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Inhibition of Gastrointestinal and Liver Hexosamine Synthesis by Sodium Salicylate and Ethanol[1] (40999)

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Abstract. The influence of oral sodium salicylate (NaSal) or ethanol (ETOH) on glucosamine synthetase (GmS) specific activity in rat fundic and antral mucosae and liver was measured. Sodium salicylate, 32 mg/kg in 0.1 *N* HCl, caused a 46% decrease in GmS specific activity in only 5 min. Recovery to 179% of control was complete by 30 min. Larger doses of NaSal caused more severe damage to the gastric mucosa and more persistent decreases in fundic GmS specific activity and also decreased liver GmS activity. ETOH, 20% (v:v) in 0.1 *N* HCl, 1.6 g/kg, caused a 29% decrease in GmS activity in the fundic mucosa in 15 min. Recovery, to 157% of control was complete in 30 min. We conclude that specific inhibition of hexosamine synthesis, not attributable to necrosis or general metabolic decline occurs during NaSal- and ETOH-induced gastric damage and may be involved jointly with acid not only in the development but also in the delayed healing of severe damage.

Deleterious effects of aspirin, sodium salicylate, and ethanol on gastrointestinal mucosa have been reported in many species including rat, dog, and man (1–5). Hypothesized mechanisms include alterations in potential difference (2), mucus secretion (3), acid back-diffusion (1) and cellular exfoliation (5), as well as vascular disturbances such as increased capillary fragility and platelet dysfunction (6).

Although considerable attention has been given to changes in mucus secretion and hexosamine content of the gastric mucosa accompanying various forms of experimentally induced ulceration, the mechanisms by which these alterations might lead to ulceration remain obscure. Furthermore, the biochemical or enzymatic mechanisms responsible for these alterations have not been fully explained.

The initial and rate-limiting enzyme in the hexosamine synthesis pathway is L-glutamine: D-fructose-6-phosphate aminotransferase (EC 2.6.1.16), commonly referred to as glucosamine synthetase (GmS). The second enzyme in the pathway is glucosamine 6-phosphate: *N*-acetylase (EC 2.3.1.4).

Perrey demonstrated that very large doses of sodium salicylate inhibit glucosamine synthetase *in vivo* in rat gastric mucosa (7). Chan *et al.* (8) used more clinically relevant doses of drug *in vitro* and also observed inhibition of this enzyme.

Kent (9), on the other hand suggested that acetyl salicylates may inhibit the *N*-acetylase reaction indirectly by interferring with the production of acetyl-CoA, one of the substrates necessary for the reaction to proceed.

The purpose of this study was to determine if clinically relevant doses of sodium salicylate or ethanol, administered *in vivo*, would cause gastrointestinal damage or alter glucosamine synthetase or glucosamine-6-PO_4: *N*-acetylase specific activity.

Materials and methods. Chemicals. Adenosine 5′-triphosphate disodium salt, L-glutamine, D-fructose-6-PO_4 sodium salt, and D-glucose-6-PO_4 disodium salt hydrate were obtained from Sigma Chemical Company, St. Louis, Missouri. *N*-Acetyl-D-glucosamine, D-glucosamine HCl, and coenzyme A trilithium salt were purchased from Calbiochem, Los Angeles, California. Sodium salicylate and absolute ethanol were obtained locally. All other chemicals were reagent grade and were obtained commercially.

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Animals and Drugs. Male, Sprague–Dawley rats, 250–300 g, were used for all studies. All animals were housed in our animal facilities under standardized conditions with controlled temperature and lighting for at least 1 week before use. Rats received commercial rat chow *ad libitum.*

Rats were fasted for 18 hr before drug administration. Water was available until drugs were given. All drugs were administered between 8 and 10 AM. Animals were killed by a blow on the head at selected times after drug administration.

