably less potent as an inhibitor of H^+ secretion.

The methyl esters of thio- and isothiocyanic acid show reasonable solubility in aqueous media. In view of the solubility problems with esters of higher alcohols and aromatic derivatives, we limited our studies to only two additional compounds, phenylisothiocyanate, and butyl thiocyanate. Both of these compounds inhibited H^+ secretion irreversibly, and also brought about the transient decrease in PD as noted in the case of MeisoSCN.

For all of the above compounds the effects on the short-circuit current were quite similar to what has been reported for SCN^-. That is, with concentrations up to 5 m*M* the steady state short-circuit current was either not affected or elevated above control values. Under conditions where mucosae are normally secreting H^+ the short-circuit current was equivalent to a current of actively transported Cl^- (12). Providing that the inhibition of H^+ secretion does not alter this relationship, it appears that the gastric Cl^- pump is not appreciably affected by the organic esters of thiocyanate, or their isomers.

The effect of SCN^-, MeSCN, and MeisoSCN on gastric microsomal ATPase activity is shown in Fig. 4. These studies indicate that MeSCN is indeed a very poor inhibitor of the ATPase; even at very high concentrations, inhibition of enzyme activity was not greater than 10–15%. Although SCN^- and MeisoSCN are relatively more effective, the concentrations required for an equivalent percentage reduction of H^+ secretion were approximately an order of magnitude less than those required for inhibition of ATPase activity.

Discussion. The SCN^- is a relatively potent, specific, and reversible inhibitor of gastric H^+ secretion and although this has been known for some time, knowledge regarding its mechanism of inhibition has remained speculative. An understanding of its mode of inhibition is of importance in the study of the mechanism of gastric HCl secretion. In model reaction systems involving a "carrier" for Cl^- and H^+, SCN^- has been suggested (2) to compete with Cl^- for free carrier; the carrier SCN^-–complex thus formed was assumed to be incapable of participating in reactions leading to H^+ secretion. The present studies show that the nonpolar anal-

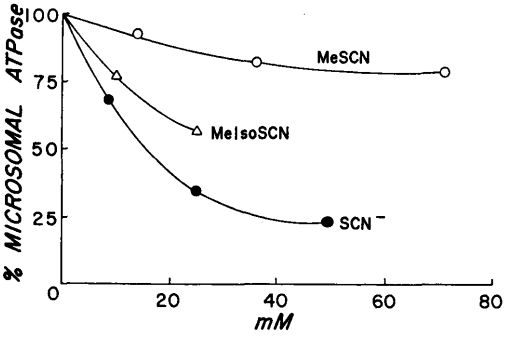

FIG. 4. Comparative effect of SCN^-, MeSCN, and MeisoSCN on ATPase activity of microsomes isolated from bullfrog gastric mucosa. The points were calculated as the % ATPase when compared to rates observed without inhibitor.

ogue of thiocyanate, MeSCN, inhibits acid secretion and that this inhibition is not the result of its conversion to the thiocyanate anion. An important underlying assumption, namely, the anionic basis of competition between Cl^- and SCN^- for the "carrier" in gastric H^+ secretion thus remains unfulfilled, and it appeared appropriate to seek other explanations for the observed effect of MeSCN.

The inhibition of gastric microsomal ATPase by SCN^- has been used as one of the bases for the postulation that this enzyme is involved in H^+ secretion (4). This interpretation also requires to be reexamined in the light of the present observations with MeSCN, since this compound inhibits H^+ secretion while having only a small inhibitory effect on the activity of the microsomal ATPase. The rationale for the participation of ATPase in the sequence of reactions leading to H^+ secretion becomes equivocal, unless further assumptions are made. One such assumption would be a nonlinear transduction between the site utilizing ATP and ion translocation in the gastric mucosa. Under such circumstances only a small decline in the available free energy, resulting from a 10–15% inhibition of ATPase activity could prevent the accumulation of large H^+ gradients at a specific membrane locus, e.g., the tubular members of smooth-surfaced endoplasmic reticulum of the acid secreting cells. A theoretical basis for such a system of nonlinear energy transduction exists (13); however, experimental verification of its operation in any membrane transport process is not available at present.

