Effect of Uridine Diphospho-*N*-acetylglucosamine and Sodium Salicylate on L-Glutamine–D-Fructose-6-phosphate Aminotransferase Activity from Rat Gastric Mucosa

JAMES CHAN and K. H. LEE ˣ

Abstract □ Mechanisms of inhibitory action of sodium salicylate on L-glutamine–D-fructose-6-phosphate aminotransferase, prepared from rat gastric mucosa, were studied. Sodium salicylate at lower concentrations (10–20 m*M*) inhibited reversibly aminotransferase activity by competing with fructose-6-phosphate. At higher concentrations, sodium salicylate inactivated the enzyme irreversibly, with an inactivation rate following first-order kinetics with respect to the enzyme concentration. Uridine-5′-diphospho-*N*-acetylglucosamine is an endogenous feedback inhibitor. It inhibited the aminotransferase-catalyzed reaction also by competing with fructose-6-phosphate but with an inhibiting activity 1000 times that of sodium salicylate. Uridine-5′-diphospho-*N*-acetylglucosamine reduced the salicylate inhibition of the enzymic reaction and protected the enzyme from salicylate-induced irreversible inactivation. At a fixed concentration of uridine-5′-diphospho-*N*-acetylglucosamine (7×10^{-6} *M*), an increase of salicylate concentration produced an increase in enzyme activity as compared with the control.

Keyphrases □ L-Glutamine–D-fructose-6-phosphate aminotransferase activity—inhibition by sodium salicylate and uridine-5′-diphospho-*N*-acetylglucosamine, rat gastric mucosa □ Sodium salicylate—inhibition of L-glutamine–D-fructose-6-phosphate aminotransferase activity from rat gastric mucosa □ Uridine-5′-diphospho-*N*-acetylglucosamine—inhibition of L-glutamine–D-fructose-6-phosphate activity from rat gastric mucosa □ Gastric mucosa—effect of uridine diphospho-*N*-acetylglucosamine and sodium salicylate on L-glutamine–D-fructose-6-phosphate aminotransferase activity

Sodium salicylate has been reported to inhibit the activities of many enzymes involved in mucopolysaccharide synthesis including L-glutamine–D-fructose-6-phosphate aminotransferase (EC 2.6.1.16) (1–4), acetylcoenzyme A synthetase (EC 6.2.1.1) (5, 6), uridine-5′-diphospho-*N*-acetylglucosamine-uridine-5′-diphospho-*N*-acetylgalactosamine epimerase (EC 5.1.3.1) (7), uridine-5′-diphosphoglucose dehydrogenase (EC 1.1.1.22) (8), and uridine-5′-diphospho glucuronyltransferase (EC 2.4.1.17) (9).

L-Glutamine–D-fructose-6-phosphate aminotransferase is located at a branch point in carbohydrate metabolism and competes for fructose-6-phosphate with other pathways utilizing hexose monophosphates such as glycolysis, glycogenesis, and the hexose monophosphate shunt. Depending on the tissue, 1.5–20% of the fructose-6-phosphate is converted to hexosamine (10, 11). The aminotransferase enzyme has the lowest extractable activity of the enzymes in the hexosamine pathway (12) and has been reported to be the rate-limiting enzyme in hexosamine biosynthesis (10).

In addition, several reports indicated that hexosamine biosynthesis was subjected to feedback regulation by uridine-5′-diphospho-*N*-acetylglucosamine (13–16). This feedback inhibition is on the initial enzyme of the pathway, catalyzing the apparent irreversible formation of glucosamine. Perrey (2) suggested that there may be a possible correlation between the inhibition of the aminotransferase enzyme in the rat gastric mucosa by sodium salicylate and gastric lesions.

In view of the potentially important role of this enzyme and implications resulting from its inhibition, this study presents the mechanisms of action of sodium salicylate and uridine-5′-diphospho-*N*-acetylglucosamine on L-glutamine–D-fructose-6-phosphate aminotransferase prepared from the rat gastric mucosa and the interaction between the two inhibitors.