Two concentrations of sodium salicylate were studied: 20 and 156 m*M;* administered at the rate of 10 ml/kg yielding dosages of 32 and 250 mg/kg, respectively. At the lower concentration the sodium salicylate was in 0.1 *N* HCl and was completely soluble. The higher dose was administered in either citrate buffer, pH 2.5, or in phosphate buffer, pH 7.5. Control animals received the appropriate solvent solutions. Since salicylic acid precipitates from a concentrated drug solution at low pH's it is possible that drug particles could cause mechanical damage to the gastric mucosa. To simulate this condition Celite, an inert, nonsoluble diatomaceous filter aid, was added to the acidic control solutions at the same milligram per milliliter of concentrations as the sodium salicylate.

Ethanol was administered as a 20% (v:v) solution in either water, phosphate buffer, pH 7.4, or in 0.1 *N* HCl, at a dose of 10 ml/mg (1.6 g/kg). Control rats received the solvent only.

Enzymatic analysis. After killing the animals a ventral midline incision was made, the vena cava was cut, the portal vein was cannulated, and the liver was perfused with ice-cold 154 m*M* KCl. Liver and GI tissues were removed. The gastric fundus and antrum were separated and the mucosal tissues were scraped free of underlying muscle tissue. Liver was homogenized using a Potter–Elvehjem glass-to-glass conical homogenizer. Several methods of preparing the GI tissues were compared. These included simple mincing with scissors and complete homogenization with either a Polytron or a Potter–Elvehjem glass-to-glass conical homogenizer. It was determined that the enzyme in the GI tissues was very easily and rapidly inactivated by any method other than fine mincing. All the tissues were then suspended separately in 1:2 (w:v) solutions of isotonic KCl containing glucose-6-PO_4 to stabilize the enzyme. Suspensions were centrifuged for 30 min in a refrigerated centrifuge at 37,000*g*. The clear, pink supernatant was withdrawn and assayed for enzyme specific activity and protein content.

Glucosamine synthetase specific activity was assayed using the method of Kornfeld (10), modified in our laboratory for use in gastric tissues (11). Glucosamine-6-PO_4, the product formed in the GmS reaction, was measured using the Levy–McAllan modification (12) of the Elson–Morgan reaction. In some experiments the specific activity of glucosamine-6-PO_4:*N*-acetylase, hereafter referred to as *N*-acetylase, was also determined. This assay was adapted from that of Kent (9) and has been described earlier (13). Product formation was measured by the Reissig modification (14) of the Morgan–Elson method for *N*-acetylated hexosamines. Protein was measured using a modified biuret (15) or by the method of Lowry *et al.* (16).

Enzyme activity data are expressed in two forms. First, all data are expressed a nanomoles of product formed per milligram of supernatant protein per hour of incubation. The mean of all values obtained in a particular study were computed and a standard error of the mean was calculated. Second, each individual study was performed several different times on different days. In some instances the mean control values differed from day to day. Therefore, the mean of all control values measured on a given day was determined. All data obtained on that day (both control and experimental) were then recalculated as percentages of the control mean for that day. The percentages calculated in this manner for all individual experiments (different days) in a given study were then averaged and the standard error of the mean was calculated. Data so calculated are expressed as percentage of the control mean.

Statistics. Data obtained from each group of experimental animals were compared with those from the appropriate control group. Student's t test was used to determine significance. The 5% probability level was chosen as an indication of significance. In only one instance (discussed later) did conversion of the data to percentage of the control mean alter the statistical significance.

Occurrence of ulceration. Each mucosa was examined visually for damage and graded according to the extent of surface area involvement of the mucosa in ulceration. In some instances, blood was present in the stomach although no lesions were apparent. Incidence of ulceration was taken to mean the number of rats in each group with visible damage of any kind.