A second assumption would be that the decrease in H^+ secretion induced by SCN^- and its nonpolar derivatives may primarily involve a delimiting or uncoupling of the close association between ATPase activity and the separation of H^+ and OH^-. The reported inhibition of the enzyme by SCN^- (4) may be merely incidental, and if a dissociation between ATPase and ion translocation process is the primary event, then a lack of inhibition of the gastric microsomal ATPase by MeSCN would not necessarily contradict the suggested participation of this enzyme in H^+ secretion.

Additional explanations are possible, but as in the above cases they require assumptions which are highly speculative and they can at best represent hypotheses for further study. However, the results do offer clear support for the hypothesis presented by LeFevre *et al.* (1) regarding the structural basis for the inhibitory action of SCN^- on H^+ secretion. All inhibitory compounds tested share the common feature of the unshared pair of electrons on the N atom, although the precise site and mode for inhibition is not understood. The negative charge of SCN^- does not appear to be an essential prerequisite for its inhibitiory action. An explanation has previously been offered (6) for the apparent discrepancy between observed competitive kinetics between Cl^- and SCN^- (2), on the basis of the charge, and the "noncompetitive" type inhibition of SCN^- (and related analogues) in enzymatic reactions leading to H^+ secretion.

The isothiocyanates, which are isomeric with thiocyanates and which also possess a nitrogen atom with an unshared pair of electrons, inhibit acid secretion; however, their inhibitory action appears to be irreversible, unlike that of thiocyantes and other compounds studied by the Birmingham group (1). Whether this irreversibility is a manifestation of the low partition coefficients of these compounds in aqueous solution, as compared to that in the membrane lipids, is not clear. In the heterogeneous cell system these nonpolar compounds may preferentially be absorbed into the lipoidal membranes, and they may resist leaching out, even after several washes. The effect of most of the nonpolar compounds studies on PD is unusual and noteworthy. The immediate depression of PD following the addition of these compounds suggests an interaction of unknown nature with the mebranes. The immediate perturbations produced, however, appear to be transient in nature, as is evidenced from a gradual restoration of PD.

Finally, it is of interest that several isothiocyanates recently were shown to inhibit the

uptake of I^- and iodination reactions in the thyroid gland (14). As with gastric mucosa the effect of isothiocyanates in reducing thyroid function is essentially irreversible. There is considerable information available concerning the various compounds (mostly anions) which alter the normal transport and metabolism of thyroidal I^- (15), and a comparative study shows that many of the same agents are effective in depressing HCl secretion (1, 16). Thus it appears that for both systems inhibition is not entirely dependent upon the anionic charge of the inhibitor. However, since active anion treatment plays a primary role in thyroid function as well as in gastric HCl secretion, some common basis is available on which to propose hypotheses for the mode of inhibition.

Summary. The effects of nonpolar analogues of thio- and isothiocyanate on H^+ secretion and microsomal ATPase of bullfrog gastric mucosa were studied and compared with those of thiocyanate anion. Methyl thiocyanate reduced H^+ secretion without any appreciable inhibitory effect on ATPase. There was no conversion of MeSCN to SCN^- in gastric mucosa. The nonpolar derivatives of isothiocyanates inhibit H^+ secretion irreversibly and are also potent inhibitors of the ATPase activity. These findings are consistent with the hypothesis that certain compounds containing a nitrogen atom with an unshared pair of electrons are inhibitors of H^+ secretion. An anionic charge however is not a prerequisite for their inhibitory action. The effects of these nonpolar compounds on gastric H^+ secretion are discussed in relation to their effects on gastric microsomal ATPase.

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