EXPERIMENTAL

Materials and Chemicals—The following were used: D-glucose-6-phosphate sodium salt, D-fructose-6-phosphate sodium salt, L-glutamine hydrochloride, uridine-5′-diphospho-*N*-acetylglucosamine[1], sodium salicylate[2], and cross-linked dextran gel[3].

Preparation of L-Glutamine–D-Fructose-6-phosphate Aminotransferase—The enzyme was prepared by the method described by Bates and Handschumaker (16) with some modifications. Sprague–Dawley male rats[4] (120–150 g) were decapitated, and the stomachs were removed and placed into an ice-cold extraction medium containing 10 m*M* glucose-6-phosphate, 20 m*M* glu-

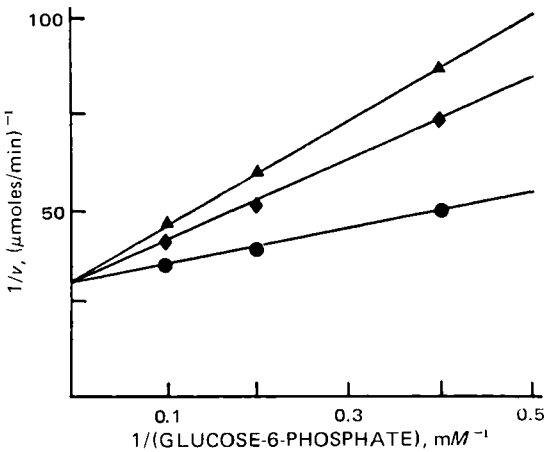

Figure 1—*Effect of salicylate on* L-*glutamine–*D-*fructose-6-phosphate aminotransferase activity as a function of the concentration of glucose-6-phosphate;* v *is expressed as micromoles of glucosamine formed per minute. Key:* ●,- *control;* ♦, 1×10^{-2} M *sodium salicylate; and* ▲, 1.5×10^{-2} M *sodium salicylate.*

[1] Sigma Chemical Co., St. Louis, Mo.
[2] J. T. Baker Chemical Co., Phillipsburg, N.J.
[3] Sephadex G-25, Pharmacia Co., Uppsala, Sweden.
[4] Simenson Laboratories, Gilroy, Calif.

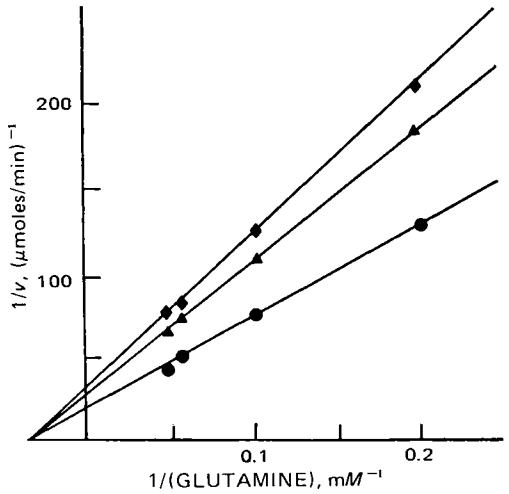

Figure 2—*Effect of salicylate on aminotransferase activity as a function of the concentration of glutamine. Key:* ●, *control;* ▲, *1* × 10^{-2} M *salicylate; and* ♦, *1.5* × 10^{-2} M *salicylate.*

tamine, 85 m*M* potassium phosphate buffer (pH 7.4), 0.1% mercaptoethanol, and 1 m*M* ethylenedinitrilotetraacetic acid disodium salt.

Each stomach was cut along its greater curvature and spread on a watchglass placed over ice, and the mucosal layer was scraped with a small spatula. The mucosal scrapings were homogenized in a hand homogenizer[5] with the volume to weight of the extraction medium. The homogenate was centrifuged at 35,000×*g* for 60 min in a refrigerated ultracentrifuge[6]. The clear supernatant fraction was passed through columns (20 × 1.5 cm) of cross-linked dextran gel[3] previously equilibrated with the extraction medium and maintained between 0 and 4°. The protein-containing fraction containing the enzyme was eluted with the extraction medium.