Results. Oral administration of sodium salicylate, 250 mg/kg in pH 2.5 citrate buffer caused hemorrhage and lesion formation in the gastric mucosa of 87.5% of the rats killed 4 hr after drug administration. Damage consisted of multiple acute erosions not penetrating the muscularis mucosa. Control animals received pH 2.5 buffer containing diatomaceous earth. This substance caused small cuts in the gastric mucosae of several animals. These cuts in no way resembled the erosions seen in the sodium salicylate-treated animals. Glucosamine synthetase specific activity was decreased from 74.4 ± 7.5 nmole/mg/hr (N = 8) to 38.7 ± 9.6 nmole/mg/hr (N = 8). This represents a decrease to 52.0 ± 12.8% of the control mean, $P < 0.025$ (Fig. 1).

Hepatic GmS specific activity was not altered 4 hr after exposure to sodium salicylate, pH 2.5.

In order to determine whether decreases in fundic GmS specific activity precede or follow lesion formation the experimental conditions were altered in several ways in an attempt to reduce the incidence of damage.

First, the time interval between drug administration and sacrifice of the rats was reduced to 2 hr. The results were similar both quantitatively and qualitatively to those observed at 4 hr after drug administration. In the fundic mucosa the incidence of damage was 85.7% and the GmS specific activity was reduced from 87.3 ± 6.5 nmole/mg/hr (N = 7) to 57.9 ± 7.6 nmole/mg/hr (N = 7). This represents a decrease to 67.8 ± 9.7% of the control mean, $P < 0.025$. As expected hepatic enzyme specific activity was not reduced (Figs. 1 and 2).

Second, sodium salicylate, 250 mg/kg, was administered in a neutral buffer solution, pH 7.5. Rats were sacrificed 4 hr after drug administration. Visible damage did not occur in the stomachs of these animals, most likely due to the absence of acid. The enzyme specific activity data collected in this study are more difficult to interpret due to marked variations in the mean control values measured on different days. However, in all instances sodium salicylate did decrease GmS specific activity. When all raw data are combined, the mean control specific activity in the gastric mucosa was 42.6 ± 5.3 nmole/mg/hr (N = 8). After sodium salicylate this value was 32.5 ± 6.3 nmole/mg/hr (N = 7), however, because of

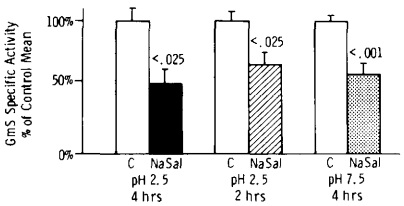

FIG. 1. Glucosamine synthetase specific activity in rat gastric fundic mucosa 2 or 4 hr after oral sodium salicylate, 250 mg/kg. All values are percentage of control mean ± SEM. P values are for comparisons to individual control groups.

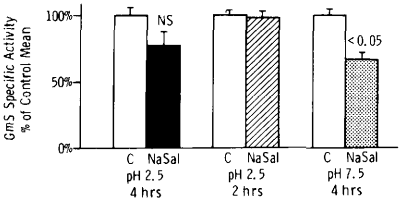

FIG. 2. Glucosamine synthetase specific activity in rat liver 2 or 4 hr after oral sodium salicylate, 250 mg/kg. All values are percentage of control mean ± SEM. P values are for comparisons to individual control groups.

the between days variability the change was not statistically significant. If, however, all measured values are converted to percentage of the simultaneously run control mean the study indicates that sodium salicylate decreases GmS specific activity from 100.0 ± 5.0 to 60.1 ± 8.2% of the control mean, $P < 0.001$. The reasons for the wide spread in control values and the overall lower control values in this particular study are not known.

No such variability was encountered in the liver. Hepatic GmS specific activity decreased from 30.9 ± 4.9 nmole/mg/hr ($N = 8$) to 17.3 ± 2.1 nmole/mg/hr ($N = 7$). This represents a decrease to 65.2 ± 12.7% of the control mean, $P < 0.05$.

N-Acetylase specific activity was altered in only one instance. Sodium salicylate, 250 mg/kg, pH 2.5, decreased *N*-acetylase activity in the liver 4 hr after drug administration.

Damage was not observed in the antrum or duodenum following sodium salicylate administration in these experiments.

Davenport has demonstrated that low (20 m*M*) concentrations of sodium salicylate are capable of breaking the gastric mucosal barrier and increasing acid back diffusion (1). Oral administration of sodium salicylate, 20 m*M*, 10 ml/kg (32 mg/kg) in 0.1 *N* HCl, caused rapid lesion formation and hemorrhage in the fundus. The incidences of damage were 37.5, 75, and 70% at 5, 15, and 30 min after drug administration. Fundic GmS specific activity was initially decreased from 117.5 ± 15.1 nmole/mg/hr ($N = 7$) to 69.4 ± 15.8 nmole/mg/hr ($N = 8$). This represents a reduction, at 5 min after drug administration to 54.7 ± 10.1% of the control mean, $P < 0.01$. Activity had begun to recover after only 15 min and at 30 min after drug administration had rebounded to 179.1 ± 11.0% of the control mean, $P < 0.001$. In absolute terms this was an increase from 117.2 ± 16.9 ($N = 11$) to 193.6 ± 11.4 nmole/mg/hr ($N = 10$) (Fig. 3).

Enzyme specific activity in the antral mucosa tended to be decreased at all time periods after drug administration but the differences were not significant. No damage was observed in the antrum. Likewise,

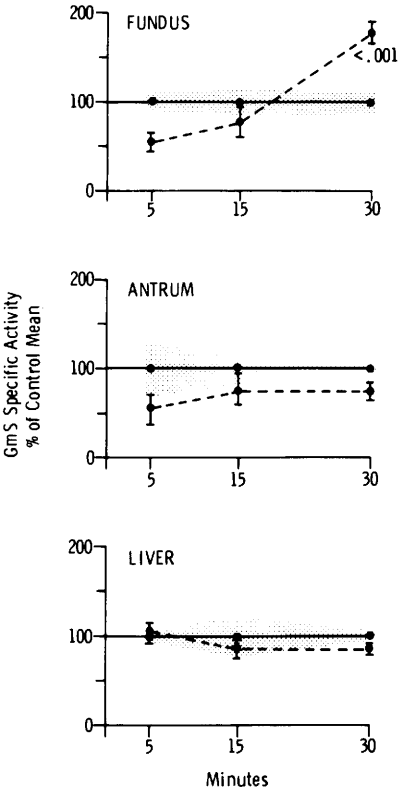

FIG. 3. Glucosamine synthetase specific activities in rat gastric fundic and antral mucosae and liver, 5, 15, and 30 min after oral sodium salicylate, 20 m*M*, 10 ml/kg. All values are percentage of control mean ± SEM. Lightly shaded areas indicate ±SEM of control values. *P* values are for comparisons to same time control groups.

GmS specific activity was not altered by the low dose of sodium salicylate in the liver.

N-Acetylase specific activity was not altered by this dose of sodium salicylate in any of the three tissues at any time period.

Ethanol. Oral administration of ethanol in 0.1 *N* HCl resulted in acute erosions in the oxyntic gland region (fundus) of the stomach within 15 min in 92% of the animals tested. GmS specific activity was decreased from 111.7 ± 10.9 nmole/mg/hr ($N = 13$) to 75.1 ± 11.8 nmole/mg/hr ($N = 11$). This represents a decrease to 70.7 ± 11.7% of control mean, $P < 0.05$. Spontaneous lesion formation occurred, or was previously present in 14% of the control animals given acid only. At 30 min the incidence of dam-

age had dropped to 43% and GmS specific activity had rebounded to 160.2 ± 23.5% of the control mean, $P < 0.05$ (30 min control 135.3 ± 17.8 nmole/mg/hr, $N = 7$; ETOH 203.9 ± 21.1 nmole/mg/hr, $N = 7$) (Fig. 4).