L-Glutamine–D-Fructose-6-phosphate Aminotransferase Assay—The activity of the enzyme was determined by the rate of formation of glucosamine. The reaction mixture contained, in 4 ml, 85 m*M* potassium phosphate buffer (pH 7.4), 1 m*M* ethylenedinitrilotetraacetic acid disodium salt, 0.1% mercaptoethanol, 1 ml of the eluted mucosal supernatant protein fraction (0.4–0.5 mg of protein/ml), and graded concentrations of glucose-6-phosphate, L-glutamine, sodium salicylate, and uridine-5′-diphospho-*N*-acetylglucosamine as indicated. The incubation was carried out in a 25-ml flask on a Dubnoff shaker at 37° for 45 min.

Enzyme activity was terminated by heating the samples in boiling water for 2 min. After cooling to room temperature, the mixtures were centrifuged at 35,000×*g* for 15 min, and the supernatant fraction was removed for glucosamine determination using the method described by Benson and Friedman (17). Protein concentrations were determined by the biuret method (18).

Inactivation Studies—The enzyme was preincubated with sodium salicylate in the absence of glucose-6-phosphate. The preincubation mixture contained, in 3 ml, 85 m*M* potassium phosphate buffer (pH 7.4), 1 m*M* ethylenedinitrilotetraacetic acid disodium salt, 0.1% mercaptoethanol, 20 m*M* glutamine, and 1 ml of the mucosal supernatant protein fraction. After the preincubation period, the samples were passed through columns (20 × 1.25 cm) of the dextran gel; the columns had been equilibrated with a solution containing 85 m*M* phosphate buffer (pH 7.4), 20 m*M* glutamine, 1 m*M* ethylenedinitrilotetraacetic acid disodium salt, and 0.1% mercaptoethanol. The procedure was carried out between 0 and 4°.

The protein fraction, characterized by a pink band, was collected and 1 ml was added to 2 ml of the incubation medium containing 85 m*M* phosphate buffer (pH 7.4), 20 m*M* glutamine, 1 m*M* ethylenedinitrilotetraacetic acid disodium salt, 0.1% mercaptoethanol, and fructose-6-phosphate to give a final concentration of 10 m*M*. This mixture was incubated for 30 min, and enzyme activity was determined by glucosamine assay.

Protection Studies—Fructose-6-phosphate, glutamine, and uridine-5′-diphospho-*N*-acetylglucosamine were studied as compounds that could afford some protective effect on salicylate-induced inactivation of the enzyme. The enzyme was preincubated with salicylate and one of these compounds. The preincubation mixture contained, in 3 ml, 85 m*M* phosphate buffer (pH 7.4), 1 m*M* ethylenedinitrilotetraacetic acid disodium salt, 0.1% mercaptoethanol, 1 ml of the mucosal supernatant protein fraction, and one of the following: 20 m*M* glutamine, 10 m*M* fructose-6-phosphate, or 3.5 × 10^{-6} *M* uridine-5′-diphospho-*N*-acetylglucosamine.

After preincubation, the samples were treated as described under *Inactivation Studies*. In experiments where preincubation in the absence of glutamine and/or fructose-6-phosphate was indicated, they were not present in the homogenizing medium and were added only to the final incubation mixture for glucosamine synthesis.

Interaction Studies—In studies involving the interaction of sodium salicylate and uridine-5′-diphospho-*N*-acetylglucosamine, the reaction mixture contained, in 4 ml, 10 m*M* glucose-6-phosphate, 20 m*M* glutamine, 85 m*M* potassium phosphate buffer (pH 7.4), 1 m*M* ethylenedinitrilotetraacetic acid disodium salt, 0.1% mercaptoethanol, 1 ml of the mucosal supernatant protein fraction, and varying concentrations of salicylate and uridine-5′-diphospho-*N*-acetylglucosamine. The incubation was carried on for 30 min, and the enzyme activity was determined by glucosamine assay.