Acidic ethanol did not cause lesion formation in the antral mucosa and GmS specific activity was not significantly altered. Values did, however, tend to decrease in this tissue 15 min after drug administration.

Ethanol, administered in 0.1 *N* HCl increased GmS specific activity in the liver from 13.9 ± 1.8 nmole/mg/hr ($N = 13$) to 19.2 ± 1.6 nmole/mg/hr ($N = 14$) in 15 min. This is an increase to 153.7 ± 20.7% of control mean, $P < 0.025$. Thirty minutes after drug administration enzyme specific activities were similar to those of the control level.

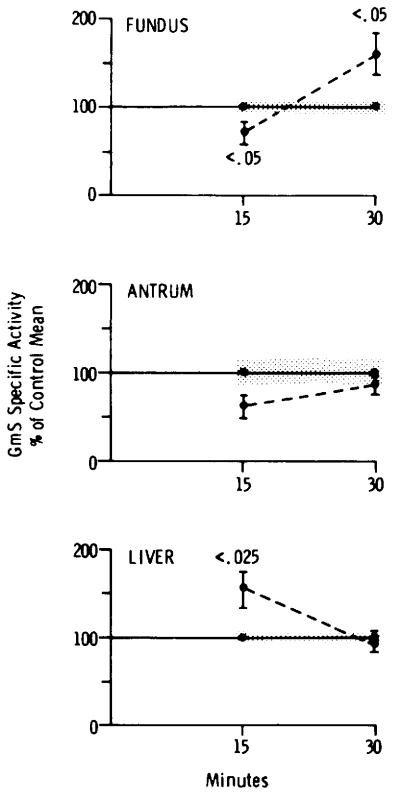

FIG. 4. Glucosamine synthetase specific activities in rat gastric fundic and antral mucosae and liver 15 and 30 min after oral ETOH, 20% (v:v), 10 ml/kg. All values are percentage of control mean ± SEM. Lightly shaded areas indicate ±SEM of control values. *P* values are for comparisons to same-time control groups.

Administration of ethanol in buffer, pH 7.5, or in water resulted in enzyme alterations similar to those observed after acidic ethanol administration.

The only alteration in *N*-acetylase specific activity induced by ethanol was a slight increase in activity in the fundus at 15 min (15 min control 46.0 ± 4.4 nmole/mg/hr, $N = 14$; ETOH 52.0 ± 4.3 nmole/mg/hr, $N = 13$, $P < 0.01$).

Discussion. The correlation between gastric damage and sodium salicylate ingestion is well established. This study demonstrates a definite relationship between sodium salicylate ingestion and the inhibition of hexosamine synthesis. Such inhibition has been previously reported by Perrey (7), Kent (9), and Chan *et al.* (8). However, these studies involved extremely high drug doses and/or *in vitro* drug administration which were not used in the present study. Salicylate ingestion in humans commonly follows one of two patterns: (a) the occasional ingestion of one to two tablets for short-term relief of multiple discomforts, (b) repeated ingestion of many tablets per day for extended periods of time to treat chronic conditions. This study attempts to simulate both of these conditions. The 20 m*M* sodium salicylate solution corresponds to two aspirin tablets in 8 oz of water. The larger 250 mg/kg dose seems high for rats on a weight basis, but, according to St. John *et al.* (18) is comparable on a surface area basis to doses given humans clinically. Additionally, this dose in rats results in a mean serum salicylate level 4 hr after drug administration which is similar to that seen clinically in salicylate-treated rheumatic disease.

All doses of sodium salicylate resulted in marked gastric bleeding, erosion formation and significant decreases in GmS specific activity in the fundic mucosa. However, interesting differences occurred in the time course of these alterations and in their pH dependency. At the lowest dose, 32 mg/kg, damage, predominantly bleeding, with or without visible lesions occurred within minutes. GmS specific activity also de-

creased immediately but recovered rapidly. At higher doses, damage, predominantly blood-filled erosions, was apparent 2 and 4 hr after the drug was given. Earlier time periods were not measured. Fundic mucosal glucosamine synthetase specific activity was decreased at both time periods suggesting delayed recovery.

There are several possible explanations for the apparent inconsistencies in the observations at these dosage levels. First, Yeomans *et al.* (19) has described two types of lesions occurring in the rat stomach after aspirin administration: (a) superficial erosions which occur within 30 min after drug administration and heal completely within 24 hr; (b) deeper erosions which reach maximal numbers by 4 hr and which heal very slowly. It is possible that the damage observed in this study after the lower dose of drug corresponds to the superficial erosions seen by Yeomans while that seen following the higher dose corresponds to the deeper erosions. Second, using an *in vitro* technique Chan *et al.* (8) demonstrated reversible inactivation of glucosamine synthetase by 10–20 m*M* sodium salicylate. At higher concentrations sodium salicylate caused irreversible inactivation of the enzyme. The rapid recovery of enzyme activity observed in this study after low drug doses may be due to recovery from reversible inactivation while the prolonged decrease in activity seen with the higher dose may be due to irreversible inactivation.

The rapidity of the reduction in enzyme activity and its recovery at low drug concentrations suggests that the alterations induced are not due to changes in enzyme synthesis which would require more time. However, Winterburn *et al.* have demonstrated that the normal regulation of glucosamine synthetase specific activity is related to the binding of the feedback inhibitor UDP-*N*-acetylglucosamine to the enzyme, a process which could occur much more rapidly (20).

The observation of decreased GmS specific activity unaccompanied by visible gastric damage after the administration of sodium salicylate in a neutral solution indicated that the decreased GmS specific activity is not a result of tissue necrosis. On the other hand, the decrease in *N*-acetylase specific activity which occurred only in the severely damaged stomach (high dose of sodium salicylate in acid) may be due to necrosis.

The inhibition of GmS activity in neutral solution also indicates that decreases in GmS activity are alone insufficient to initiate damage. This is consistent with the observation that acid in the stomach is required for the development of gastric lesions although acid alone is also insufficient to initiate damage. It may well be that both decreased GmS specific activity and acid are required simultaneously for damage to occur. Additionally, a low pH in the stomach would enhance gastric absorption of salicylate.

It is quite unlikely that alterations in liver enzyme activities are related in any way to the gastric events. These enzymes in the liver are involved primarily in plasma glycoprotein synthesis. The inhibition of enzyme activities in the liver with higher doses of sodium salicylate suggests widespread systemic action on hexosamine synthesis. The apparent pH dependence of this inhibition is most probably the result of experimental variation. At a pH of 7.5 the GmS specific activity was decreased to 65% of control. At pH 2.5 there was also a decrease in specific activity which was, however, not significant.

The rapid decrease and subsequent recovery of glucosamine specific activity in the gastric fundic mucosa following ethanol administration parallels the alterations caused by low doses of sodium salicylate. Similarly, damage and enzyme inhibition were not observed in the antrum following ethanol.

Restraint and hydrocortisone-induced gastric ulceration have also been shown to be accompanied by decreases in GmS specific activity (11, 13).

The overall importance of the inhibition of hexosamine synthesis in ulcerogenesis remains to be explained. It is unlikely that the extremely rapid and reversible inactivation of GmS by low doses of sodium salicylate or ethanol is causally related to ulceration. However, the apparently irreversible inactivation of GmS by higher

doses of sodium salicylate may contribute to delayed healing of the severe lesions caused by the higher doses of sodium salicylate and may also be involved jointly with acid in their development. Hexosamine produced by the pathway examined is not only a constituent of gastric mucus but also of cell membrane glycoproteins and glycolipids. Interference with the normal production of gastric mucus and plasma membranes may well be one of the many factors involved in drug-induced ulcerations.

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