No correction was applied for the fact that glucosamine-6-phosphate, on a molar basis, gave 85% of the color produced by glucosamine (19). Throughout this work, it was assumed that the isomerization of glucose-6-phosphate to fructose-6-phosphate was not rate limiting due to the presence of hexose isomerase in the enzyme extracts (16). In fact, Pogell and Gryder (20, 21) found that glucose-6-phosphate was a better substrate for the crude enzyme. Experiments using glucose-6-phosphate and fructose-6-phosphate gave essentially identical results. The production of glucosamine-6-phosphate was proportional to time for 60 min under experimental conditions, and the presence of inhibitors did not alter this linearity of product formation.

RESULTS

Figure 1 shows the effect of salicylate on L-glutamine–D-fruc-

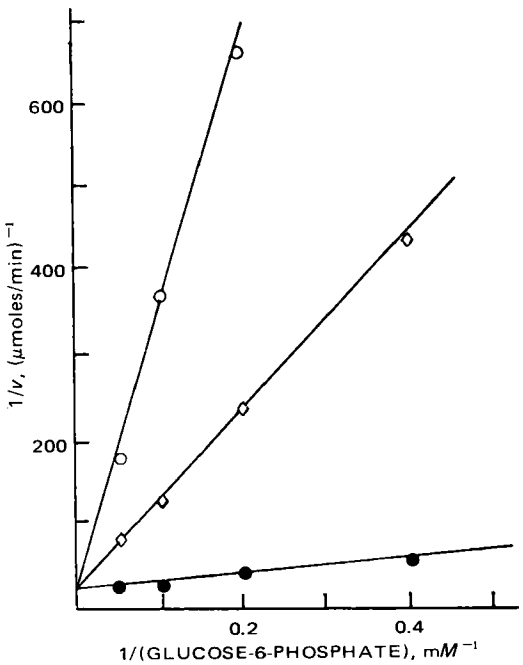

Figure 3—*Effect of uridine-5′-diphospho-*N*-acetylglucosamine on aminotransferase activity as a function of the concentration of glucose-6-phosphate. Key:* ●, *control;* ◊, *1* × 10^{-5} M *uridine-5′-diphospho-*N*-acetylglucosamine; and* ○, *5* × 10^{-5} M *uridine-5′-diphospho-*N*-acetylglucosamine.*

[5] Corning Glass Works, Corning, NY 14830
[6] Spinco, Class A, Beckman Instruments, Palo Alto, CA 94304

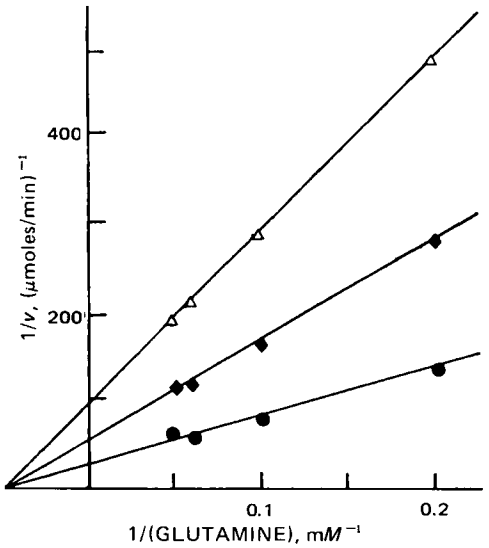

Figure 4—*Effect of uridine-5′-diphospho-*N*-acetylglucosamine on aminotransferase activity as a function of the concentration of glutamine. Key:* ●, *control;* ♦, *4* × 10^{-6} M *uridine-5′-diphospho-*N*-acetylglucosamine; and* △, *1* × 10^{-5} M *uridine-5′-diphospho-*N*-acetylglucosamine.*

tose-6-phosphate aminotransferase activity as a function of the glucose-6-phosphate concentration. The resultant double reciprocal plot indicated that salicylate acted as a competitive inhibitor with respect to glucose-6-phosphate. A double reciprocal plot of the aminotransferase activity as a function of the glutamine concentration is shown in Fig. 2. The results indicated that salicylate

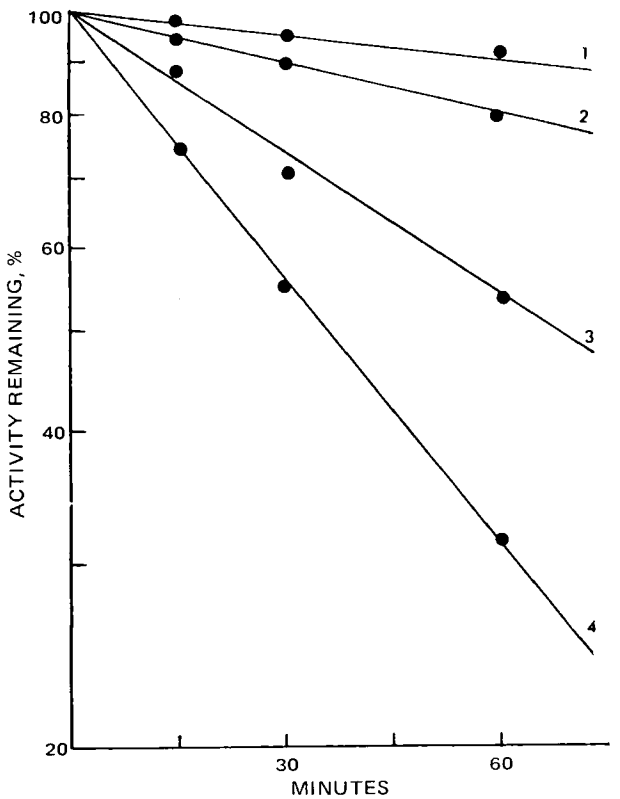

Figure 5—*Effect of varying concentrations of salicylate on the aminotransferase activity as a function of the preincubation time. Concentrations of salicylate are as follows: curve 1, 15 m*M; *curve 2, 25 m*M; *curve 3, 30 m*M; *and curve 4, 35 m*M. *Enzyme activity is expressed as percent of the control.*

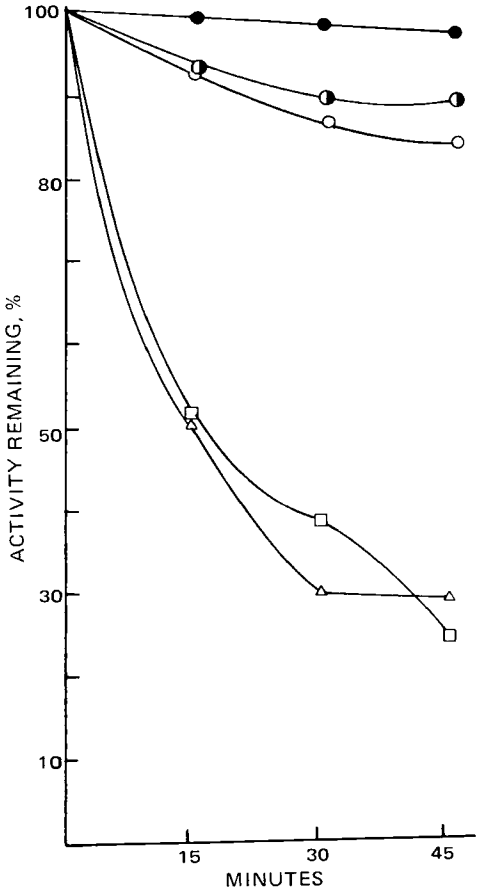

Figure 6—*Protective effect of* D-*fructose-6-phosphate,* L-*glutamine, and uridine-5′-diphospho-*N*-acetylglucosamine on salicylate-induced enzyme inactivation. Enzyme activity is expressed as percent of the control. Key:* ●, *control;* ◑, *40 m*M *salicylate and 3.5* × 10^{-6} M *uridine-5′-diphospho-*N*-acetylglucosamine;* ○, *40 m*M *salicylate and 10 m*M *fructose-6-phosphate;* □, *40 m*M *salicylate and 20 m*M *glutamine; and* △, *40 m*M *salicylate.*

acted as a noncompetitive inhibitor with respect to glutamine. Using uridine-5′-diphospho-*N*-acetylglucosamine as the inhibitor, similar double reciprocal plots depict this compound to be a competitive inhibitor with respect to glucose-6-phosphate (Fig. 3) and a noncompetitive inhibitor with respect to glutamine (Fig. 4).

It appears that both sodium salicylate and uridine-5′-diphospho-*N*-acetylglucosamine acted as competitive inhibitors of the enzyme with respect to glucose-6-phosphate and as noncompetitive inhibitors with respect to glutamine. The inhibitor dissociation constants (K_i) for competitive inhibition for salicylate and uridine-5′-diphospho-*N*-acetylglucosamine were 7.5 × 10^{-3} *M* and 1 × 10^{-6} *M*, respectively, indicating about a 700,000-fold difference in activity.

Salicylate was found to be capable of inactivating L-glutamine-D-fructose-6-phosphate aminotransferase at higher concentrations and longer exposure times. This inactivation was apparently irreversible since passage through columns of the dextran gel did not restore original enzyme activity. The results indicated that the rate of inactivation (Fig. 5) appears to follow first-order kinetics with respect to the enzyme and a much higher order with respect to the salicylate concentration. The results further indicate that under the conditions where reversible inhibition was studied (salicylate concentrations of 10–15 m*M* and 45-min exposure time), no significant inactivation of the enzyme occurred. Therefore, in Figs. 1 and 2 the observed inhibition resulted only from a reversible inhibition of the enzymatic reaction.

The two substrates, fructose-6-phosphate and glutamine, as well as the endogenous feedback inhibitor, uridine-5′-diphospho-*N*-acetylglucosamine, were studied as compounds that may afford protection for the enzyme from inactivation by sodium salicylate.

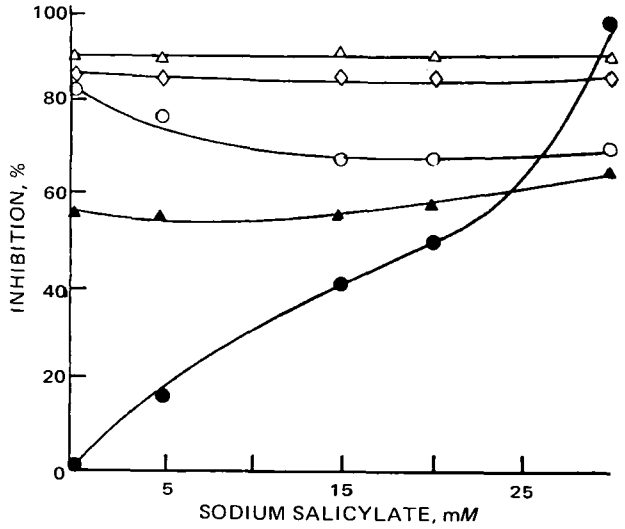

Figure 7—*Effect of sodium salicylate on the activity of* L-*glutamine*–D-*fructose-6-phosphate aminotransferase activity in the absence and presence of uridine-5′-diphospho-*N*-acetylglucosamine. Concentrations of uridine-5′-diphospho-*N*-acetylglucosamine are as follows:* ●, *none;* ▲, *3.5* × 10^{-6} M; ○, *7* × 10^{-6} M; ◇, *1.4* × 10^{-5} M; *and* △, *2.8* × 10^{-5} M.

The results (Fig. 6) indicated that fructose-6-phosphate at 1 × 10^{-2} *M* and uridine-5′-diphospho-*N*-acetylglucosamine at 3.5 × 10^{-6} *M* can protect the enzyme against inactivation by 40 m*M* of salicylate. Glutamine up to 20 m*M* showed no ability to protect the enzyme.

The addition of sodium salicylate to a system inhibited by uridine-5′-diphospho-*N*-acetylglucosamine modified the inhibition (Fig. 7). At a fixed concentration of uridine-5′-diphospho-*N*-acetylglucosamine (7 × 10^{-6} *M*), an increase in salicylate concentration gave rise to activation. Since both salicylate and uridine-5′-diphospho-*N*-acetylglucosamine inhibited the enzyme in a similar manner, such an activation effect caused by two inhibitors acting in concert may be interpreted as a competition for a common site.

DISCUSSION

The results indicated that sodium salicylate can inhibit L-glutamine–D-fructose-6-phosphate aminotransferase either by inhibiting the enzyme-catalyzed reaction by a reversible competitive inhibition with respect to fructose-6-phosphate or by an irreversible inactivation of the enzyme *via* a yet undefined mechanism. In the range of 10–15 m*M*, the inhibition observed was reversible; at concentrations of 30 m*M* or greater, irreversible inactivation was observed also.

Fructose-6-phosphate and uridine-5′-diphospho-*N*-acetylglucosamine were capable of protecting the enzyme against salicylate-induced inactivation as well as of modifying the reversible inhibition of the enzyme. The results further indicated that uridine-5′-diphospho-*N*-acetylglucosamine and sodium salicylate apparently bind to the same site on the enzyme. Based on the consideration of the chemical structures of the two compounds, one can conceive of salicylate binding to the same site occupied by the pyrimidine ring of uridine. Other uridine diphospho sugars such as uridine diphospho galactose and glucose have been reported to inhibit the enzyme at concentrations $\frac{1}{100}$ that of uridine-5′-diphospho-*N*-acetylglucosamine (13).

Kornfeld (13) was able partially to desensitize the enzyme to inhibition by uridine-5′-diphospho-*N*-acetylglucosamine, suggesting that the inhibitor site was distinct from the active site. This finding further suggests that the binding of salicylate and uridine-5′-diphospho-*N*-acetylglucosamine to the "inhibitor" site resulted in the increase in K_m of fructose-6-phosphate, which is characteristic of competitive inhibition (22). The enzyme has a reported molecular weight of 360,000–400,000 but does not exhibit the sigmoid dependence of velocity and modifier concentration, a property in common with the classical allosteric enzymes (23).

This study indicates that for salicylate to inhibit L-glutamine–D-fructose-6-phosphate aminotransferase *in vivo*, the *in vivo* concentration of the endogenous feedback inhibitor, uridine-5′-diphospho-*N*-acetylglucosamine, as well as of fructose-6-phosphate and salicylate would be important. Work by Hardingham and Phelps (10) and Winterburn and Phelps (14) indicated that the enzyme was inhibited in excess of 90% by the feedback inhibitor *in vivo* in the rat skin and rat liver. If this type of inhibition occurred in the general gastric mucosa, it would mean that the binding site for salicylate would be occupied by the feedback inhibitor *in vivo*. In addition, the presence of a feedback loop tends to correct for any deviation from the normal metabolic flux of uridine-5′-diphospho-*N*-acetylglucosamine. If salicylate should inhibit the formation of glucosamine-6-phosphate, it would reduce the level of uridine-5′-diphospho-*N*-acetylglucosamine which, in turn, would release more enzyme from inhibition, restoring the original metabolic flux of glucosamine-6-phosphate. These factors seem to suggest that L-glutamine–D-fructose-6-phosphate aminotransferase would not be a sensitive site for salicylate inhibition *in vivo*.

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[x] To whom inquiries should be directed.